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Effects of Bile Acids and Fatty Acids on Cell Signalling in Human Colorectal Cancer Cells

Thesis for the degree of Doctor of Philosophy

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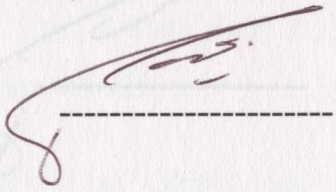

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PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

Increased urinary nitrite, a marker of nitric oxide, in active inflammatory bowel disease. M.G. Goggins, S.A. Shah, J. Goh, D. Kelleher, N. Mahmud. *Mediators of Inflammation* 2001; 10:71-000.

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MANUSCRIPT IN PREPARATION

Ursodeoxycholic Acid Inhibits Deoxycholic Acid-induced Translocation of Protein Kinase C beta 1, PKC epsilon and PKC delta in Human Colonic Cancer. Submitted to *EJC*

Ursodeoxycholic Acid inhibits Interleukin beta 1 and Deoxycholic Acid-induced Activation of NF-κB and AP-1 in Human Colon Cancer Cells. Submitted to *international journal of Cancer*

Chronic but Not Acute Treatment with Conjugated Linoleic Acid (CLA) Isomers (cis-9, trans-11 CLA and trans-10, cis-12 CLA) inhibits DCA-induced Protein Kinase C and DCA and IL-1β- induced NF-κB activation in Human Colorectal Cancer cells. To be submitted to *European journal of cancer prevention*

PRESENTATIONS

Oral Presentation 'Ursodeoxycholic acid inhibits Deoxycholic acid–induced Protein Kinase C beta 1 and PKC epsilon activation in Human Colon cancer' at Irish Society of Gastroenterology Conference at West Port in April 2002, winning first prize for the *best Oral Presentation*

Oral Presentation 'Ursodeoxycholic acid inhibits Deoxycholic acid–induced Protein Kinase C beta 1 and PKC epsilon activation in Human Colon cancer' at the Registrar Prize Competition, organised by the Royal Academy of Medicine at RCPI, winning Prize for the *second best oral presentation*

Poster presentation 'Ursodeoxycholic acid inhibits Deoxycholic acid–induced Protein Kinase C beta 1 and PKC epsilon activation in Human Colon cancer' at 10TH United European Gastroenterology Week at Geneva October 2002.

Oral presentation 'Ursodeoxycholic acid inhibits Deoxycholic acid-induced translocation of protein kinase C beta 1, PKC epsilon and PKC delta in human colonic cancer' at Irish Society of Immunology/ St Vincent's University Hospital Conference in November 2002.

Poster Presentation 'Differential Effects of Tumour Promoting Bile Acid Deoxycholic Acid and Chemoprotective Ursodeoxycholic Acid on Protein Kinase C and Transcription Factors NF-κB and AP-1 in Human Colon Cancer at American Gastroenterology Association in Digestive Disease Week 2003 (DDW) Orlando 2003.

Oral presentation 'Ursodeoxycholic Acid inhibits Deoxycholic Acid and Interleukin beta 1-induced NF-κB and AP-1 activation in Human Colorectal Cancer' 11th United European Gastroenterology Week at Madrid November 2003.

Oral Presentation Ursodeoxycholic Acid Inhibits Deoxycholic Acid and Interleukin Beta-1 induced NF-κB and AP-1 activation in Human Colorectal Cancer Cells at Irish Society of Gastroenterology, Spring Meeting June 2003 in Belfast

Oral presentation Chronic but Not Acute Treatment with Conjugated Linoleic Acid (CLA) Isomers (cis-9, trans-11 CLA and trans-10, cis-12 CLA) inhibit DCA-induced Protein Kinase C and DCA and IL-1β- induced NF-κB activation in Human Colorectal Cancer cells. Royal Academy of Medicine Meeting, March 2004.

Oral presentation Chronic but Not Acute Conjugated Linoleic Acid (CLA) Isomers (cis-9, trans-11 CLA and trans-10, cis-12 CLA treatment) inhibit DCA-induced Protein Kinase C and DCA and NF-κB activation in Human Colorectal Cancer cells. ISG Kilkenny May 2004.

Oral presentation 'Chronic but Not Acute Conjugated Linoleic Acid (CLA) Isomers (cis-9, trans-11 CLA and trans-10, cis-12 CLA treatment) inhibit DCA-induced Protein Kinase C and DCA and NF-κB activation in Human Colorectal Cancer cells at 12th United European Gastroenterology Week at Prague September 2004.

SUMMARY

Colorectal cancer is a multifactorial disease. The interplay between genetic and environmental factors leads to the development of CRC. There have been great advancements in the knowledge of genetic alterations leading to CRC and adenoma-carcinoma sequence with corresponding histopathological changes has clearly been defined. The genetic mutations in the hereditary forms of colon cancer can now be analysed by genetic testing in individuals at risk but vast majority of CRC are sporadic in nature with no clear genetic association. The geographic epidemiology of the CRC suggests that environmental factors play a significant role in its pathogenesis. This view is strengthened by the fact that incidence of CRC increases when populations move from low incidence to high incidence regions. High fat diet and particularly that from animal sources have long been blamed for increase in the incidence of CRC in affluent countries. The role of fatty diet in the causation of CRC is still controversial. One hypothesis is that high fat diet increases the concentrations of the toxic secondary bile acids in the colon milieu, as high amounts of detergents are required for the digestion of fats. Bile acids are the endogenous environmental factors, designated as tumour promoters in CRC. These bile acids have been reported to cause hyperproliferation of the colonic mucosa and aberrant crypt proliferation. Bile acids have also been reported to activate cell signalling mechanisms responsible for cell proliferation, cell growth and apoptosis and may eventually give rise to genetic mutations responsible for CRC. On the other hand, a tertiary bile acid, ursodeoxycholic acid is known to have cytoprotective, antioxidant and immunomodulator effects. It is a non expensive and non toxic drug. UDCA has recently been reported to reduce the incidence of colonic dysplasia in patients with ulcerative colitis. Furthermore, many animal studies have outlined UDCA's role in the protection against CRC. Our study concentrated on some of the signalling mechanisms involved in the pathogenesis of CRC and their interactions with bile acids.

First aim of the study was to investigate the effects of secondary bile acids deoxycholic acid and UDCA on expression of protein kinase C (PKC) and the transcription factors, NF-κB and AP-1. PKC and the transcription factors NF-κB and AP-1 have been implicated in the pathogenesis of CRC with effects on cell proliferation, cell migration and apoptosis. Many of the PKC isoforms disappear early in colorectal carcinogenesis and overexpression of the PKC isoforms have been associated with increased growth and oncogenesis. Similarly high

levels of NF-κB have been associated with increased proliferation and resistance to apoptosis in cancer cells. Secondary bile acids have been shown to activate all three molecules. We studied the effects of bile acids DCA and UDCA on PKC translocation and the activation of these transcription factors to delineate the tumour promoting and and chemoprotective effects of DCA and UDCA with regard to these signalling molecules respectively. Researchers in the past have used techniques like western blotting to examine the effects of bile acids on PKC. As the dynamics of serine-threonine, kinases are difficult to examine in the living cells because of the intermittent nature of activation of these signalling molecules and downregulation by phosphorylation. We employed innovative molecular biology techniques to study these mechanisms. The effects of the bile acids were first studied by immunofluorescence of the endogenous PKC isoenzymes. The tagging of PKC isoenzymes with green fluorescent protein have made it possible to investigate these enzymes in living cells and replicate the alterations in real time. We studied these PKC dynamics under direct visualization and examine these alterations. The study shows the differential effects of the bile acids on PKC translocation. DCA caused translocation of the PKC isoenzymes while UDCA had no effect on the subcellular localization of these PKC isoenzymes. Pretreatment of the cells with UDCA inhibited DCA-induced activation of PKC isoenzymes. Subcellular fractionation and Western blot analyses of the treated cells confirmed these data.

The effects of bile acids on the transcription factors NF-κB and AP-1 were examined with electrophoretic mobility shift assays, Western blotting and immunofluorescence. UDCA in contrast with DCA did not activate these transcription factors but also inhibited induction of these transcription factors by DCA. The effects of the bile acids on IκB-degradation were also examined. DCA in contrast to UDCA reduced IκB-α levels, which were restored by pre-treatment with UDCA. The use of PKC inhibitors provided us with further information about the interactions between PKC and the downstream transcription factors.

Lastly, the effects conjugated linoleic acid (CLA) on PKC and NF-κB expression were examined in colorectal cells. CLAs are the isomers of linoleic acid, found in dairy products and meat from animal sources. Many epidemiological and animal studies have outlined the role of CLA in the prevention of CRC. However, the precise molecular mechanisms by which CLA may produce remain unclear. In our study the two CLA isomers *c-9, t-11,* and *t-10, c-12* inhibited DCA-induced PKC and NF-κB DNA binding in the colon cancer cells.

In conclusion both UDCA and CLA inhibited DCA-induced PKC translocation and NF-κB DNA binding in colon cancer cells. The mechanisms through which UDCA and CLA might act in CRC chemoprevention are explored.

DEDICATION

To my Parents

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REFERENCES

Appendix: Reagents and buffers

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ABBREVIATIONS

AO	acridine orange
ACF	aberrant crypt foci
AP-1	activator protein 1
APC	adenomatous polyposis coli
APS	ammonium persulphate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CI	confidence interval
CLA	conjugated linoeic acid
COX-2	cyclooxygenase
CRC	colorectal cancer
DAG	diacylglycerol
DCA	deoxycholic acid
DMSO	dimethly sulphoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EB	ethidium bromide
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGFP	enhanced Green Fluorescent Protein
EGF	epidermal growth factor
EGTA	ethyleneglycol bis-(aminoethlether)tetra-acetic acid
EMSA	electrophoretic mobility shift assay
FAP	familial adenomatous polyposis
FCS	foetal calf serum
FITC	fluorescine isothiocynate
FXR	farnesoid X receptor
GSK	glycogen synthase kinase
HBSS	hank's balanced salts solution
HCL	hydrochloric acid
HNPCC	hereditary non-polyposis colorectal cancer
HRT	hormone-replacement therapy
H_20_2	hydrogen peroxide
IκB-α	Ikappa B-alpha
IKK	IkappaB kinase complex
IL-1	Interleukin-1
INOS	inducible nitric oxide synthetase
JNK	c-Jun NH_2-terminal kinase
LB	l-broth medium
kDa	kilodaltons
LPS	lipopolysacharide
LXR	liver X receptor
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
Mab	monoclonal antibody

$MgCl_2$	magnesium chloride
MHC	major histocompatibility antigen
MMP	matrix-mettaloproteinases
MMR	mismatch repair
MSI	microsatellite instability
NAC	n-acetylecysteine
NaCl	sodium chloride
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa B
NIK	NF-κB-inducing-enzyme
Np40	nonidet p40
NSIADs	nonsteroidal anti-inflammatory drugs
PBC	primary biliar cirrhosis
PI 3-K	phosphoinositide 3-kinase
PKC	protein kinase C
PMA	phorbol-12-myristate-13 acetate
PMSF	phenyl methyle sulphonyl fluoride
PVDF	polyvinylidene difluoride
SRE	serum response elements
PAGE	polyacramide gel electrophoresis
SDS	sodium dodecyl sulphate
Ser	serine
SOD	superoxide dismutase
SRE	serum response element
TAE	tris-acetate EDTA buffer
TBE	tris-borate EDT A buffer
TBS	tris-borate saline buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethyl ethylene-diamine
TGF	transforming growth factor
thr	threonine
TNBS	trinitrobenzenesulfonic acid
TNF-α	tumour necrosis factor-alpha
TRAF	TNF receptor associated factor
TRADD	TNF receptor associated death domaine
Tris	tris (hydroxymethyl) aminomethane
UDCA	ursodeoxycholic acid
UV	ultraviolet

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER I

GENERAL INTRODUCTION

1.1 COLORECTAL CANCER

1.1.1 Background

Colorectal cancer (CRC) is the leading cause of cancer related deaths in the Western world. It is second in incidence only to breast cancer in women, and prostate cancer in men. In Ireland between 1994 and 1998, colorectal cancer accounted for 10% of new cancers in men and 7% in women and for 12% and 13% of cancer deaths in women and men respectively (National Cancer Registry in Ireland, 2002). Colorectal cancer is an important health problem in England and Wales with approximately 29, 000 individuals being diagnosed with colorectal cancer each year in, of whom 15, 000 will die (Hayne *et al.*, 2001). Worldwide CRC represents 9.4% of all incident cancer in men and 10.1% in women. The incidence of CRC varies with the geographical distribution. In Westernized countries, including North America, Europe, Australia and New Zealand CRC represents 12.6% of all incident cancer in men and 14.1 % in women. Elsewhere CRC represents 7.7 % in men 7.9 % in women of all cancer incidence. The incidence of CRC in population changes with changes in life style and with geographical distribution. The groups of migrants from low incidence region quickly acquire the patterns of incidence of new community. Colorectal cancer has a recognised pre-malignant phase, the adenoma, which over time can progress to invasive carcinoma. This transformation from adenoma to carcinoma can take a significant time to complete. The latent periods for the development of cancer, form a polyp may range from 5 to 15 years (Koretz *et al.*, 1993; Muto *et al.*, 1975).This phenomenon provides a window of opportunity for the application of surveillance protocols for the early detection of colorectal carcinomas as well as strategies for the prevention of this dreadful malignancy.

Colon cancer is associated with a sequence of genetic alterations and many genetic tools are being exploited for early diagnosis of the cancer. There are a number of familial forms of CRC with clear associations with genetic alterations though approximately 95% of colorectal cancers are sporadic and occur in individuals with no family history of the disease. (Ilyas *et al.*, 1999)

1.1.2 Familial adenomatous polyposis

Familial adenomatous polyposis (FAP) is a hereditary form of colorectal cancer. It is a dominantly inherited syndrome and characterized by the development of hundreds of

adenomatous colorectal polyps. FAP affects 1: 10, 000 individuals per year and approximately twice as many individuals are at risk (Stern *et al.*, 1995). It is a rare disease and responsible for less than 0.5% of all cases of colorectal cancer. FAP occurs in younger people aged 20-30 years. Because of the high risk of development of CRC in individuals and families with FAP, the identification of gene carriers is essential. The germline mutations of the tumour suppressor gene, adenomatous polyposis coli (*APC*) gene are located on chromosome 5q21 in FAP syndrome patients (Leppert *et al.*, 1987; Bodmer *et al.*, 1987; Groden *et al.*, 1991). In view of the fact that a single gene is responsible for the disease, genetic analysis can be easily performed with relative accuracy for FAP. The children from high-risk families are now offered direct mutation testing at about 13-14 years of age. This is much less invasive than sigmoidoscopy and endoscopic surveillance can now be restricted to individuals from families with mutations where these mutations are undetectable. The detection of mutations in *APC* at different loci not only provides with the diagnosis of FAP but also predict the phenotype such as extracolonic manifestations and the course of the disease process such as severity of the disease (Vasen *et al.*, 1996).

1.1.3 Hereditary non-polyposis colon cancer (HNPCC)

Hereditary non-polyposis colorectal cancer (HNPCC) is the second major hereditary form of colorectal cancer. It is dominantly inherited disorder and is characterised by the onset of CRC at an average age of 45 years. HNPCC is the most common Mendelian disorders with the incidence of between 0.5 and 5 percent (Mecklin *et al.*, 1987; Aaltonen *et al.*, 1994; Ponz de Leon *et al.*, 1993; Westlake *et al.*, 1991). It is relatively common compared with FAP and accounts for 2–4% of all colorectal cancers in the Western world (Lynch *et al.*, 1996). Other cancers such as uterine, ovarian, gastric, and upper urinary tract also occur frequently with HNPCC. The error in DNA mismatch repair (MMR) system is the basic genetic defect underlying HNPCC. These defects in MMR can lead to secondary mutations in tumour suppressor gene, APC as well as the oncogenes, which ultimately leads to tumour development (Lazar *et al.*, 1994). Microsatellite instability (MSI) replication errors are the other genetic defects described in HNPCC. The MSI are the instabilities in DNA at short tandem repeat sequences and affect the DNA of individuals with HNPCC. At least four genes located at various chromosomes at different loci participate in DNA mismatch repair system. These are *MSH2* on chromosome 2p (Leach *et al.*, 1993; Liu *et al.*, 1994; Fishel *at al.*, 1993),

MLH1 on chromosome 3p (Han *et al.*, 1995; Bronner *et al.*, 1994), *PMS1* on chromosome 2q and *PMS2* on chromosome 7p (Nicolaides *et al.*, 1994). Approximately 50% of patients with HNPCC carry mutations in *MSH2* while another 30% show mutations in *MLH1* (Liu *et al.*, 1994; Han *et al.*, 1995). Approximately 10% of individuals with this familial form of colonic cancer have an affected first degree relative but not all carriers of HNPCC are identified because of lack of pathognomonic features, early death of relatives and incomplete gene penetrance (St John *et al.*, 1993; Dunlop *et al.*, 1992).

1.1.4 Sporadic colorectal cancer

Colon cancer is a common malignancy and 1 in 20 persons will develop colorectal neoplasia. Three specific patterns of colon cancer are observed: sporadic, inherited, or familial. Sporadic colorectal cancer accounts for approximately 70% of disease and there is no familial or inherited predisposition. It is common in persons older than 50 years of age. Environmental and dietary factors as well as normal aging have been implicated in the pathogenesis of the sporadic disease. Environmental factors are thought to play important role in the aetiology of CRC. This is because populations in different geographic area and those living in geographic proximity but with different life-styles vary in the incidence of CRC (Potter 1999). Epidemiological and animal studies have reported links between CRC pathogenesis and fats, vegetables, fibre and micronutrients. Diets high in animal fat and red meat have been associated with an increased risk of these colonic adenomas and colorectal cancer (Giovannucci *et al.*, 1992; Willett *et al.*, 1990). High fat diet is thought to increase the concentrations of secondary bile acids within the colon, which may act as carcinogens or tumour promoters (Nagengast *et al.*, 1995). Total calorie intake from fat in Western countries is 40-45 percent, whereas in low-risk populations it is 10-15 percent of dietary calories (Shike *at al.*, 1990). Recent cohort studies and combined analyses of 13 case-control studies have provided a strong evidence for association between dietary fat and CRC (Howe *et al.*, 1997). A number of studies in animal have also reported a role for fat in CRC pathogenesis. Rodents fed on diets rich in polyunsaturated and saturated fats, injected with a carcinogen, 1, 2-dimethylhydrazine (DMH) or azoxymethane (AOM) developed larger number adenocarcinomas than those on low-fat diets (Rao *et al.*, 1996). The chronic inflammatory conditions such as inflammatory bowel disease (IBD) have also been associated with the development of colorectal cancer (Potter 1999). Patients with ulcerative colitis have an approximate 50% lifetime risk of developing colorectal cancer. This suggests

a causal relationship between ulcerative colitis and the development of CRC. Patients with crohns disease are also at an increased risk. This hypothesis is supported by the observation that the increased risk begins after the first 10 years of the disease with the highest risk in patients who have extensive or continuous disease. Genotoxic carcinogens have long been implicated in the pathogenesis of CRC and mutagenic activity is frequently present in the faeces of groups at high risk of CRC. One of these agents is the highly reactive compound synthesised by colonic bacteria, fecapentaenes. Other compound resembling heterocylclic amines, known to cause colon cancer in rodents are found in charbroiled and fried meat and fish. Beer and ale drinking may also increase the risk of CRC (Kindall *et al.*, 1989; Baron *et al.*, 1998). Germline genetic mutations are not present in patients with sporadic colorectal cancer. Patients acquire somatic mutations during their life as a result of exposure to various carcinogens. Some of the agents mentioned above may induce somatic mutations, with a variety of mechanisms that induce DNA damage (Nagao *et al.*, 1998). However, some of the genetic alterations observed in familial forms of CRC may also be present in somatic form of sporadic CRC. For example, APC is mutated in more than 70% of all colorectal cancers including sporadic CRC.

1.1.5 The adenoma–carcinoma sequence

Colorectal carcinomas arise as a result of well-characterized histopathological changes associated with specific genetic defects in oncogenes and tumour-suppressor genes (**Fig. 1.1**). This stepwise model of colorectal tumorigenesis involves early mutations in the tumour-suppressor gene adenomatous polyposis coli (*APC*), occurring during the development of polyps, oncogenic *K-ras* mutations during the adenomatous stage, and mutations of *p53* and deletions on chromosome 18q coinciding with the transition to malignancy (Fearon *et al.*, 1990). Additional genetic events also occur in the regulation of cell proliferation and apoptosis in neoplastic cells.

At least four sequential genetic changes, three tumour-suppressor genes (*APC*, *SMAD4* and *p53*) and oncogene (*K-ras*) are necessary for colorectal cancer evolution. One oncogenic mutation at *K-ras* and six additional mutations affecting both the alleles of the tumour-suppressor genes are required for development of CRC because of the dominant or recessive nature of these genes (Kinzler *et al.*, 1996; Fearon et *al.*, 1990). Most tumours carry mutations in tumour-suppressor genes, whereas *K-ras* mutations are found in approximately 50% of cases (**Table 1.1**).

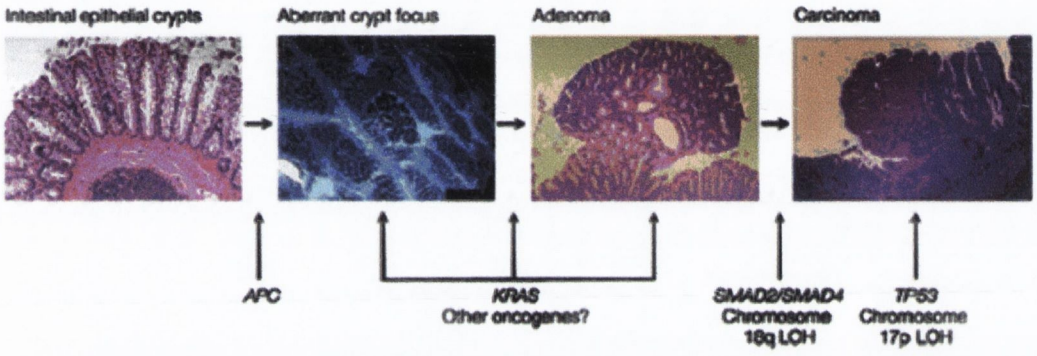


Fig. 1.1 Schematic presentation of the chromosomal and genetic events associated with histopathological alterations from normal colonic epithelium to metastatic colorectal carcinoma. (Adapted from Fodde *et al.*, 2001).

Table 1.1 Prevalence of genetic mutations in colorectal cancer
(Reproduced from Chung 2000)

Gene	Chromosomal location	Prevalence of mutations	Comment
Oncogenes			
KRAS2	12p	50%	Consensus activating point mutations at codons 12 and 13
CTNNB1	3p22	4%–1.5%	Identified in 50% of tumours without APC mutations; most mutations in exon 3 at GSK-3β phosphorylation sites Identified only in metastatic tumours
SRC	20q11	2%	
Tumour-suppressor genes			
APC	5q21	70%	Most mutations are truncating and occur early at adenoma stage
TP53	17p13	50%–70%	Rarely identified in benign adenomas; mutant p53 protein detectable immunohistochemically
SMAD4	18q21	16%	>70% of colorectal tumours show allelic loss on 18q; many potential tumour-suppressor gene targets including *SMAD4, SMAD2*, and *DCC*
SMAD2	18q21	6%	
DCC	18q21	3%	
DNA mismatch repair genes			
hMSH2	2p21		>90% of identified germline mutations are in *hMSH2* or *hMLH1;* limited mutational data in sporadic tumours, but maximum of 26% with MSI have somatic *hMSH2* mutations
hMLH1	3p21		
hPMS1	2q31–33	15%[a]	
hPMS2	7p22		
hMSH6	2p21		

[a] Overall, 15% of tumours display MSI.

1.1.6 Tumour suppressor genes

1.1.6.1 *APC*

The mutations in *APC* gene are the earliest genetic alterations in the colorectal tumours (Powell *et al.*, 1992). The smallest lesions found in the very earliest stages of the adenoma–carcinoma sequence, aberrant crypt foci (ACF), contain *APC* mutations (Kinzler *et al.*, 1996; Jen *at al.*, 1994). The types of defects involved in chromosome 5q21 where *APC* is localized, are allelic losses and point mutations (Powell *et al.*, 1992; Okamoto *et al.*, 1988; Solomon et al., 1987). The somatic mutations in *APC* are clustered at the 5' end of exon 15 between amino acid residues 1280-1500, though germline inactivating mutations may be distributed throughout the entire gene (Miyake *et al.*, 1994). The somatic mutations in *APC* frequently overlap with the 15-amino acid repeat domains between residues 1020 and 1169 and 20-amino acid repeat domains between residues 1324 and 2075, the region implicated in β-catenin and *APC* interactions (Su *et al.*, 1993; Rubinfeld *et al.*, 1993). The SAMP (Ser-Ala-Met-Pro) domains located between the 20-amino acid repeats that interact with axin are also involved in these mutations (Behrens *et al.*, 1998). Mutations in one copy of *APC* are typically coupled with chromosomal deletions of the second wild-type allele. However, in some tumours from FAP patients carrying germline *APC* mutation, deletions of the wild-type *APC* allele are not observed. Deletions of the inherited mutant allele may also occur, accompanied by new somatic mutations in the remaining allele (Miyake *et al.*, 1994; Spirio *et al.*, 1998). Whatever the mechanism, more than 90% of *APC* mutations result in a truncated gene product through premature stop codon (Miyake *et al.*, 1994; Miyoshi *et al.*, 1992). Commonly used clinical assay for *APC* gene testing exploits this fact in the *in vitro* synthesized protein truncation test (Powell *et al.*, 1993). Many intracellular proteins including Wingless/Wnt signalling proteins, α-catenin, β-catenin, glycogen synthase kinase (GSK)-3β, axin and tubulin interact with the *APC* gene product and these interactions play a significant role in the adenoma- carcinoma sequence in CRC (Kinzler *et al.*, 1996).

1.1.6.2 *p53*

The *p53* has been labelled the 'guardian of the genome'. This is because *p53* can block cell proliferation in the presence of DNA damage, stimulate DNA repair and promote apoptotic cell death if repair is inefficient (Lane 1992). The *p53* gene is located on chromosome p17 and is most frequently mutated gene in human cancers (Caron de Fromentel *et al.*, 1992). It

functions as a sequence-specific DNA-binding protein and transcription factor and controls the expression of a large number of genes (Oren *et al.*, 1999; May *et al.*, 1999). The consequence of loss of p53 function is the propagation of damaged DNA to daughter cells (Carder *at al.*, 1995). *p53* mutations precede aneuploid clonal divergence though crude aneuploidy can occur independent of *p53* alterations and chromosomal aberrations have been observed in colorectal cancers with only wild-type *p53* (Reid *et al.*, 1996; De Angelis *et al.*, 1999; Cums *et al.*, 2000). Furthermore, a mutant *p53* has been found in chromosomally stable cell lines (Eshelman *et al.*, 1998). This is because not all the mutations have similar downstream effects and various types of mutations can result in the development of different tumour phenotypes. For example, the missense mutations may result in functional inactivation of *p53* and oncogenic viral or cellular protein interaction may also result in loss of function (Momand *et al.*, 1992; Mietz *et al.*, 1992). The alteration in *p53* or 17p allelic loss has been reported in 4-26 % of adenomas, in 50 % of invasive foci within adenomatous polyps and in 50-75 % of adenocarcinomas (Vogelstein *et al.*, 1988; Hardingham *et al.*, 1998; Scott *et al.*, 1993; Rashid *et al.*, 1999; Darmon *et al.*, 1994; Ohue *et al.*, 1994; Yamaguchi *et al.*, 1994; Kaklamanis *et al.*, 1993; Kaserer *et al.*, 2000; Boland *et al.*, 1995). Mutant *p53* has an abnormally long half-life due to resistant to proteolysis by mdm-2--ubiquitin and result in the accumulation of mutant *p53* in the tumour cells. This accumulated protein can be detected by immunohistochemistry techniques (Midgley *et al.*, 1997; Rodrigues *et al.*, 1990). The mutations and functional inactivation of *p53* protein is an essential initiating step in the transition from adenoma to carcinoma.

1.1.6.3 18q loss

The allelic loss at chromosome 18q is the second common alteration and occurs in approximately 70 % of CRC cases. It occurs in 10-30 % of early adenomas and may rise to 60 % in late adenomas (Vogelstein *et al.*, 1988; Boland *et al.*, 1995). A tumour suppressor gene 'Deleted in colorectal cancer' *(DCC)* gene was initially thought to be the gene involved but a component of a receptor complex that mediates the effects of netrin-l is now considered as the gene involved (Fearon *et al.*, 1990; Keino-Masu *et al.*, 1996). The role *DCC* have been questioned after the experiments with mouse homologue failed to support a tumour suppressor function for *DCC* (Fazeli *et al.*, 1997) and because mutant alleles of *DCC* are rarely seen in colorectal tumours showing 18q allelic loss (Cho *et al.*, 1994). The other two tumour suppressor genes *SMAD2* and *SMAD4* have also been identified in this region (Hahn *et al.*, 1996). The protein products of these genes are the regulators of the inhibitory

transforming growth factor (TGF-β) signalling pathway, which regulates cell growth, differentiation and apoptosis (Heldin *et al.*, 1997; Duff *et al.*, 1998). Several human cancers including colorectal carcinomas carry mutations in *SMAD2* and *SMAD4* (Riggins *et al.*, 1997; Riggins *et al.*, 1996, 1997; Thiagalingam *et al.*, 1996; Eppert *et al.*, 1996). The deletion of *SMAD4* in human CRC cell lines is reported to disrupt TGF-β signalling (Zhou *et al.*, 1998; Xu *et al.*, 2000). Germline mutations of *SMAD4* are thought to be prevalent in sporadic adenomas (Howe *et al.*, 1998).

1.1.7 Oncogene-*K-ras*

Approximately 50% of colorectal tumours carry *K-ras* mutations. The *K-ras* is small GTP-binding protein and mediates the early events in the signal transduction pathways of many mitogenic stimuli. The oncogene *K-ras* controls signal pathways from extracellular growth factors to regulate cell cycle and cell proliferation. (Rommel *et al.*, 1998; Pronk *et al.*, 1994). Mutations in *K-ras* lead to constitutive activation and unregulation of signals mimicking continuous growth stimulatory response. This inappropriate signal transduction from the mutant ras protein is responsible for the neoplastic change. The activating point mutations occur at codons 12, 13 and 61 in *K-ras* and are associated with up-regulation of DNA methyltransferase, cyclin D1, and gastrin. (Guan *et al.*, 1999; Aktas *et al.*, 1997; Nakata *et al.*, 1998). The *K-ras* mutations are observed in 90 % of nondysplastic or hyperplastic aberrant crypt foci and 57 % of dysplastic or adenomatous aberrant crypt foci (Takayama *et al.*, 1998). It has been suggested that activating *K-ras* mutations are responsible for the development of up to 30 % of human cancers (Forrester *et al.*, 1987; Bos *et al.*, 1987). The evidence for *K-ras* in carcinogenesis comes for the experiments in which transfection of mutated *K-ras* genes, resulted in neoplastic transformation of those cells (Barbacid *et al.*, 1987). *K-ras* gene mutations have been observed in 50% of colorectal carcinomas and small adenomas (Forrester *et al.*, 1987; Bos *et al.*, 1987; Barbacid *et al.*, 1987; Vogelstein *et al.*, 1987). These mutations commonly occur in the transition of small adenomas to larger more dysplastic tumours but may initiate neoplasia in a subset of colorectal tumours. Mutations of *K-ras* genes occur early in the adenoma to carcinoma sequence and adenomas with these mutations are more likely to progress to carcinoma than adenomas without *K-ras* gene mutations (Fearon *et al.*, 1990). In contrast to these findings, *K-ras* mutations are also prevalent in colonic lesions with little or no malignant potential (Pretlow *et al.*, 1993). Thus, the synergistic action of the mutated *APC* and *K-ras* genes seems to be necessary for clonal expansion and dysplasia in the colorectal tumour. However, up to 50 % of

colorectal cancers do not have *K-ras* mutations, suggesting that mutations in other yet unknown oncogenes may be responsible in a substantial proportion of cases. In the absence of the activation of *K-ras,* other factors such as epigenetic mechanisms may promote *APC*-driven carcinogenesis.

1.1.8 The DNA mismatch repair pathway

Microsatellite instability (MSI) has recently been described in colorectal tumorigenesis. Microsatellites are a type of DNA that consists of tandem repeats, usually between one and five base pairs, repeated many times (Wheeler *et al.*, 2000). Thousands of microsatellites are found interspersed throughout the human genome and are particularly prone to errors during DNA replication. The mismatch repair (MMR) proteins will ordinarily repair such errors but if MMR is defective, these microsatellites errors accumulate (Wheeler *et al.*, 2000). In case of defective MMR, not only microsatellites error will replicate but also all the nucleotide repeat sequences at important coding regions of key regulatory genes will replicate. Hence, MSI can be interpreted as a 'mutator phenotype (Parsons *et al.*, 1993; Loeb *et al.*, 1991). Mutations in MMR involving five different genes (*hMSH2, hMSH6, hMLH1, hPMS1*, and *hPMS2*) have now been discovered in HNPCC (Chung *at al.*, 1995), but the most commonly implicated genes are *hMSH2* or *hMLH1* which occur in as many as 45%-70% of HNPCC families (Wijnen *et al.*, 1998; Liu *et al.*, 1996). These five genes encode a protein involved in DNA mismatch repair. These genes complex with each other to complete repair and different combinations of the genes have been reported. The *MSH2* complexes with *MSH6* to correct single base pair mismatches and for larger base pair insertions or deletions *MSH2* complexes with *MSH3* (Kolodner *et al.*, 1996). The mismatched nucleotide is then excised from the newly synthesised DNA strand by *MLH1* and *PMS2* gene products. A defective MMR system fails to correct errors in DNA replication and displays a mutator phenotype. A consensus panel of five mononucleotide and dinucleotide microsatellite markers for MSI testing has been proposed recently. Tumours can be classified as demonstrating high frequency *MSI* (MSI-H, 2 markers unstable), low frequency *MSI* (MSI-L, 1 marker unstable), or microsatellite stability (MSS, 0 markers unstable) based on this system (Boland *et al.*, 1998). Up to 15% of sporadic colorectal tumours and virtually all of tumours in HNPCC display MSI. But these MSI may not correspond with somatic mutations and only 4 %–26 % of these tumours have somatic mutations in *hMSH2* and only 6 %–10 % of sporadic cases with MSI carry germline mutations in *hMSH2* or *hMLH1* (Borresen *et al.* 1995; Bubb *et al.*, 1996; Liu *et al.*, 1995; Wu *et al.*, 1997). For MSI-H, phenotype

inactivation of both alleles of *hMSH2* or *hMLH1* is necessary. This inactivation can occur through point mutations or loss of heterozygosity (LOH) of *hMSH2* or *hMLH1* (Liu *et al.*, 1995). The other suggested mechanisms are the epigenetic mechanisms as *hMLH1* can be silenced through hypermethylation of CpG islands in its promoter. Hypermethylation of the *hMLH1* promoter is observed in 80 % of MSI-positive sporadic colorectal tumours (Herman *et al.*, 1998). Further evidence for this hypothesis comes from the study in which both alleles of *hMLH1* were hypermethylated in five of six MSI-positive colon cancer cell lines with no identifiable mismatch repair gene mutations. In addition, the demethylating agent 5-azacytidine was able to restore the expression of both *hMLH1* alleles as well as functional mismatch repair activity (Herman *et al.*, 1998; Veigl *et al.*, 1998).

1.1.9 Epigenetic mechanisms

As described above colorectal tumorigenesis results from mutations in tumour suppressor genes and oncogenes but other processes such as DNA methylation or epigenetic pathways may also play a significant role. Epigenetic mechanisms are involved in genomic imprinting in normal embryonic development but its role in tumorigenesis is now being discovered (Razin *et al.*, 1994; Li *et al.*, 1992; Counts *et al.*, 1995; Jones *et al.*, 1996). The DNA from both benign and malignant colonic tumours is found to display hypomethylation. DNA methyltransferase is an enzyme responsible for the methylation of the DNA. Carcinogenic properties of 5-azacytidine which causes hypomethylaton of the DNA through inactivation of DNA methyltransferase have been reported (Goelz *et al.*, 1985; Feinberg *et al.*, 1988; Landolph *et al.*, 1982). By contrast overexpression of DNA methyltransferase can also cause neoplastic transformation in mouse and high levels of DNA methyltransferase have been found in colorectal adenomas and carcinomas and the reduction of DNA methyltransferase activity inhibits adenoma formation in mice (el-Deiry *et al.*, 1991; Wu *et al.*, 1993; Laird *et al.*, 1995). Hypermethylation of specific sequences of DNA in the promoter region of several tumour suppressor genes is associated with transcriptional silencing of these genes (Herman et *al.*, 1995; Herman *et al.*, 1998; Veigl *et al.*, 1998; Wheeler *et al.*, 1999; Herman *et al.*, 1994; Wheeler *et al.*, 2001; Esteller *et al.*, 2000). Hypermethylated promoter region of *APC* gene with loss of *APC* expression is found in 18 % of sporadic carcinomas and adenomas (Esteller *et al.*, 2000). Hypermethylation of DNA has also been reported in association with MSI, particularly of the *hMLHl* gene in sporadic colorectal cancer (Herman *et al.*, 1998; Wheeler *et al.*, 1999; Ahuja *et al.*, 1997). These data underline the importance of the methlyation status of DNA of the genes involved in CRC.

1.1.10 Chemoprevention of Colorectal Cancer

Chemoprevention is defined as the use of synthetic or natural compounds to prevent the development of benign or malignant tumours (Hakama *et al.*, 1998). Colorectal cancers result from complex interactions between environmental factors and genetic predisposition that occur during multistage process of carcinogenesis. Screening of CRC with faecal occult blood testing and surveillance endoscopies have been employed for early detection and treatment of the tumour. However, the difficulty with the surveillance programmes is the cost, the compliance and the invasiveness of the procedures. Another problem is that flat and depressed tumours are more common and are difficult to detect endoscopically. These programmes detect tumour, which has already developed or is developing. Hence, the importance of the strategies against prevention of CRC cannot be overemphasized. There is significant interest in chemoprevention for colorectal cancer and the list of agents with potential chemoprotective effects in CRC have been growing over the past decade. Some of these agents are briefly discussed.

1.1.10.1 Non-steroidal anti-inflammatory drugs

Many epidemiological studies have reported significantly lower rates of colorectal cancer with the use of aspirin and other non-steroidal anti-inflammatory drugs (NSAIDs). Some retrospective and prospective studies have reported 30-50 % reductions in the rates of colorectal cancer in humans with a regular intake of aspirin or NSAIDs (Giovannucci *et al.*, 1994, 1995; Kune *et al.*, 1988; Muscat *et al.*, 1994; Smalley *et al.*, 1999; Thun *et al.*, 1991; Janne *et al.*, 2000). One such study of 635 patients with a previous history of colorectal cancer, randomized to receive either 325 mg of aspirin daily or placebo, reported a significantly reduction in the risk of adenoma development in the aspirin-treated group (Sandler *et al.*, 2003*)*. A variety of potential mechanisms have been suggested by which NSAIDs might act in the prevention of CRC. One of the proposed mechanism is the induction of apoptosis by the NSAIDs and it has been suggested that they may exert their chemopreventive effects in colon cancer by restoring the defective apoptotic mechanisms in the CRC to normal (Chan *et al.*, 2002). Cyclooxygenase (COX) is involved in the metabolism of arachidonic acid and the production of prostaglandins. Arachidonic acid has been shown to be a critical signal for apoptosis in cancer cells. Aspirin and NSAIDs act by inhibiting the COX enzymes. Two isoforms of COX have been recognized. COX-1 is constitutively active, is responsible for protecting upper gastrointestinal mucosa from injury, whereas COX-2 is an inducible isoform, and is expressed at sites of inflammation (Smith *et*

al., 1996). COX-2 is not ordinarily expressed in normal colorectal epithelium but high levels area detected in neoplastic colonic mucosa. Overexpression of COX-2 has been associated with the progression of colonic tumours (Cao *et al.*, 2000). NSAIDs in general are non-selective COX inhibitors and inhibit the activity of both isoforms and hence the metabolism of arachidonic acid through COX-dependent pathways. This allows accumulation of arachidonic acid and triggers apoptosis in the tumour cells. The major issue with NSAIDs and aspirin use in chemoprevention in CRC is its potential toxicity in long-term use.

1.1.10.2 Cyclooxygenase inhibitors

By contrast, with NSAIDs which inhibit both the isoforms of COX, other agents with selective COX-2 inhibitory effects have recently been developed and these selective COX-2 inhibitors have the potential as chemoprotective agents in CRC. As COX-2 is thought to play a key role during the early stages of polyp formation, the selective COX-2 inhibitors may have a protective role against the development of theses polyps (Ota *et al.*, 2002). One such selective COX-2 inhibitor in clinical use is Celecoxib. Celecoxib has shown promise as it decreased the mean number of polyps by 28 % in patients with familial adenomatous polyposis (Steinbach *et al.*, 2000).

A number of natural substances such as curcumin (from turmeric), Resveratrol (from grapes) and the omega-3 fatty acids (from oily fish) have recently been emerging as chemopreventitive agents in the CRC. All these substances are found to have COX-2 inhibitory properties (Goel *et al.*, 2001; Subbaramaiah *et al.*, 1999; Dommels *et al.*, 2003). These substances are reported to have growth inhibitory effects in human CRC with the added advantage of safety. One study with omega-3 fatty acids has shown up to 90 % inhibition of tumour growth in human colon carcinoma xenografts in athymic mice (Kato *et al.*, 2002).

1.1.10.3 Ursodeoxycholic acid

Ursodeoxycholic acid (UDCA) is the tertiary bile acid that is primarily used in cholestatic liver diseases. Recently it has shown promise as chemoprotective agent in CRC. UDCA is an antioxidant that stabilizes the mitochondrial membrane preventing oxidative injury to DNA (Mitsuyoshi *et al.*, 1999). Rats with normal liver function and normal colons that are fed ursodiol, reported a significant reduction in carcinogen-induced adenomas and colon cancers compared with rats fed cholic acid (Wali *et al.*, 1995; Ikegami *et al.*, 1998). In a recent study in humans, 52 patients with ulcerative

colitis and primary sclerosing cholangitis were found with a relative risk of 0.26 (95% CI, 0.06-0.92; $P < 0.03$) for the development of colorectal dysplasia or cancer in the ursodeoxycholic acid-treated group. (Pardi *et al.*, 2003). Another study found a significantly reduction in risk of adenoma recurrence in patients with primary biliary cirrhosis undergoing colonoscopic surveillance and taking ursodeoxycholic acid (Serfaty *et al.*, 2003). The mechanisms involved in the chemoprotective effects are not fully understood .One reported potential mechanism for this chemopreventive effect of ursodeoxycholic acid is the dilution of secondary bile acid, deoxycholic acid in the colon (Batta *et al.*, 1998; Rodrigues *at al.*, 1995). A major multi-centre trial is now underway to determine whether ursodeoxycholic acid can prevent sporadic adenoma formation in humans (Brentnall 2003).The role of UDCA in CRC will be discussed in detail in later sections.

1.1.10.4 Folic acid

Folic acid has been used to prevent neural tube defects during pregnancy. Long-term use of folic acid is associated with the reduction in the risk of colorectal adenomas and cancer. A large European case-control study found a significantly reduced risk of colorectal cancer when comparing the highest versus the lowest intakes of folic acid (La Vecchia *et al.*, 2002). In the Nurses Health Study, effects of dietary intake of folic acid were analysed in 88, 756 women who were free of cancer in 1980. Updated dietary assessments, including multivitamin use were assessed for 1980-1994 (Giovannucci *et al.*, 1998). It was found that women who took regular multivitamins supplements containing folic acid for 15 years had the greatest reduction in the risk of developing colonic cancer, with a relative risk of 0.25 (95% CI, 0.13-0.51; $P = 0.0003$). Interestingly no effect was observed in the risk of rectal cancer with long-term multivitamin use. However, this was an observational rather than a randomized study, and therefore the results cannot definitely be attributed to folate. In another study the effects of folate intake in 295 cases of CRC and 5334 randomly selected controls from 56, 837 women, were investigated between 1980 and 1985. (Terry *et al.*, 2002). A 40% lower risk of CRC was observed amongst women in the highest compared with the lowest quintile level of folate intake (Terry *et al.*, 2002).

1.1.10.5 Hormone-Replacement Therapy

During the past 20 years, a slight reduction in mortality from colorectal cancer in women is attributed to the increasing use of postmenopausal hormone-replacement

therapy (HRT). The hypothesis is that the oestrogens in HRT may protect against colorectal cancer by reducing the production of, bile acids and insulin-like growth factor 1. It may also exert direct effects on the colorectal epithelium. The Cancer Prevention Study II found a significant decrease in mortality from colon cancer with the use of hormone-replacement therapy (Calle *et al.*, 1995). The effect was stronger in women currently receiving HRT therapy and in those who had received continuous therapy for more than 11 years. The beneficial effects of HRT were observed in larger adenomas, suggesting that it probably acts at later stages of CRC.

1.1.10.6 Dietary fibre

Dietary fibre is not a chemoprotective agent by definition, but has the potential to reduce the risk of development of colorectal cancer. The mechanisms described for dietary fibre action in CRC include dilution of potential carcinogens, binding potential carcinogens in the intestinal lumen and promoting a favourable colonic microflora (Kim 2000). Another mechanism is production of short-chain fatty acids, such as butyrate by the fermentation of dietary fibre by colonic bacteria, which is shown to have anti-carcinogenic properties (Kim 2000). A large study investigated the association between dietary fibre intake and the incidence of colorectal cancer in 519, 978 individuals from 10 European countries. Participants were followed up for CRC incidence. Follow-up for an average 4.5 years reported 1065 cases of colorectal cancer in the cohort. An inverse relation was found between intake of dietary fibre and the incidence of large bowel cancer (Bingham *at al.*, 2003). The meta-analyses of case-control studies have revealed a 50 % reduction in the risk of development of colorectal cancer in individuals with the high fibre intake (Kim 2000). However, prospective cohort studies have revealed equivocal results with dietary fibre intake (Fuchs *et al.*, 1999; Giovannucci *et al.*, 1994; Steinmetz *et al.*, 1994). In prospective, randomized controlled trials, wheat-bran fibre supplementation was found to have no protective effect on the incidence of recurrent adenomas (Alberts 2000; MacLennan *et al.*, 1995; McKeown-Eyssen *et al.*, 1994).

1.1.10.7 α-difluoromethylornithine

The naturally occurring polyamines putrescine, spermidine and spermine are necessary for normal cellular proliferation and these compounds are found in increasing concentrations in rapidly growing tissues (Tabor *et al.*, 1984; Pegg *et al.*, 1982). The biosynthesis of polyamines starts with the decarboxylation of ornithine to form the diamine putrescine. The enzyme, ornithine decarboxylase (ODC) catalyses this rate

limiting step (Saydjari *et al.*, 1989). Elevated activity of ODC has been found in the colonic mucosa in patients with familial adenomatous polyposis (Luk *et al.*, 1984). Significantly, high ODC activity has also been reported in the mucosa of sporadic colorectal adenomas and carcinomas (Berdinskikh *et al.*, 1991; Hixson *et al.*, 1993; LaMuraglia *et al.*, 1986; Lawson *et al.*, 1989; Linsalata *et al.*, 1993; Narisawa *et al.*, 1989; Porter *et al.*, 1987; Porter *et al.*, 1987; Rozhin *et al.*, 1984; Freitas *et al.*, 2002). α-Difluoromethylornithine is an enzyme-activated, irreversible inhibitor of ODC and results in the depletion of intracellular levels of putrescine and its derivative spermidine (Pegg *et al.*, 1988). Thrombocytopenia and reversible audiotoxicity are the major dose-limiting toxic side effects of α-difluoromethylornithine (Abeloff *et al.*, 1984). Phase II chemoprevention trials are underway using α-difluoromethylornithine with sulindac and celecoxib in patients with familial adenomatous polyposis (Gwyn *et al.*, 2002).

1.1.10.8 Epidermal growth factor receptor inhibitors

The epidermal growth factor receptor (EGFR) is involved in signal transduction pathways leading to cell proliferation, differentiation, migration, adhesion, resistance to apoptosis and enhanced survival (Ritter *et al.*, 2003). High levels of epidermal growth factor and transforming growth factor-α are found in many human colon cancer cells. Furthermore, up to 70% of colonic carcinomas are reported to overexpress EGFR (O'dwyer *et al.*, 2002). Both TGF-α and EGF bind to EGFR and play an important role in the growth of colon cancer. There is considerable interest in EGFR as a target for drug therapy as its inhibition has a potential in both the treatment and prevention of solid neoplasia (Ritter *et al.*, 2003). The possibility of antibody against EGFR and EGFR-tyrosine kinase inhibitors have been explored (Sirotnak *et al.*, 2003). A selective and reversible inhibitor of EGFR, Gefitinib (ZD1839, Iressa) that inhibits the binding of adenosine triphosphate required for kinase activation is the potential chemoprotective agents with anti-EGFR activity (Dancey *et al.*, 2003). Anti-tumour activity of ZD1839 against human colon carcinoma xenografts in mice has been reported (Douglass *et al.*, 2003). Results from phase I and II clinical trials have shown clinical efficacy and good tolerability with ZD1839, in patients with advanced colorectal disease and further trials are ongoing to explore its role in the CRC prevention (Douglass *et al.*, 2003).

1.1.10.9 Selenium

Selenium is an essential trace element of plant origin, found in bread, cereals, fish, poultry and meat (Rayman *et al.*, 1997, 2000). It is used as selenomethionine for

chemoprevention against CRC (Ganther *et al.*, 1999). The glutathione peroxidase enzymes that reduce free radicals are dependent on selenium for their acitivity (Rayman *et al.*, 1997, 2000). Recently, it was shown that selenomethionine could activate the DNA repair branch of the *p53* pathway (Seo *et al.*, 2002). Epidemiological studies have provided evidence for an inverse relation between selenium intake and cancer mortality (Rayman *et al.*, 2000). In animal models of colorectal carcinogenesis, selenium compounds have been shown to inhibit the development of adenocarcinomas (Reddy *et al.*, 2000). Clark *et al.* (1996) undertook a chemoprevention trial in 1312 patients with previously resected non-melanoma skin cancers using 200 μg of selenium daily. The total cancer incidence was significantly lower in the selenium group versus placebo and there was a significant reduction in the risk of developing colorectal cancer in the selenium group (relative risk, 0.42; 95% CI, 0.18-0.95; $P = 0.03$).

1.1.10.10 Calcium

The dietary nutrient calcium is reported to have potential anticancer properties in CRC. It is hypothesized that calcium binds to toxic bile acids in the colon, inhibiting their proliferative and carcinogenic effects. Calcium supplementation has been reported to reduce carcinogen-induced colonic epithelial hyperproliferation and the formation of tumours in animal models (Newmark *et al.*, 1984). Some studies in humans have shown decreased proliferation of colorectal epithelial cells and decreased faecal water cytotoxicity with calcium supplements. In one of the study, 930 patients with a history of colorectal adenomas were randomly assigned to receive either daily supplementation with 3 g of calcium carbonate or placebo. The results were obtained with serial endoscopic examination at 1 and 4 year intervals. A moderate but significant reduction (relative risk, 0.85; 95% CI, 0.74-0.98; $P = 0.03$) in the development of further adenomas in the group was found (Baron *et al.*, 1999).

1.2 Bile Acids

1.2.1 Background and Structure

Bile acids are metabolites of cholesterol and detergents for the absorption of fat and fat-soluble vitamins. The primary bile acids cholic acid (CA) and chenodeoxycholic acid (CDCA) are metabolised in the liver and conjugated with glycine or taurine and secreted in the bile (**Fig. 1.2**). Cholesterol 7α-hydroxylase (CYP7a1) and sterol 12α-hydroxylase (CYP8b1) are the rate-limiting enzymes involved in the synthesis of bile acids. The ratio of CA and CDCA determines the hydrophobocity of the circulating bile

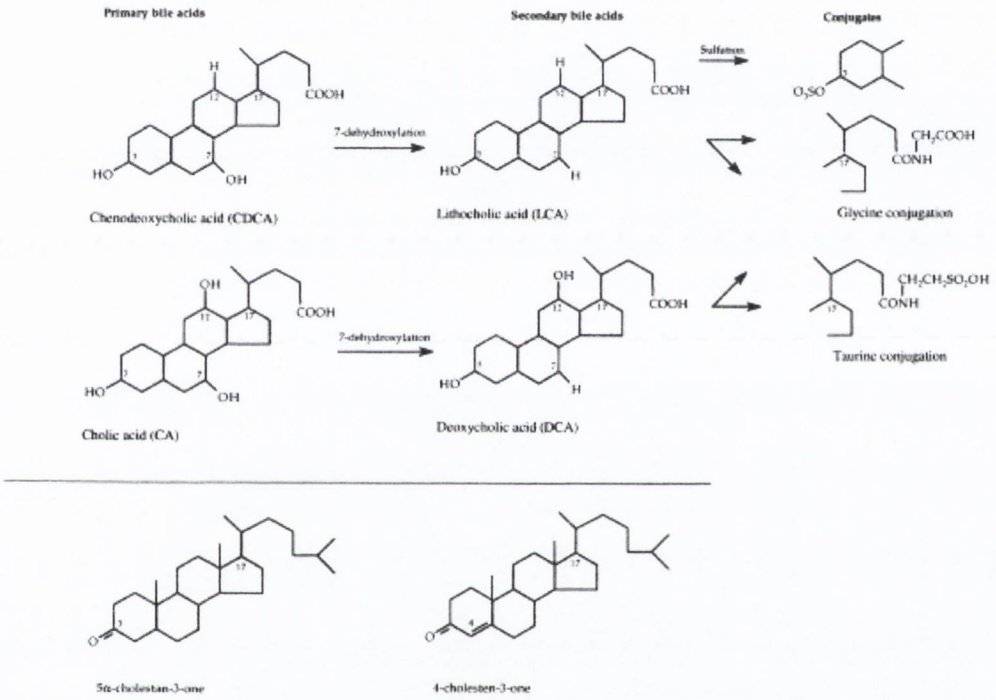


Fig. 1.2 Chemical structures of primary, secondary and tertiary bile acids that are present in human bile and of their amino acid (R) conjugants. (Reproduced from de Kok *et al.*, 2000)

acid (Vlahcevic *et al.*, 2000; Gustafsson 1999). Up to 90% of the secreted bile acids is absorbed in the small intestine. Active transport involving the Na^+-coupled co-transport across the brush-border by the Na^+K-ATPase at the membrane is involved in the uptake of unconjugated bile acids. Passive diffusion of the bile acids in mixed micelles of bile acids, fatty acids and monoglyceride also takes place and depends upon hydrophobocity. About 15 % of the secreted bile acids reach the colon. The anaerobic bacterial flora in the colon helps deconjugation of conjugated CA forming the secondary bile acids such as deoxycholic acid (DCA) and 7α-dehydroxylation of CDCA results in the formation of lithocholic acid (LCA). Subsequent epimerization of CDCA results in the formation of tertiary bile ursodeoxycholic acid (UDCA) and sulphation of LCA results in the formation of sulpho-LCA (SLCA). Approximately 10% of the secreted bile acids, mainly the deconjugated dihydroxy bile acid DCA, are reabsorbed in the colon through passive diffusion and about 2-5% of the secreted bile acids, are excreted in the stool. Absorption of bile acids occurs solely into portal blood resulting in a concentration of 0.01-0.02 mmol/l. This completes the enterohepatic circulation, which is the reuptake of most of the bile acids by the liver for re-use. The total amount of the circulating bile acid pool contains about 30-40 % CA and CDCA, 20-30 % DCA and <5% of LCA. The bile acid pool depends upon the number of meals per day and the type of meals (Nagengast *et al.*, 1995; Moseley 1999; Hofmann 1992). A negative feedback loop controls the synthesis of bile acids and reabsorbed hydrophobic bile acids inhibit the transcription of the enzymes CYP7a1 and CYP8b1 by binding to the nuclear farnesoid X receptor (FXR) (Vlahcevic *et al.*, 2000; Gustafsson *et al.*, 1999). Cholesterol in diet enhances transcription of CYP7a1 through liver X receptor (LXR) (Gustafsson 1999; Kliewer *et al.*, 1999). The major components of the faecal bile acids are DCA and LCA.

1.2.2 Bile acids and cancer

Bile acids have long been implicated in the pathogenesis of CRC. They resemble the carcinogenic polycyclic aromatic hydrocarbons in structure. Cook and Kennaway reported in 1932, bile acids as aetiological agents in the process of carcinogenesis. This hypothesis was strengthened by the observation that DCA caused "malignant spindle-celled tumours" upon subcutaneous injection into mice (Cook *et al.*, 1940). Bile acids were initially considered as mutagens or co-carcinogens but they were unable to cause tumours in animal models administered without a carcinogen (Narisawa *et al.*, 1974;

Reddy *et al.*, 1977). A number of studies have reported a link between bile acids and colorectal cancer. In rat intrarectal instillation of bile acids, such as CA, CDCA, DCA and LCA increased the development of colorectal adenomas and adenocarcinomas (Narisawa *et al.*, 1974; Reddy *et al.*, 1977). The populations where the incidence of CRC is higher, were found to have higher concentrations of faecal steroid and secondary bile acids (Hill *et al.*, 1971). Patients with colorectal adenomas had higher faecal and serum levels of DCA than control (Bayerdorffer *et al.*, 1993; Kishida *et al.*, 1997; Owen *at al.*, 1997). Patients who had cholecystectomy were found to have higher risk of CRC as cholecystectomy results in a higher faecal loss of bile acids (McMichael *et al.*, 1985). Additionally bile acid-specific binding sites have been identified in mouse and human colorectal cancers (Summerton *et al.*, 1983, 1985).

1.2.3 Bile acids as tumour promoters

Exposure of cells to carcinogens such as dimethylhydrazine or *N*-methyl-*N*'nitro-N-nitrosoguanidin with bile acids supplements have demonstrated the tumour promoting effect of secondary bile acids (Narisawa *et al.*, 1974). Co-mutagenicity to known colon carcinogens has been reported in microbial test systems as the Salmonella mutagenicity assay and in animal models (Wilpart *et al.*, 1983; Silverman *et al.*, 1977; Wilpart *et al.*, 1986; Kelsey *et al.*, 1979; Nigro *et al.*, 1973; Narisawa *et al.*, 1974; Reddy *et al.*, 1976; Cohen *et al.*, 1980; Martin *et al.*, 1981). A number of researchers have demonstrated tumour promoting effects of the secondary bile acids (Reddy *et al.*, 1977; McSherry *et al.*, 1989). Increased cell proliferation rates of the colon epithelium are reported after intrarectal instillation of bile acids and fatty acids (Kelsey 1979; Deschner *et al.*, 1981; Bull *et al.*, 1983; Wargovich *et al.*, 1983; Bird *et al.*, 1986). The detergent action of bile acids is thought to cause cell lysis and alteration in membrane structure of the colon with resultant compensatory cell-proliferation and tumour formation by other mutagens. In animal studies, feeding rats with bile acids caused increased cell proliferation in colonic crypt cells in azoxymethane treated rats (Hori *et al.*, 1998). DCA is known to induce the proto-oncogene, *c-fos* that regulates cell proliferation and differentiation and caused hyperproliferation rat colon (Velazquez *et al.*, 1996). Excessive apoptosis in colon mucosa cells by the bile acids may result in apoptosis resistant cells. This results in damaged DNA being replicated in the daughter cells leading to mutation and neoplasia (Payne *et al.*, 1995, 1998). Feeding rats a diet supplemented with DCA induced

colonocyte proliferation and though it contrasts with other studies that that bile salts do not act as direct mitogens on colonic epithelial cells (Barone *et al.*, 2002).

1.2.4 Mechanisms of Bile Acids in Cancer

1.2.4.1 Bile acids and DNA Damage

Bile acids were initially thought to be mutagens or carcinogens in CRC. Later it was reported that bile acids act as tumour promoters during the later stages of the adenoma carcinoma sequence rather than mutagens. Direct mutagenicity of several bile acids in the standard Ames/Salmonella mutagenicity assay showed negative results (Silverman *et al.*, 1977; McKillop *et al.*, 1983; MacDonald *et al.*, 1978). It has been proposed that even though bile acids reveal only weak mutagenic effects, continuous exposure of the gut to bile acids might still increase the risk of CRC with age. Using a M13 transfection assay, the transfection efficiency of single stranded circular DNA of phage M13 declined strongly after interaction with bile acids, suggesting that bile acids CDCA and DCA are DNA-damaging faecal compounds (Cheah *et al.*, 1990). It was reported that single strand breaks in DNA could result after treatment of L1210 cells with LCA. The incubation of isolated naked DNA with LCA did not induce DNA breaks suggesting that bile acid either induced a nuclease or were converted into another metabolite, which could damage DNA (Kulkarni *et al.*, 1982, 1985). Bile acids have been reported to induce the formation of oxygen free radicals and also induce NF-κB (Craven *et al.*, 1986; Blakeborough *et al.*, 1989). Both these responses may be protective against oxidant stress-induced apoptosis and result in DNA damage by the bile acids. We will explore the role of DCA on NF-κB and compare it with UDCA and examine if UDCA has different effects on this transcription factors and its antiapoptotic effects reported in previous studies.

1.2.4.2 Bile acids and colonic epithelial cells

Bile acids may damage the colonic epithelium with effects on the protective barriers against toxic substances. Mucin secretion by the colonic epithelial cells protects it from faecal toxins and other potentially carcinogenic substances. Bile acids influence mucin production by colonic cells, and thereby predispose the colonic epithelium to other faecal toxicants exposure (Shekels *et al.*, 1996). The production of mucin by HT-29 and Caco-2 colon cancer cells was significantly decreased by non-toxic concentrations of DCA. Another substance, alkaline sphingomyelinase (SMase) is a brush border enzyme

and an important inhibitor of tumorigenesis. It hydrolyzes sphingomyelin, which in turn generates ceramide, reported to inhibit cell proliferation and to induce apoptosis. The sphingomyeline and the alkaline SMase signalling in cancer development has been described (Merrill *et al.*, 1995). Conjugated bile acids dissociate alkaline SMase from rat intestinal mucosa and decrease its levels (Duan *et al.*, 1998).

1.2.4.3 Bile acids and calcium

Calcium plays an important role in colonic cell homeostasis and cell signalling. The conjugated bile acids are reported to increase the intracellular Ca^{2+} concentrations, bypassing the regulatory systems that maintain cellular Ca^{2+} homeostasis (Zimniak *et al.*, 1991). Calcium regulates PKC signalling and this may be one of the mechanism by which bile acids increase the risk of CRC.

1.2.4.4 Bile acids and cell proliferation

Colon cancer results from histopathologic changes in the colonic mucosa, which eventually transform normal mucosa into invasive carcinoma. This multistep process is accompanied by a number of specific genetic changes. The first stage of these histopathological changes is the conversion of normal epithelium into hyperproliferative epithelium followed by the formation of aberrant cryptic foci then small adenoma, large adenoma, carcinoma in situ and, finally, invasive and metastatic cancer (Debruyne *et al.*, 1999; Kinzler *et al.*, 1996; Janne *et al.*, 2000; Hanahan *et al.*, 2000). Bile acids play an important role in these colonic mucosal alterations. DCA is reported to induce proliferation in human colonic biopsies *in vitro* (Bartram *et al.*, 1993). Studies have demonstrated that colonic epithelial proliferation rates in human colonic biopsies positively correlate with serum levels of DCA (Ochsenkuhn *et al.*, 1999). Mice treated with LCA enema and given concomitant dimethylhydrazine injections showed suppressed apoptosis and increased proliferation of colonic epithelium (Kozoni *et al.*, 2000). Rats injected with azoxymethane and fed CA diet selectively increased the proliferation of aberrant crypts, but not of normal crypts (Corpet *et al.*, 1997). DCA and CDCA caused an increase in cell proliferation and a decrease in butyrate-induced apoptosis in AA/C1 human colonic adenoma cells in vitro (McMillan *et al.*, 2000). An enhanced proliferation rate of the colonic epithelium could result in an increased susceptibility to mutagens. Hence, tumour promotion by bile acids may result from growth stimulation of already initiated adenoma cells.

1.2.4.5 Bile Acids and cell invasion

Cell invasion and migration are critical for progression and metastases of the CRC. Bile acids have been reported to play a role in cell migration and invasion of colorectal cancer cells. Studies have demonstrated that treatment of human colorectal cancer Caco-2 cells by LCA resulted in an enhanced secretion of matrix metalloproteinase 2 (MMP-2) and an enhanced chemotaxis (Halvorsen *et al.*, 2000). Bile salts at physiologic conditions have been reported to increase cell migration after injury (Strauch *et al.*, 2003).

1.2.5 Bile acids and cell Signalling

1.2.5.1 Interaction with kinases

Bile acids have been implicated in the modulation of many important cell signalling pathways (**Fig 1.3**). The best studied of these signalling pathways is the protein kinase C (PKC) signalling cascade in colorectal cancer (Pongracz *et al.*, 1995; Huang *et al.*, 1992; Rao *et al.*, 1997; Hirano *et al.*, 1996). Evidence suggests that DCA exerts its tumour promoter effect through intracellular signalling pathways including PKC and mitogen-activated protein kinase (MAPK) (Qiao *et al.* 2000; Huang *et al.*, 1992). Growth and inflammation regulatory transcription factors AP-1 and NF-κB are regulated by PKC and MAPK activity (Qiao *et al.*, 2000). We compared and contrast the effects of DCA with UDCA on these signalling mechanisms and showed that these bile acids have differential and distinct effects on PKC NF-κB as well as AP-1 in colorectal cancer cells , hence providing one possible explanation for the chemoprotective effects of UDCA reported in many studies. Bile acids activate PKC probably through DAG or increasing calcium concentrations. Bile acids may stimulate PI-PLC to increase DAG formation, as evidenced by addition of DCA to extracts from human colon cancer tissue and by the increased DAG concentrations in cultures of rat hepatocytes that were treated with taurine-conjugated bile acids (Nomoto *et al.*, 1994; Rao *et al.*, 1997). In the absence of DAG, bile acids may increase PKC activity by facilitating its association with phospholipids (Rao *et al.*, 1997). DAG might in its turn activate the Ras/MEK/ERK kinase pathway through PKC (Sozeri *et al.*, 1992). This could then explain the activation of the extracellular signal-regulated kinases (ERKs) as observed in HCT-116 human colorectal cancer cells (Qiao *et al.*, 2000). Small G proteins are also the other targets for the bile acids. Involvement of Rho-like GTPases in

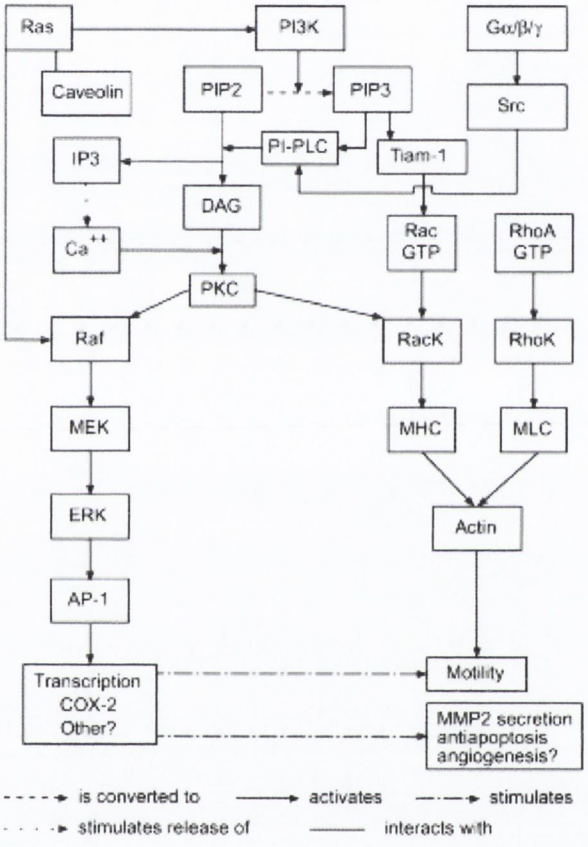


Fig. 1.3 Bile acid signalling in cancer cells. PKC and ERK activate AP-1 which targets COX-2. Activation of phosphatidylinositol 3-kinase (PI3K) is implicated upstream of phosphoinositide-specific phospholipase C (PI-PLC). Heterotrimeric G-proteins (Gα/β/γ) activate PKC through the Src tyrosine kinase. Ras and caveolins are upstream regulators of PI3K and Raf. (Reproduced from Debruyne *et al.*, 2001).

bile acid signalling pathways is suggested by the rounding of the HCT-8/E11 cells upon treatment with LCA (Scita *et al.*, 2000; Zondag *et al.*, 2000). Monomeric Rho-like GTPases regulate organization of the actin cytoskeleton and cell migration through downstream effectors as Rac- and Rho-kinase (RacK and RhoK) and myosin heavy and light chains (MHC and MLC) (Zuber *et al.*, 2000).

1.2.5.2 Bile acids AP-1

Bile acids activate the transcription factor, activator protein-1 (AP-1) proto-oncogene through PKCs and extracellular signal-regulated kinases (ERKs) in HCT-116 human colorectal cancer cells (Qiao *et al.*, 2000). The AP-1 complex activated by bile acids consists of *JunD, Fra-1* and *c-Fos*, the oncogenic components of the AP-1 and is involved in invasion pathways (Ozanne *et al.*, 2000). Another component of the AP-1 complex is the *FRA-1*, which is a target gene of the β-catenin-T cell-factor/lymphoid-enhancer-factor (TCF/Lef) complex in human colorectal carcinomas (Mann *et al.*, 1999). Increased motility and invasiveness of mammary carcinoma cells is associated with increased expression of exogenous *Fra-1* (Kustikova *et al.*, 1998). In a carcinogen-induced animal model of colorectal carcinogenesis, increased *c-Fos* expression resulted in increased mucosal hyperproliferation by DCA treatment (Velazquez *et al.*, 1996).

1.2.5.3 Bile acids and COX-2

Cyclooxygenase 2 (COX-2) is the stress response protein and the most important target gene of bile acid signalling. COX-2 may contribute to carcinogenesis in several ways. COX-2 inhibits apoptosis, stimulates colorectal cancer cells to secrete proangiogenic prostaglandins and it stimulates expression of matrix-metalloproteinases (MMP-2) involved in invasion of human colorectal cancer cells (Tsujii *et al.*, 1995, 1997, 1998). It has been shown that the dihydroxy bile acids DCA and CDCA induced a marked increase in the levels of the COX-2 mRNA and protein in oesophageal cells (Zhang *et al.*, 1998). These findings, in combination with the fact that chronic use of known COX-2 inhibitors like aspirin is associated with decreased colorectal cancer risk suggest that dihydroxy bile acid mediated induction of COX-2 may play a role in the promotion of colorectal adenomas (Gann *et al.*, 1993).

1.2.5.4 Bile acids and nuclear receptors

The nuclear receptor FXR, have been reported as targets for bile acid signalling pathway (Makishima *et al.*, 1999; Parks *et al.*, 1999; Wang and Chen 1999). This FXR

receptor functions as a heterodimer with the retinoid X receptor (RXR) and is capable of binding bile acid responsive elements in the promoter of some genes (Makishima *et al.*, 1999). Differences in potency to stimulate invasion of colorectal cancer cells between different bile acids might be related to the absence of bile acid transporters, which are necessary for the uptake of conjugated bile acids, or to preferential recruitment of specific nuclear receptor coactivators (Wang and Chen 1999).

1.3 Ursodeoxycholic acid

1.3.1 Background

Ursodeoxycholic acid (UDCA) was first identified in 1902 as hydrophilic dihydroxylated bile acid in the bile of the Chinese black bear and was named after this species. (Hammarsten 1901; Shoda 1927). Dried bear bile has been used for centuries as a remedy for liver disease in China on an empirical basis, with a long-standing belief that bear bile had curative properties (Beuers *et al.*, 1998). In 1936, Iwasaki was first to determine chemical structure of UDCA as 3α, 7β-dihydroxy-5 β -cholanoic acid (Iwasaki 1936). In 1954, an efficient and economical method of synthesizing UDCA was discovered (Kanazawa *et al.*, 1995). The Tokyo Tanabe Pharmaceutical Company launched it as a choleretic in 1957 (Makino *et al.*, 1998). UDCA constitutes 3 % of the total bile acid pool where it is formed by 7 β-epimerization of the primary bile acid chenodeoxycholic acid in the gut by intestinal bacteria (Hofmann 1994; Poupon 1995). Since then it is being used for various hepatic cholestatic and digestive diseases. Reports from Japan and Europe first revealed that UDCA was able to dissolve gallstones with equal efficacy to chenodeoxycholate, but with the added advantage that it was not hepatotoxic (Sugata *et al.*, 1974; Bachrach *et al.*, 1982). These initial reports prompted further studies on the use of UDCA, mainly in chronic cholestatic disorders (Poupon 1995).

1.3.2 Pharmacodynamics of ursodeoxycholic acid

UDCA is a tertiary bile acid and it is directly synthesized from cholesterol. After oral intake, UDCA is conjugated in the liver with glycine and taurine. Conjugated UDCA is secreted into the biliary tree and intestine and undergoes enterohepatic (Hofmann 1994). Approximately 30-60 % of the dose of unconjugated UDCA is absorbed by passive diffusion in the small and large intestines (Hofmann 1994; Rubin *et al.*, 1994).) At pharmacological doses (10-15 mg/kg/day) UDCA becomes the predominant bile acid in the liver and the

systemic circulation, comprising 40-60% of the circulating bile acid pool (Hofmann 1994; Rubin *et al.*, 1994). In patients with ileal resections, the absorption of UDCA is inefficient.

1.3.3 Mechanism of Action of Uodeoxycholic Aid

A number of mechanisms for UDCA action have been proposed as described below.

1.3.3.1 Dilution and mobilisation of toxic bile acids

UDCA is a hydrophilic non-toxic bile acid, and after oral administration, it becomes the predominant bile acid in serum and bile accounting for approximately 40-60% of the bile acids in the bile acid pool. UDCA does that by competitively inhibiting the ileal absorption of other endogenous bile salts (Beuers *et al.*, 1992; Batta *et al.*, 1989; Crosignani *et al.*, 1991; Stiehl *et al.*, 1990; Rudolph *et al.*, 1993). This alteration of the bile acid pool is considered as the most important mode of action of UDCA (Stiehl *et al.*, 1999). Apart from the dilution of the toxic hydrophobic bile acids, other effects of UDCA on bile acids have also been described. In cholestatic liver disease, some of the potentially toxic biliary constituents are retained in the liver and the systemic circulation. UDCA treatment is thought to improve mobilization of these toxic constituents (Poupon 1995; Reichen *et al.*, 1994). UDCA treatment is reported to lower the serum levels of endogenous bile acids and bilirubin in patients with PBC and primary scleroing cholingitis (Beuers *et al.*, 1992; Heathcote *et al.*, 1994; Lindor *et al.*, 1994, 1997). It has been demonstrated that UDCA prevents cholestasis induced by more hydrophobic bile acids in rat liver (Heuman *et al.*, 1991; Kitani *et al.*, 1982; Schoelmerich *et al.*, 1990). UDCA may also stimulate the secretion of bile acids and other organic anions in isolated hepatocytes (Ohiwa *et al.*, 1993).

1.3.3.2 Antioxidant effect

Lipid peroxidation by the highly reactive oxygen species secreted by the Kupffer's cells in response to hydrophobic and toxic bile salts can damage hepatocytes (Sokol *et al.*, 1993). Ursodeoxycholic acid inhibits the activation of Kupffer's cells by toxic bile salts. Another antioxidant action of UDCA is to increase the levels of glutathione and thiol-containing proteins in the hepatocytes, hence protecting hepatocytes against oxidative injury (Mitsuyoshi *et al.*, 1999).

1.3.3.3 Cytoprotective effects of UDCA

UDCA is thought to protect hepatocytes and bile ducts against the necrosis and apoptosis caused by the hydrophobic bile acids through its direct membrane-stabilizing and anti-

apoptotic effects. UDCA protects against membrane damaging effects of more hydrophobic bile acids at millimolar concentrations (Heuman *et al.*, 1991, 1994; Galle *et al.*, 1990; Guldutuna *et al.*, 1993). However, other studies have disputed these claims and suggested that the membrane-stabilizing effects of UDCA may be the result of its effects on mixed micelle formation rather than from direct membrane interaction (Heuman *et al.*, 1996). Studies have suggested that UDCA might also protect against ethanol-induced cell injury in rats and a human Hep G2 cell line (Neuman *et al.*, 1995; Vendemiale *et al.*, 1998). In addition, UDCA has also been reported to inhibit the platelet-derived growth factor-induced fibroproliferative activity in the human fibroblasts (Peterson *et al.*, 1998).

1.3.3.4 Imunomodulator effects

In patients with primary biliary cirrhosis (PBC) and extra-hepatic cholestasis the expression of HLA class I and II antigens increases in both hepatocytes and bile ducts (Calmus *et al.*, 1990, 1992). MHC class I and II genes are directly upregulate by the hydrophobic bile acids (Hirano *et al.*, 1993; Hillaire *et al.*, 1994). UDCA attenuates the stimulatory effect of hydrophobic bile acids on MHC genes by dilution of the hydrophobic bile acids. UDCA also inhibits the production of abnormal immunoglobulins and cytokines from peripheral blood mononuclear cells (Yoshikawa *et al.*, 1992). It has been demonstrated tha UDCA inhibits interferon-γ-induced activation of MHC class II promoter through the activation of GR and significantly reduces the expression of HLA class I and II in PBC patients (Tanaka *et al.*, 1996; Taresaki *et al.*, 1991; Hirano *et al.*, 1996). The immune modulation offered by UDCA may lead to reduced T cell-mediated cytotoxicity in these patients.

1.3.3.5 Anti-apoptotic effects

Hydrophobic bile acids induce apoptosis in hepatocytes through activation of variety of pro-apoptotic pathways including activation of Fas-receptor, caspases and nuclear translocation of cathepsin B (Faubion *et al.*, 1999). UDCA also has been reported to reduce the mitochondrial membrane permeability associated with toxic bile acid levels (Lemasters 1998). UDCA has been found to reduce bile acid-induced apoptosis and cytolysis in cultured rat hepatocytes *in vitro* (Benz *et al.*, 1998; Rodrigues *et al.*, 1998). Rodrigues *et al.* (1998), demonstrated that administration of UDCA markedly inhibited apoptosis in hepatocytes and various non-hepatocyte cell lines caused by hydrophobic bile acids by 50-100 percent.

1.4 PROTEIN KINASE C

1.4.1 Background

PKC were discovered almost 20 years ago as the major cellular receptor for the phorbol ester class of tumour promoters (Castagna *et al.*, 1982). They were originally described by Nishizuka and co-workers as histone kinase activity from rat brain, which could be activated by limited proteolysis (Inoue *et al.*, 1977; Takai *et al.*, 1977). It was followed by the discovery that the PKC could also be activated by diacylglycerol (DAG), phosphatidylserine (PS), calcium and also by tumour-promoting phorbol esters such as PMA (Nishizuka 1984). PKC are lipid-dependent serine-threonine kinases, established as key elements in signal transduction involved in cell proliferation, differentiation and apoptosis. The understanding of the PKC superfamily is complex, not only because large number of isoforms are involved in the mammalian cells, but also because of their expression in a variety of cells and their role in regulating diverse functions in cells.

1.4.2 PKC structure

PKC isoenzymes consist of a single polypeptide chain that contains 20-70 kDa amino-terminal regulatory domains and approximately 45 kDa carboxy-terminal kinase catalytic domains (**Fig. 1.4**). The catalytic and regulatory domain separated by hinge region v3 which is subject to proteolysis (Kishimoto *et al.*, 1983). Depending on class of PKC iosenzyme, the regulatory domain can include a calcium binding and DAG-phorbol ester-binding site. Activation of the PKC isoenzymes requires interaction with phospholipids (Kikkawa *et al.*, 1988; Lee *et al.*, 1991). The regulatory half of PKCs contains an autoinhibitory domain called pseudosubstrate and one or two membrane-targeting motifs, the C1 domain present in all isoenzymes, and the C2 domain, present in conventional and novel PKCs. The Cl domain is defined by the presence of two repeated zinc finger motifs, Cla and Clb. Each motif has a conserved pattern of cysteine and histidine residues (H-X_{I2}-C-X_2-C-$X_{13/14}$-C-X_2-C-X_4-H-X_2-C-X_7-C) and is responsible for the coordination of two Zn ions. The C1 domain binds DAG and phorbol esters in all but atypical PKCs, which are not responsive to phorbol esters, but contain a single zinc finger motif. Mutational studies have shown that the two zinc fingers in a C1 domain are not equivalent. Mutation of a highly conserved proline residue in the C1a motif of PKC δ had little effect on responsiveness to phorbol ester, whereas mutation of the equivalent amino acid residue in C1b resulted in a 125-fold decrease in phorbol-ester-binding affinity (Szallasi *et al.*, 1996). C1 domains are not specific to PKC and occur in several mammalian proteins other than PKC. In some proteins, such as DAG kinase, the domain binds to neither PMA nor DAG (Sakane *et al.*, 1996). Other proteins activated by phorbol esters including protein kinase D (PKD), a human phorbol-ester- and DAG

stimulated protein kinase and members of the chimaerin family, the GTPase activating proteins (GAPs) for the small GTPase Rac. The C2 domain is found in the cPKCs immediately medial to the C1 domain. Like the C1 domain, the C2 domain is found to be present in many other proteins, including synaptotagmins, rabphilin-3A, phospholipases and GAPs. As many of these proteins bind phospholipid in a Ca^{2+}-dependent manner, it is assumed that the C2 domain confers Ca^{2+}/PS binding to the cPKCs. It is shown that the isolated C2 domain from PKC does indeed bind to phospholipid vesicles in a Ca^{2+}-dependent fashion (Shao *et al.*, 1996). Juxtaposed to the N-terminus of the C1 domains of the cPKCs, aPKCs and nPKCs is a sequence that retains the hallmarks of a phosphorylation site, but has an alanine at the predicted serine/threonine phosphorylation site (House *et al.*, 1987). Mutation of this region confers activity on the mutant protein, which is independent of effector (Pears *et al.*, 1990). The autoinhibitory, pseudosubstrate site interacts with the catalytic domain and is responsible for the suppression of catalytic activity.

1.4.3 PKC isoenzymes

PKC consists of a family of 12 isoenzymes and are classified according to cofactor requirements in to three classes (**Fig 1.4**). Classical PKC (cPKCs) are regulated by 1, 2-diacylglycerol (DAG), and calcium and include α, β1, β2 and γ. Novel PKC's (nPKC's) δ, ε, η, μ and θ are activated by DAG & are calcium independent. Atypical (aPKCs) ι, λ and ζ are calcium & DAG independent (Saxon *et al.* 1994, Nishizuka 1988). PKCs are involved in cell growth cell, cycle progression, differentiation and & apoptosis (Black *et al.* 2000; Clemens *et al.* 1992). A further PKC class has also been described. These are PKC-related kinases (PRKs) consisting of three members, PRKs 1-3. Like the aPKCs, PRKs are independent of calcium, DAG and phorbol esters. However, PRKl has been shown to bind to the activated RhoA GTPase, which leads to a 4-fold activation of the kinase *in vitro* (Armano *et al.*, 1996; Watanabe *et al.*, 1996). It has also been shown that the other fully cloned member of the PRK subfamily, PRK2, is also capable of binding RhoA (Vincent *et al.*, 1997), suggesting that this may be the general property of this group.

1.4.4 Regulation of PKC

1.4.4.1 Regulation by lipid messengers

PKC activation is a complex process and multiple substrates and conformational changes are involved in this process. Lipid second messengers are reported to activate PKC isoenzymes both *in vitro* and *in vivo*. Classical PKCs (cPKC) are the most studied and best understood as far as mechanism of activation of PKC is concerned. All PKCs

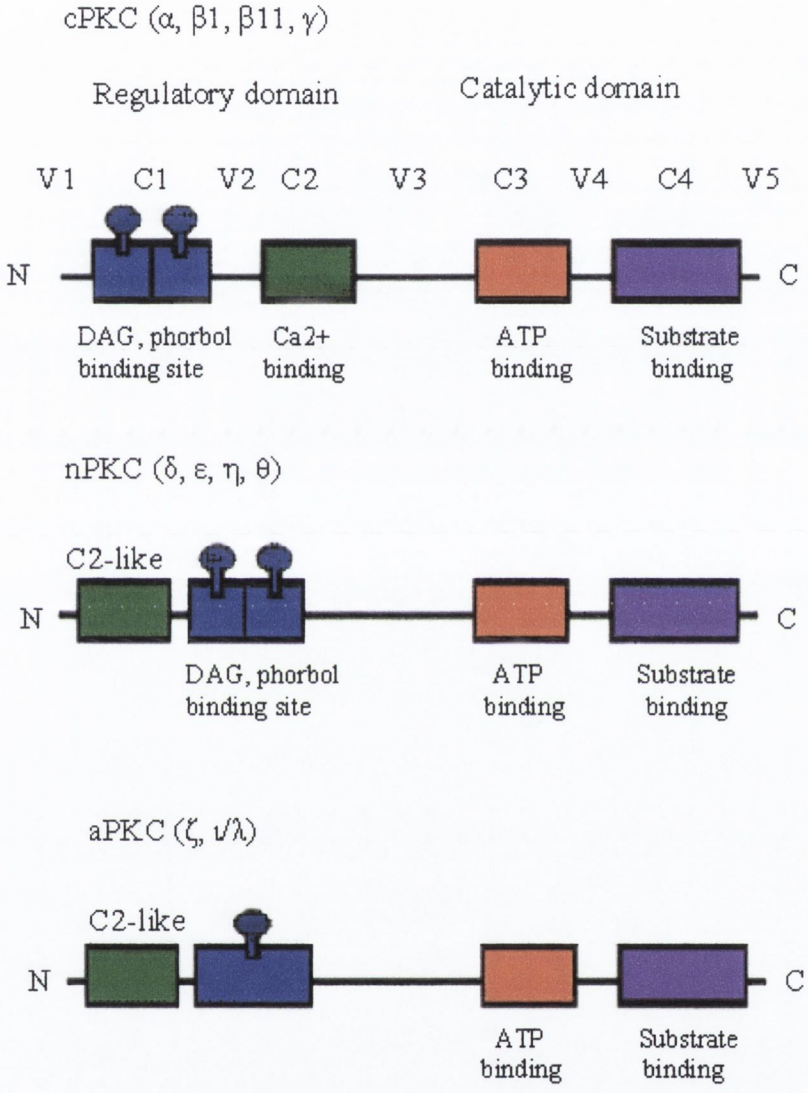


Fig.1.4 Structure of PKC. The PKC consist of N-terminal (N) regulatory domain and a C-terminal (C) catalytic domain. The conventional (cPKC) structure comprises four conserved regions (C1–C4) and five variable regions (V1–V5).The C1 region (C1A and C1B) is involved in binding to diacylglycerol (DAG) and phorbol-ester activating compounds, whereas the C2 region is binds to Ca2+. C3 and C4 are the binding sites for ATP and the PKC substrate, respectively. The novel PKC (nPKC) group and atypical (aPKC) group both possess a C2-like region within V1. Many activating agents act at the DAG/phorbol-ester binding region (or C1). (Reproduced from Way 2000).

have auto-inhibitory N-terminal pseudosubstrate domain. The pseudosubstrate domain binds to the substrate-binding site in the inactive molecule. Activation requires the removal of autoinhibitory pseudosubstrate domain from the active site. Activation of phospholipases type C (PLC) β, γ and δ, results in the hydrolysis of membrane inositol phospholipids. This leads to the generation of 1,2-sn-diacylglycerol (DAG) and soluble inositol phosphates (e.g., InsP, Ins-1, 4-P2 or Ins-1, 4, 5-P3). The latter then causes the release of calcium from intracellular stores. The generation of DAG or addition of exogenous phorbol esters that bind to the C1 domain relieves autoinhibition leading to activation and phosphorylation of relevant substrates. The cPKC C1 domain comprises a cysteine-rich motif, capable of binding two zinc ions. Binding of the co-factor phosphatidyl-L-serine (PS) to the regulatory domain is also required to promote the conformational change that results in removal of the pseudosubstrate from the catalytic site. The binding of calcium greatly increases the affinity of the enzyme for acidic phospholipids such as PS (Newton *et al.*, 1995). However, an isolated C2 domain from PKC betaII is shown not to bind to PS in a calcium-dependent manner (Johnson *et al.*, 1997). Thus, calcium/PS synergize with DAG or PMA to promote a remarkably high affinity interaction of PKCs with membrane. The precise binding site for phosphatidylserine on PKCs remains elusive. Novel PKCs such as delta and epsilon also require DAG and PS for activation, but are calcium independent. Although the classical C2 domain is absent in this family, a C2-like domain has been identified in novel PKCs at the very N-terminus of the enzyme (Sossin *et al.*, 1993). The C2-like domain of nPKCs may exist in a conformation similar to that found in the C2-calcium complex in cPKCs. Atypical PKCs only have one C1 domain and are not activated by either DAG or PMA. Similarly, they do not have a C2 domain and thus are unresponsive to calcium. It is not established whether the isolated C1 domain can act as a membrane-targeting module; a PKC delta mutant with only the second C1 domain is efficiently translocated in *vivo* in response to PMA (Szallasi *et al.*, 1996).

Many other lipid second messengers are reported to activate PKCs both *in vitro* and *in vivo*. In particular, the phosphoinositide 3-kinase (PI 3-K) products PtdIns-3, 4-P2 and PtdIns-3, 4-5-P3 has been shown to activate both the novel (delta, epsilon and eta) and the atypical (zeta) PKCs in *vitro* (Nakanishi *et al.*, 1993; Toker *et al.*, 1994; Singh *et al.*, 1993; Palmer *et al.*, 1995). There is considerable evidence that certain PKCs may be activated downstream of PI 3-K *in vivo*. The domain or residues responsible for the

phosphoinositide-PKC interaction are not yet known. These lipids may mimic PS and increase the affinity for PKC with the membrane. Other lipid mediators known to activate PKCs include unsaturated fatty acids, most notably arachidonic acid (Nishizuka 1995). These lipids activate PKC by potentiating the effects of DAG and have no effect on PKC if either DAG or PMA are absent. Ceramide, produced in TNF-alpha stimulated cells, activates and binds to both the atypical PKC zeta family member (Muller *et al.*, 1995) and to the related Raf-1 kinase (Muller *et al.*, 1998) in *vivo*. There is considerable heterogeneity in the lipid-dependent regulation of PKCs and this is further complicated by the role of phosphorylation in the activation of PKC.

1.4.4.2 Regulation by phosphorylation

It has been increasingly realised that phosphorylation plays a significant role in the activation of PKC (**Fig. 1.5**). Three key residues in the catalytic domain (C4) are mapped, and these are likely to represent the major in *vivo* phosphorylation sites. It has been demonstrated in PKC beta II that the initial, rate-limiting step is phosphorylation of the activation loop Thr500 and Thr641 and Ser660 at the carboxyl terminus (Keranen *et al.*, 1995). PKC alpha has a similar activation loop (Cazaubon *et al.*, 1994). TheThr residue is highly conserved in all PKCs and phosphorylation of this site is usually required for protein kinase activity. But, it is also found in a large number of other protein kinases. Substitution of PKC beta II Thr500 to an Ala residue results in an inactivatable enzyme (Orr *et al.*, 1994). The PKC activation loop threonine cannot undergo autophosphorylation, and there is evidence that a PKC upstream kinase, the phosphoinositide-dependent kinase-1 (PDK-1) carries out this reaction (Chou *et al.*, 1998; Le Good *et al.*, 1998). PKC zeta and PKC delta are also phosphorylated at the corresponding Thr410 at Thr505 residues, and this is sufficient to activate the PKCs both in *vitro* and in *vivo* (Chou *et al.*, 1998; Le Good *et al.*, 1998). In stimulated cells, PDK-1 binds with high affinity to the PI 3-K lipids PtdIns-3, 4-P2 and PtdIns-3, 4-5-P3, resulting in translocation from the cytosol to the membrane (Anderson *et al.*, 1998). The PDK-1 is the universal PKC upstream kinase for PKC, though this has not been proven. The binding of DAG and other lipids unmasks the activation loop Thr for the phosphorylation and hence activation of PKC. Two further phosphorylations occur in the catalytic domain of PKC and in the case of PKC beta II; these have been shown to be due to autophosphorylation of Thr641 (Keranen *et al.*, 1995). Both of these residues are conserved in all mammalian PKCs except for PKC mu. In the case of conventional

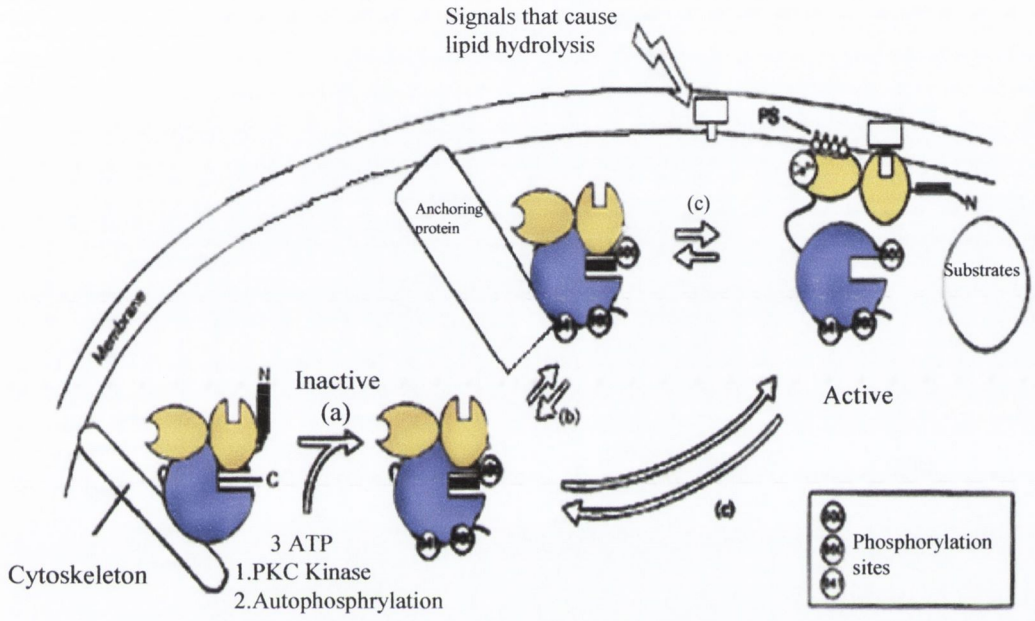


Figure 1.5 Model for the regulation of protein kinase C by phosphatylation. Newly synthesized PKC associates with the cytoskeleton (far left) which exposes the activation loop (top right). (a) Phosphorylation by a PKC kinase on the exposed activation loop (at Thr5OO in protein kinase C β11) correctly aligns residues for catalysis, allowing autophosphorylation at two carboxy-terminal positions (Thr641 and Ser660 in protein kinase C β11). Phosphorylation at the first of these (Thr641 in PKC locks the kinase in a catalytically competent conformation. Subsequent phosphorylation at the second carboxy-tenninal site releases mature PKC into the cytosol. (b) Isozyme-specific targeting of mature protein kinase C may be regulated interaction with anchoring proteins for rapid response to its second messenger, diacylglycerol (DAG). (c) Binding of DAG to the C1 domain and phosphatidylserine (PS) to the C2 domain results in removal of the pseudosubstrate from the active site. (From Newton 1997).

PKCs, phosphorylation of the activation loop threonine appears to be constitutive (Keranen *et al*, 1995). In contrast, atypical PKCs such as PKC zeta are phosphorylated at this residue by PDK-1 in a mitogen-dependent manner (Chou *et al.*, 1998).

1.4.4.3 Regulation by localization

PKC isoenzymes are located in the cytoplasm of the cell and translocate to different subcellular sites on activation (Jaken 1996). This isozyme-specific regulation by subcellular targeting and interaction with specific proteins provides an important mechanism for PKC (**Fig. 1.5**). PKC bind to a number of proteins including the receptors for activated kinase C (RACKs), a group of proteins that are proposed to bind to the active conformation of PKC (Mochly-Rosen 1995). In addition, PKC α and PKC β bind a multi-enzyme scaffold protein, for a kinase-anchoring protein (AKAP 79) (Klauck *et al.*, 1996). This AKAP 79 also regulates two other signal transducers, namely protein kinase A and the Calcium/calmodulin-dependent phosphatase, calcineurin. Furthermore, isozyme-specific binding to a number of proteins is also reported. For example, PKC ε binds to 14-3-3 proteins (Acs *et al*, 1995) and to cytoskeletal components actin via a sequence between the first and second Cl domains via its hinge or pseudosubstrate (Lehel *et al.*, 1995; Prekeris *et al.*, 1996). PKC β11 binds to actin, in this case via its carboxyl terminus (Blobe *et al.*, 1996). This positioning of the PKC at specific locations in the cell is probably central to its ability to have ready access to its substrates and to respond efficiently to second messengers for example DAG. Activated PKC isoenzymes translocate to a variety of intracellular sites including cell membrane, nucleus and membrane associated cytoskeleton (Nishizuka 1984). A number of cytoskeleton proteins including vinculin, vimentin, talin and filamentous actin (F-actin) target PKC translocation and may serve as PKC substrates and regulate membrane and cytoskeleton interactions as an integral part of PKC signalling. Furthermore, changes in localisation of PKC are associated with cell differentiation and transformation, indicating that determinants of subcellular localisation of PKC can modify cell growth, differentiation and transformation (Jaken 1996; Frey *et al.*, 2000). PKC ε in particular has been shown to play a significant role in cell adhesion (Haller *et al.*, 1998) and has been immunolocalized to the junctional region in gastrointestinal epithelia (Saxon *et al.*, 1994).

1.4.5 PKC and carcinogenesis

The PKC family is involved in regulation of three key cellular processes disrupted in malignant cells, i.e., cell growth/cell cycle progression, differentiation, and apoptosis, (Black

2000; Clemens *et al.*, 1992; Musashi *et al.*, 2000) and there is evidence for changes in the expression and/or activity of PKC isozymes in a variety of malignancies. Enterocytes and colonocytes express multiple members of the PKC family, including PKC α, βI, βII, δ, ε, η, θ, ζ, and ι, suggesting that this enzyme system plays an important role in maintenance of normal intestinal homeostasis. PKC isoenzymes have been implicated in the regulation of the normal colonic cellular processes and neoplastic transformation in the colon both in humans and in experimental animals. Studies have reported decreased expression of total PKC in human colonic carcinomas and adenomas as well as preneoplastic mucosa compared with colonic mucosa from normal controls (Kahl-Rainer *et al.*, 1994; Guillem *et al*, 1987; Kopp *et al.*, 1991). It has been reported that PKC and calcium-dependent protein kinase specific activities are reduced in human colon carcinomas when compared to their normal adjacent colon mucosa (Guillem *et al.*, 1987). Contradictory findings of increased levels of PKC isoforms in adenocarcinomas as compared with normal colonic tissue have also been described (Davidson *et al.*, 1994). Alterations in expression of specific PKC isoenzymes have also been described. PKC α, βI, and ζ seem to be lost early during intestinal carcinogenesis as shown in adenomas of the APC^{min} mouse where their expression is markedly reduced (Klein *et al.*, 2000).

1.4.5.1 PKC alpha and beta

Studies have demonstrated a reduced expression of PKC α protein in human colonic tumour (Kahl-Rainer *et al.*, 1994; Kahl-Rainer *et al.*, 1996). Carcinogen-induced colonic tumours in animals have also revealed attenuated levels of PKC α (Craven *et al.*, 1992; Wali *et al.*, 1995). Researchers have examined the effects of alterations in PKC α expression in Caco2 cells, derived from human colonic adenocarcinoma (Scaglione-Sewell *et al.*, 1998). The Caco2 cell line has no mutations in *K-ras* genes. PKC activation is reported to relate to invasiveness of the cancer cells in colorectal and other cancer cells (Batlle *et al.*, 1998; Shimao *et al.*, 1999; Johnson *et al.*, 1999). Stable PKC α sense or antisense complementary DNA (cDNA) transfectants were use in these studies. Sense transfected cells demonstrated decreased proliferation and enhanced differentiation. PKC α protein expression was increased 3-fold in these cells. In contrast, antisense transfected cells, in which PKC α expression was decreased by 95 %, showed enhanced cell growth, decreased differentiation, and a more aggressive transformed phenotype compared with empty vector-transfected control cells. However, opposite observations were made with PKC β1 in HT29 cells, in which overexpression of PKC β1 isoform was associated with suppression of cell

proliferation (Choi *et al.*, 1990). Fray *et al.* (1997) showed that growth of IEC-18 cells, derived from immature crypt cell line of rat ileum, was inhibited by phorbol esters with G 1 cell cycle arrest, concomitant with activation of PKC α. Moreover, this arrest was accompanied by an increased expression of the cyclin-dependent kinase inhibitors p21 (waf1/cip1) and p27 (kip1). These observations demonstrate that PKC α is intimately involved in the control of proliferation and differentiation in colonic cells. Several other studies have reported changes in PKC activity in colon adenomas and carcinomas. Reduced expression of PKC α, δ, ε, and ζ have been reported in colorectal tumours (Kahl-Rainer *et al.*, 1994; Doi *et al.*, 1994; Craven *et al.*, 1992; Kahl-Rainer *et al.*, 1996; Wali *et al.*, 1995). PKC β1 and βII levels are reported reduced in some studies (Levy *et al.*, 1993; Doi *et al.*, 1994), up regulated in other studies (Craven *et al.*, 1992; Wali *et al.*, 1995) and unchanged levels have also been reported (Kahl-Rainer *et al.*, 1994). Additional evidence for complex role of PKC enzyme system in colon carcinogenesis comes from the finding that overexpression of PKC β1 in HT29 and SW 480 human colon cancer cells can cause growth inhibition and tumour suppression in nude mice (Choi *et al.*, 1990; Goldstein *et al.*, 1995). This contrasts with the previous studies obtained with rodent fibroblasts, where stable overexpression of PKC β1 and γ isoforms resulted in increased growth rate, high saturation densities, and anchorage-independent growth and enhanced tumorigenicity, in nude mice (Housey *et al.*, 1988; Pearsons *et al.*, 1988: Krass *et al.*, 1989). PKC β1 overexpression has been associated with resistance to apoptosis (Cesaro *et al.*, 2001). It has been reported that transgenic mice expressing elevated PKC betaII in the colonic epithelium exhibit a trend toward increased colon tumour formation after exposure to azoxymethane (Gokmen-Polar *et al.*, 2001). Although PKC βI expression is modestly decreased in aberrant crypt foci, it is markedly reduced in *APC^{min}* mouse adenomas and in azoxymethane-induced colon carcinomas in mice (Gokmen-Polar *et al.*, 2001; Klein *et al.*, 2000). Thus, PKC seems to exert both positive and negative regulatory effects on cell growth depending on the cell type, the stage of carcinogenesis and species type.

1.4.5.2 PKC epsilon and delta

PKC epsilon and PKC delta are involved in the cell growth, morphology, apoptosis and tumorigenicity. PKCs ε and PKC δ are novel PKC family members which respond to both DAG and PS in vivo. In addition, PKC epsilon is also activated by the PI 3-K lipids PtdIns-3,4-P2 and PtdIns-3,4-5-P3 *in vitro* (Toker *et al.*, 1994, Moriya *et al.*, 1996). Overexpression of PKC ε is reported to be oncogenic in a rat colonic epithelial cell line and is associated with

increase in cell growth rates (Perletti *et al.*, 1998; Marras *et al.*, 2001; Weller *et al.*, 1999; Mischak *et al.*, 1993). It has also been reported that R6 cells overexpressing high levels of PKC ε display morphologic and growth properties that are consistent with malignant transformation and the cells are tumorigenic in nude mice (Cacace *et al.*, 1993). PKC ε is upstream of the Raf-1 protein in the Ras-Raf-l-MEK-MAP kinase signal transduction pathway (Cacace *et al*, 1996). The oncogenic potential of PKC epsilon has also been observed in rat colonic epithelial cells, where overexpression leads to anchorage-independent colony formation in soft agar and formation of tumours in nude mice (Perletti *et al.*, 1996, 1998). PKC epsilon regulates the transcription factors N-FAT-1 and AP-1 and NF-κB in activated T cells and its effects are similar to ras (Genot *et al.*, 1995). Protein kinase C δ is the most thoroughly studied member of the nPKC subfamily. PKC δ has distinct effects on cell growth. Overexpression of PKC δ is associated with reduction in growth rate and cell density in NIH 3T3 cells and it may inhibit the G2/M transition by downregulating Cdc25 phosphatase (Livneh *et al.*, 1997). Reports also indicate that PKC δ may inhibit cell proliferation in src-transformed cells (Perletti *et al.*, 1999). These findings suggest that PKC δ may suppress tumour growth and progression in certain cell types. Other researcher have refuted these findings and reported that overexpression of a truncated PKC δ containing only the regulatory domain inhibited anchorage-independent growth (Liao *et al.*, 1994) and proposed that PKC δ regulatory domain may have acted as a dominant negative mutant of PKC δ with the result that it may have a positive role in cell growth. Unlike other PKC isoforms, PKC δ can also be phosphorylated on tyrosine with either inhibitory or stimulatory effects on PKC δ (Li *et al.*, 1994). Further, PKC δ downregulates several receptors with tyrosine kinase activity, including the epidermal growth factor (EGF) (Downward *et al.*, 1985; Friedman *et al.*, 1984) and insulin like growth receptors (Chin *et al.*, 1993). Thus, it is possible that depleting cells of PKC δ could help sustain an active growth factor-induced signal by preventing downregulation of an activated receptor. Besides the involvement of PKC δ in the regulation of cell growth and differentiation, this PKC isoform might play a role also in apoptosis and tumour development (Emoto *et al.*, 1995; 1996, Ghayur *et al.*, 1996). Bharti *et al.* (1998), reported on an interaction of PKC δ with the DNA-dependent protein kinase (DNA-PK) resulting in the inactivation of this kinase. DNA-PK is essential in the repair of DNA double-strand breaks. Thus, interaction of PKC δ and DNA-PK may contribute to DNA damage-induced apoptosis. Reddig *et al.* (1999) demonstrated that transgenic mice overexpressing PKC δ in the epidermis showed a dramatic reduction in the formation of skin tumours upon treatment with 7,12-dimethylbenz[*a*]anthracene/TPA.

1.4.5.3 PKC zeta

PKC ζ is the critical mediator of mitogenic signal transduction. Berra *et al.* (1993), demonstrated that this PKC isoform is required for maturation of Xenopus oocytes and also for DNA synthesis in fibroblasts. This was the first indication that PKC ζ participates in mitogenic signalling. Nakanishi and Exton reported that PKC ζ could be activated in vitro by the lipid product of PI 3-K, PtdIns-3,4-5-P3 (Nakanishi *et al.*, 1993). PKC ζ is downstream effector of p21ras, involved in mitogenic signal transduction (Berra *et al.*,, 1993; Coleman *et al.*, 1994). Reduced expression of PKC ζ has been reported in both human and experimental colon cancers (Wali *et al.*,, 1995, 1996; Kahl-Rainer *et al.*, 1994). Moreover, in the azoxymethane(AOM) model of colon carcinogenesis, three structurally unrelated agents, ursodeoxycholic acid piroxicam and F6-D3 (a fluorinated analogue of 1,25-dihydroxyvitamin D3), have each been shown to prevent the expected decrease in PKC ζ in AOM-induced adenomas while markedly reducing the frequency of AOM-induced carcinomas (Wali *et al.*, 1996). PKC ζ participates in activation of NF-kappa B-like activity in Xenopus oocytes and mouse fibroblasts (Diaz-Meco *et al.*, 1993; Dominguez *et al.*, 1993). The outcome is the activation of NF-kappa B-dependent gene transcription and cell proliferation. A number of studies have provided conflicting evidence as to the role of PKC ζ in cell transformation and proliferation. Firstly, Montaner *et al.*, (1995) were unable to detect any differences in the growth properties of NIH 3T3 fibroblasts transfected with wild-type PKC ζ or any differences in the regulation of NF-kappa B (Montaner *et al.*, 1995), as originally reported (Diaz-Meco *et al.*, 1993). However, contradictory results have been reported in which a direct interaction between PKC ζ and *ras* was not detected (Warne *et al*,. 1993). Moreover, PKC ζ has been reported to suppress the neoplastic transformation of fibroblasts mediated by the v-raf oncogene (Kieser *et al.*, 1996). A recent study has suggested that the concerted action of a hierarchical signalling cascade involving PKC lambda, PKC epsilon and PKC ζ may be required to mediate transcriptional activation of the *c-fos* promoter in cells expressing oncogenic Ha-ras (Kampfer *et al.*, 1998).

1.4.6 PKC and cytoskeletal structures

PKC are one of the most important regulators of the cytoskeleton functions in cells. Certain PKC substrates such as the MARCKS protein and pleckstrin associate with the cytoskeleton in a PKC-dependent manner (Brumell *et al.*, 1997; Hartwig *et al.*, 1992; Myat *et al.*, 1997). A separate and distinct actin-binding motif has also been identified

in the PKC beta II isoform (Blobe *et al.*, 1996) and PKC zeta has also been shown to interact with actin in the presence of Zn^{2+} (Gomez *et al.*, 1996). Data indicates that PKC epsilon plays an important role in cytoskeletal organization. A unique actin- binding motif is identified in PKC epsilon (Prekeris *et al.*, 1996). PKC epsilon translocates to the membrane fraction when HeLa cells, plated on a gelatin substratum, but not when cells are plated on plastic (Chun *et al.*, 1996). Inhibition of PKC epsilon with antisense oligodinucleotides inhibits cykeleton-dependent cell spreading on a fibronectin matrix (Haller *et al.*, 1998). PKC epsilon translocates to focal adhesions following integrin activation. Thus, PKC epsilon may be involved in the regulation of adhesion of cells to the extracellular matrix.

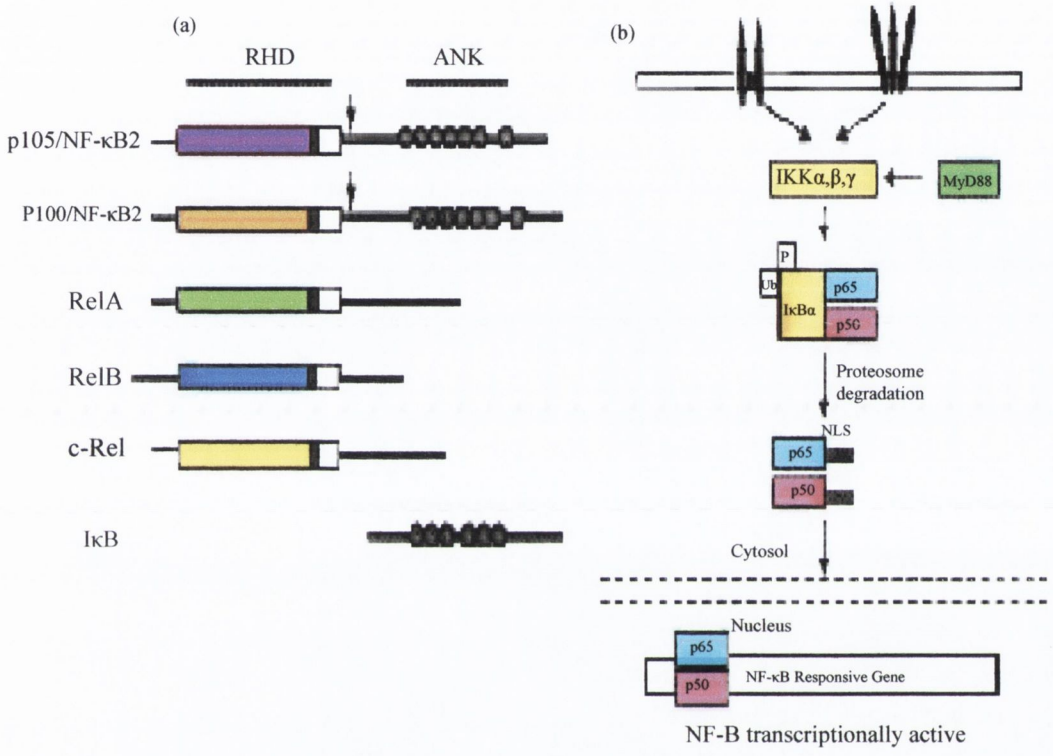


Fig. 1.6 Members of the Rel/NF-κB and IκB families of proteins. (a) The arrows indicate the endoproteolytic cleavage sites of p105 and p100, which give rise to p50 and p52, respectively. (b) In unstimulated cells, the Rel/NF-κB associate with members inhibitor proteins IκB and remain inactive in the cytoplasm. Upon stimulation, IκB-kinases (IKK) rapidly phosphorylate IκB molecules, which lead to their ubiquitination, and degradation. This unmasks nuclear localisation sequences (NLS) allows NF-κB dimers to translocate to the nucleus and regulate transcription through binding to κB sites in specific gene promoters. RHD, Rel homology domain; ANK, ankyrin repeat. (Reproduced from Shapira *et al.*, 2004)

1.5 TRANSCRIPTION FACTORS

1.5.1 NUCLEAR FACTOR KAPPA B (NF-κB)

NF-κB/Rel are structurally related eukaryotic transcription factors, involved in the control of a large number of normal cellular processes, such as immune and inflammatory responses, growth, development and apoptosis. They were originally identified as proteins that bound to a specific decameric DNA sequence (ggg ACTTTC C), within the intronic enhancer of the immunoglobulin kappa light chain in mature B- and plasma cells but not pre B-cells (Sen and Baltimore 1986). Later, it was shown that NF-κB DNA binding activity is induced by a variety of exogenous stimuli, and that this activation is independent of de-novo protein synthesis. NF-κB has been detected in most cell types, and specific NF-κB binding sites (with the consensus sequence: ggg RNN YYC C, R=purine Y=pyrimidine) have been identified in promoters and enhancers of a large number of inducible genes.

1.5.1.1 NF-κB and IκB proteins

NF-κB resides in the cytoplasm of the cells as homo- or heterodimers of a family of structurally related proteins (Baldwin 1996; Kopp *et al.* 1995). Each member of this family contains a conserved N-terminal region called the Rel-homology domain (RHD) within which lies the DNA-binding and dimerization domains and the nuclear localization signal (NLS) (Baldwin 1996; Kopp *et al.*, 1995). Five proteins belonging to the NF-κB family have been identified in mammalian cells: p65, c-Rel, RelB, p50/p105 and p52/p100 (**Fig 1.6**). NF-κB dimers are sequestered in the cytosol of unstimulated cells bound to a class of inhibitory proteins called IκBs. These inhibitory proteins also comprise a structurally and functionally related family of molecules. Seven IκB molecules are identified: IκB-α, IκB-β, IκB-γ, IκB-ε, Bcl-3, p100 and p105. All known IκB proteins contain multiple copies of a 30–33 amino acid sequence known as ankyrin repeats; and the specific interaction between the ankyrin repeats of IκB and the RHD of NF-κB is the defining feature of the association between NF-κB and IκB. The NLS of NF-κB is mask by IκB molecules to prevent its nuclear translocation. The dissociation and subsequent degradation of IκB proteins causes NF-κB to become active, allowing it to enter the nucleus and induce gene expression. The function of NF-κB is that of a second messenger molecule through its ability to transduce upstream signals from the cytoplasm into the nucleus in activated cells (Baldwin 1996; Kopp *et al.*, 1995; Thanos *et al.*, 1995; Verma *et al.*, 1995).

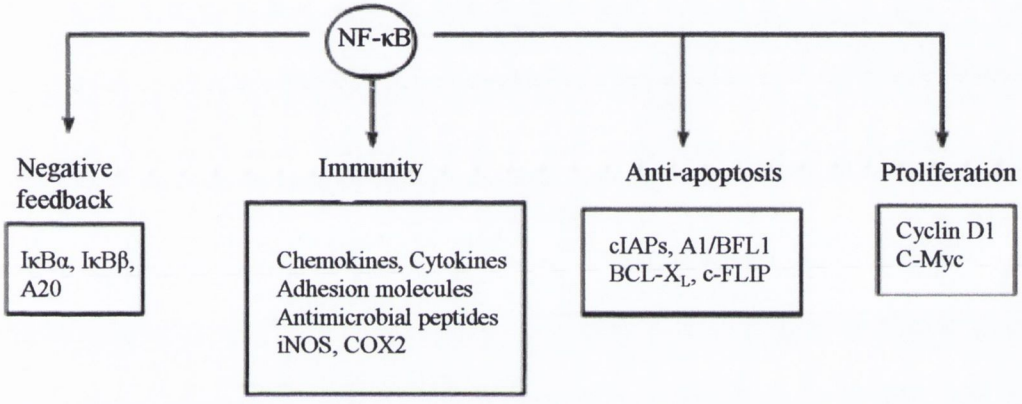


Figure 1.7 Classes of genes induced by NF-κB (Reproduced from Karin *et al.*, 2002).

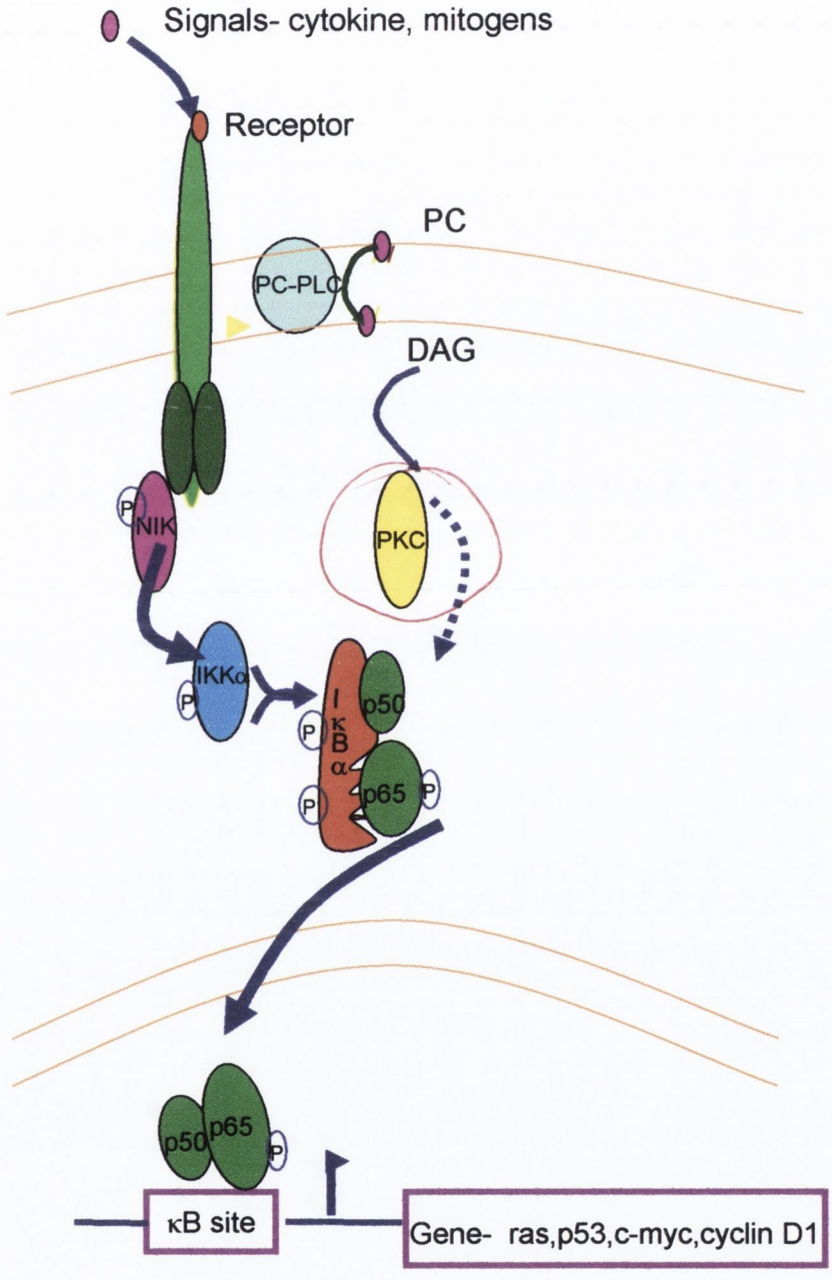


Fig. 1.8 NF-κB signalling pathway (Reproduced and Modified from Gilmore 1996).

NF-κB plays an important role in the regulation of a large number of inducible genes involved in a variety of cellular process such as to stress response, immunity and inflammation, apoptosis and cell proliferation (Kopp *et al.*, 1995) (**Fig. 1.7**). Several induction mechanisms involved in the activation of NF-κB converge on the cytosolic NF-κB-IκB complex. These include cytokines such as interleukin 1 (IL-1) and tumour necrosis factor (TNF); bacterial and viral products such as lipopolysaccharide (LPS), sphingomyelinase, double-stranded RNA and pro-apoptotic and necrotic stimuli such as oxygen free radicals, UV light and γ-irradiation.

1.5.1.2 IκB-kinase

A number of different kinases, including PKC, PKA, Raf-1, double-stranded-RNA-dependent kinase (PKR) and p90-ribosomal S6 protein kinase (p90-RSK), are suggested to take part in the specific phosphorylation of Ser32 and Ser36 and initiation of IκB-α degradation and dissociation of the NF-κB-IκB-α complex *in vitro* (Ghosh *at al.*, 1990; Diaz-Meco *et al.*, 1994; Finco *at al.*, 1993; Kumar *et al.*, 1994; Schouten *et al.*,1997) (**Fig 1.8**). However, none of these kinases phosphorylates specifically both of the N-terminal serine residues of IκB α is required for activation *in vivo*. Studies have revealed that substitution of the serine residues with threonines is sufficient to prevent NF-κB activation in response to numerous signals, demonstrating that an intact substrate sequence is required (Brown *et al.*, 1995; DiDonato *et al.*, 1996). This indicates that a novel and serine-specific kinase may be required and there must be a point where all inducers of NF-κB converge. This novel and converging kinase responsible for the phosphrylation of IκB-α the IκB-kinase. The IκB-kinase was first described as a high-molecular-weight kinase complex that phosphorylates specifically Ser32 and Ser36 of IκB-α and is dependent on ubiquitination for activity (Chen 1996; Lee *et al.*, 1997). Two IκB-specific kinases associated with a high-molecular-weight cytoplasmic IκB-kinase complex have been characterised which are not regulated by ubiquitination (DiDonato *et al.*, 1997; Mercurio *et al.* 1997; Regnier *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). One of these kinases described is Ser/Thr kinase of unknown function named as IKKα (IκB-kinase α-subunit) (Connelly *et al.*, 1995). Another kinase exhibiting 52 percent homology to IKKα is IKKβ. Both IKKα and IKKβ are ubiquitously expressed and are similar in structure but some differences in phosphorylation patterns of each subunit may result in different patterns of NF-κB activation (Zandi *et al.*, 1997). It was previously reported that the kinase NF-κB-

inducing kinase (NIK) was required for NF-κB activation (Malinin *et al.*, 1997). Later it was discovered IKKα and IKKβ, associate with NIK (Regnier *et al.*, 1997; Woronicz *et al.*, 1997). Immunoprecipitation studies have demonstrated that IKKα and IKKβ can form homo- or heterodimers. Transfection studies involving overexpression of these kinases have demonstrated that each subunit is sufficient to cause the activation of NF-κB. The IKKβ is constitutively active, whereas the activity of IKKα requires costimulation with an inducer to become fully active (Mercurio *et al.*, 1997; Woronicz *et al.*, 1997; Zandi *et al.*, 1997). Both kinases are involved in proinflammatory cytokine-induced activation of NF-κB and both have the ability to phosphorylate IκB proteins except IκB-ε isoform.

1.5.1.3 Regulation of IκB-kinase activity

The precise mechanisms by which the activity of IκB-kinase is regulated and the upstream regulators remain unclear. It is proposed that multiple, as-yet-unidentified components of the high-molecular-weight kinase complex may be responsible for such regulation (Chen and parent 1996; DiDonato *et al.*, 1997; Mercurio *et al.*, 1997). Several studies have demonstrated that protein kinase, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEKK-1) may be involved in TNF-α -induced NF-κB activation (Hirano *et al.*, 1996; Meyer *et al.*, 1996; Read *et al.*, 1997),while others have disputed a role for MEKK-1 in NF-κB activation (Liu *et al.*, 1996). It has been suggested that NIK by interaction with the TNF-receptor-associated factor 2 (TRAF-2), stimulates IκB-α degradation and may play an important role in NF-κB activation (Malinin *et al.*, 1997). Other members of the TRAF family of molecules (TRAF-5 and -6) which can associate with several of the TNF receptor family of proteins may act as upstream activators of NF-κB (Aizawa *et al.*, 1997, Cheng *et al.*, 1995, 1996; Ishida *et al.*, 1996; Nakano *et al,.* 1996; Rothe *et al.*, 1995; Shu *et al.*, 1996; Cao *et al.*, 1996). Interleukin-1 (IL-1)-induced NF-κB activation requires IL-1 receptor accessory protein (IL-1RAcP) and two Ser/Thr kinases named IRAK (IL-1 receptor-associated kinase) (Greenfeder *et al.*, 1995; Wesche *et al.*, 1997; Muzio *et al.*, 1997; Cao *et al.*, 1996). Post-translational modifications in the inducible transcription factors are necessary for their regulation. It is reported that post-translational modification of NF-κB activity involves cAMP-independent activation of PKA (Zhong *et al.* 1997). A consensus PKA phosphorylation site has been discovered in amino acid sequence of the p65 subunit of NF-κB and mutational analysis has demonstrated that

Table 1.2 NF-κB-dependent genes (Modified and Reproduced from Bharti 2002)

Immunoregulatory	MCH class 1 and 11
	TCRα and β
	TAPI
	β_2–microglobulin
	Igk
Adhesion molecules	ICAM-1
	VCAM-1
	MAdCAM-1
	E-selectin
Cytokines	IL-1 α and β
	1L2,-3, -6, -8, -12
	TNF α
	LT α
	IFN-β
	G-CSF
	M-CSF
Stress proteins	SAA
	Complement factors B, C3 and C4
Anti-apoptotic genes	Bcl-2
	Bcl-x_l
	TNF receptor-associated factor 1 (TRAF1)
	TRAF2
	Cyclin D1
	cIAP-2
	xIAP
	A20
	Cyclooxygenase-2
	c-Myc
Genes involved in cancer	Epidermal growth factor receptor (EGFR)
	Cell adhesion molecules
	iNOS
	VEGF
	Interleukin-6
	Matrix metalloproteinase-9 (MMP-9)
	MMP-2
	Interleukin-8
	Cyclooxygenase-2
	c-Myc

phosphorylation by PKA within this sequence (at Ser276) regulates the transcriptional activity of p65. The conclusion is that interactions of IκB kinases and NF-κB activation is a complex process and different subunits of IκB kinase may be targets for separate signalling cascades.

1.5.2 NF-κB and cancer

NF-κB activation has been implicated in the development and progression of many human cancers. Constitutive NF-κB activation associated with a mutation in the gene encoding the I-κB-α inhibitor has been observed in Hodgkin's disease, (Wood *et al.*, 1998; Krappmann *et al.*, 1999). The v-*rel* oncogene of the reticuloendotheliosis virus T (Rev-T) was the first member of the Rel/NF-κB family to be discovered (Gilmore *et al.*, 1991, 1999). Further evidence for role of NF-κB in carcinogenesis comes from the observation in chickens injected with the Rev-T virus developed aggressive lymphomas and also that many genes belonging to the NF-κB and IκB are amplified or translocated in human cancers (Rayet *et al.*, 1991). It is thought the genetic modifications in NF-κB targeted genes may lead to increased NF-κB transcriptional activity with implication for cellular transformation and cancer progression. Another argument for its role in carcinogenesis is that fact that many carcinogens and tumour promoters such as benzo[a]pyrene, NNK, UV radiation, and phorbol esters induce NF-κB activation (Pahl 1999; Yan *et al.*, 2000; Li *et al.* 1998; Baeuerle *et al.*, 1988). Inhibition of NF-κB is reported to block sunburn-induced damage caused by UV radiation a factor that predisposes to skin cancer (Abeyama *et al.*, 2000). Aberrant and constitutive expression of NF-κB is reported in many cancers including leukaemia, lymphoma, myeloma, melanoma, prostate, colon, breast, pancreas, and head and neck squamous cell carcinoma cell (Mukhopadhyay *et al.*, 2001; Mori *et al.*, 1999; Bargou *et al.*, 1997; Bours *et al.*, 1994). Finally a number genes involved in tumour cell invasion and angiogenesis are regulated by NF-κB including the cell adhesion molecules (ICAM-1, VCAM-1, ELAM-1), COX-2, iNOS, uPA, MMP-9, MMP-2, VEGF, chemokines, and inflammatory cytokines (Ambs *et al.*, 1998; Marrogi *et al.*, 2000; Chan *et al.*, 1998; Philip *et al.*, 2001; Heiss *et al.*, 2001; Oshima *et al.*, 2001; Wang *et al.*, 1999) (**Table 1.2**).

1.5.2.1 Cell proliferation and NF-κB

NF-κB regulates many cellular processes including cell proliferation. This observation was reported in studies in which overexpression of activated NF-κB induced cell proliferation

and while its suppression caused inhibition of cell proliferation (Rath *et al.*, 2001; Mukhopadhyay *et al.*, 2001; Garg *et al.*, 2002). NF-κB activation has also been implicated in cell survival and its downregulation is reported to increase sensitivity the cells to chemotherapeutic-induced apoptosis (Wu *et al.*, 1996; Beg *et al.*, 1996; Wang and Mayo 1996; Van Antwerp 1996). Furthermore, NF-κB regulates many important genes involved in cell survival including *bcl-2*, *bcl-x_L*, *cIAP*, *xIAP*, *TRAF1* and *TRAF2* **(Table 1.2).**

1.5.2.2 Apoptosis and NF-κB

NF-κB activation is associated with resistance to apoptosis and many proapoptotic stimuli induce NF-κB activation. Many researchers have reported a role of the NF-κB in the control of pro- and antiapoptotic pathways in normal and cancer cells (Liu and Hsu 1996; Beg *et al.*, 1996; Wang and Mayo 1996). It has been reported that NF-κB activation prevents apoptosis in several untransformed and tumour cell types (Barkett and Gilmore 1999). NF-κB activation regulates several antiapoptotic molecules including those coding for TNF receptor-associated factor 1 (TRAF1) and TRAF2, cIAPs, manganese superoxide dismutase (MnSOD), A20, and IEX-1L (Pahl 1999; Wu *et al.*, 1998; Stehlik *et al.*, 1998; Krikos *et al.*, 1992; Jones *et al.*, 1997). Moreover, NF-κB also controls the expression of two antiapoptotic proteins from the Bcl-2 family, Bfl-1/A1 and Bcl-xL, (Zong *et al.*, 1999; Grumont *et al.*, 1999; Chen *et al.*, 1999; Lee *et al.*, 1999). It has been reported that NF-κB activity influences Bcl-2 expression in some experimental systems (Tamatani *et al.*, 1999; Feuillard *et al.*, 2000). NF-κB has been reported to interfere with *p53* transcriptional activity through the competition for cofactors and this constitutes a second potential mechanism for the NF-κB antiapoptotic effect (Webster *et al.*, 1999; Ravi *et al.*, 1998; Wadgaonkar *et al.*, 1999). Constitutive NF-κB activation has been observed in many cancer cells and NF-κB inhibition induces apoptosis in these cells (Sovak *et al.*, 1997; Bargou *et al.*, 1997). By contrast, it is reported that inhibition of NF-κB does not cause apoptosis in response to anticancer drugs (Bentires-Alj *et al.*, 1999). However, other studies have reported that NF-κB acts as proapoptotic agent in response to oxidative stress, ischemia, or even to some cytotoxic drugs (Barkett *et al.*, 1999, Dumont *et al.*, 1999; Grilli *et al.*, 1996; Lin *et al.*, 1995).

1.5.2.3 NF-κB and chemotherapeutic agents

Chemotherapeutic agents have been shown to induce as well as block NF-κB activation. For example, taxol, doxorubicin, daunorubicin, etoposide, vincristin, vinblastin,

JUN FAMILY
c-jun
Jun B
Jun D

FOS FAMILY
c-fos
Fra 1
Fra 2
Fos B

Fig. 1.9 The members of the AP-1 transcription factor.
(From Ozanne *at al.*, 2000)

anthralin, AZT, ciprofirate, cisplatin, temoxifen, and camptothecin are reported to activate NF-κB (Pahl 1999). Gamma irradiation, commonly used to treat cancer patients, has also been found to activate NF-κB (Prasad *et al.*, 1994). NF-κB activation has antiapoptotic effects leading to chemoresistance experienced with chemotherapy or radiation therapy.

1.5.2.4 NF-κB suppression by chemopreventive agent

Certain chemopreventitive agents also target NF-κB for their action. These include curcumin, resveratrol, emodin, green tea polyphenols, silymarin, β-lapachone, caffeic acid phenethyl ester, and sulindac which suppress the activation of NF-κB (Mukhopadhyay *et al.*, 2001; Han *et al.*, 1999; Clement *et al.*, 1998).

1.6 ACTIVATOR PROTEIN-1

1.6.1 Background

Activator protein 1 (AP-1) is the collective term used the dimeric transcription factors composed of Jun (*v-Jun, c-Jun, JunB, JunD*), *Fos* (*v-Fos, c-Fos, FosB, Fra1, Fra2*) or activating transcription factor (ATF2) and MAF (musculoaponeurotic fibrosarcoma) protein families. All these proteins bind to common DNA site called AP-1 binding sites (**Fig. 1.9**) but different dimer combinations recognize different sequence elements in the promoters and enhancers of target genes. The main DNA response-element is the TPA-responsive element (TRE) with the base sequence TGACTCA. This element was first identified in the promoter and enhancer elements of the metallothionein I gene and simian virus 40 (SV40) (Angel *et al.*, 1991). The others are cAMP-response element (CRE) with the base sequencc TGACGTCA), the MAF-recognition elements (MAREs) and the antioxidant-response elements (AREs) (Chinenov *et al.*, 2001; Hai *et al.*, 1991). The main AP-1 proteins in mammalian cells are *Fos* and *Jun*. AP-1 is implicated in cell proliferation, survival, differentiation and transformation as tumour promoters, growth factors, cytokines and oncoproteins all induce the TRE sequence (Angel 1991).

1.6.2 Regulation and function of AP-1 genes

The activity of AP-I proteins is regulated through extracellular stimuli, transcription of the genes and interaction with ancillary proteins (Karin 1995). MAP and JNK kinases induce phosphorylation of serines and threonines at the amino-terminal activation domain of the AP-1 followed by ubiquitination and degradation (Musti *et al.*, 1997). Among the ancillary proteins that interact with AP-1 and have clinical importance are nuclear receptors such as

the glucocorticoid receptor (GR) and the retinoic-acid receptor (RAR), both of which can inhibit AP-1 target-gene transcription by transrepression (Chinenov *et al.*, 2001; Wagner *et al.*, 2001; Herrlich 2001). However, in certain tissues or cell types, AP-1 and GR may have synergistic effect to activate common target genes through composite DNA response elements. The composition of AP-1 determines whether the crosstalk with GR is positive or negative. The transcriptional activity of the *c-jun* and *c-fos* components of AP-1 as well as NF-κB is inhibited by the interferon-inducible p202, whish is reported to interact directly with *c-jun, c-fos* and the p50 and p65 subunits of NF-κB (Min *et al.*, 1996).

1.6.2.1 *C-FOS*

Stimulation of cells with various stimuli including neurotransmitters and polypeptide hormones induce *c-fos* transcription very rapidly (Piechaczyk *et al.*, 1994; Treisman 1992). The CRE, CRE-binding protein (CREB) or ATF proteins, all mediate *c-fos* induction via cAMP- and CaZ+-dependenc signalling pathways (Sheng *et al.*, 1991). A number of *cis* elements are also involved in the regulation of *c-fos*. One such *cis* element a Sis-inducible enhancer (SIE) which regulates *c-fos* function through signal transducer and activator of transcription (*STAT*) group of transcription factors (Darnell *et al.*, 1994). Another *cis* element called serum-response element (SRE), is recognized by a dimer of the serum-response factor (SRF) and recruits important mediators of *c-fos* induction such as monomeric ternary complex factors (TCFs) (Treisman 1992). Extracellular signal regulated kinase (ERK) takes part in mitogen-stimulated phosphorylation of one of the TCFs called Elk-I (Treisman 1994; Gille *et al.*, 1992; Matais *et al.*, 1993).

The role for *c-fos* in cell proliferation is emphasised by its induction in response to growth factors but experiments with the knockout of the mouse *c-fos* gene have demonstrated that *c-fos* is not essential for the proliferation and differentiation of the cells (Johnson *et al.*, 1992; Piechaczyk *et al.*, 1994). Conversely, overexpressing *c-fos* in bone tissue resulted in development of osteosarcomas and overexpression of *c-jun* and *c-fos* in bone enhanced the rate of osteosarcoma formation, indicating *in vivo* cooperation between *c-jun* and *c-fos* in tumour development (Van Dam *et al.*, 1993; Wang *et al.*, 1995).

1.6.2.2 *C-JUN*

C-jun is the major component of all AP-l complexes, expressed in many cell types and its expression is increased in response to many stimuli, including growth factors, cytokines and DV irradiation (Karin 1995). The function of *c-jun* is mediated through the TRE, recognized by *c-jun-ATF2* heterodimers (Van Dam *et al.*, 1993). Many kinases such as JNK, p38 and

MAPKs are thought to play role in proinflammatory cytokines or growth factors induction of *c-jun* (Hibi *et al.*, 1993; Raingeaud *et al.*, 1995; Derijard *et al.*, 1994). Members of MAPKs, Jun kinases are shown to interact with the delta region of the amino-terminal activation domain of c-Jun (Hibi *et al.*, 1993; Derijard *et al.*, 1994). Among the JNKs, JNK-2 exhibits the highest affinity for *c-jun*, known to contain a putative loop region that interacts with the JNK-docking site on *c-jun* (Kallunki *et al.*, 1994). Many members of the jun family form heterodimers and this heterodimerization not only affects their abilities to bind DNA but also influences their recognition by protein kinases and other enzymes. The phosphorylation of *c-jun* at serine 73 and, to a lesser extent, at serine 63 enhances its ability to activate transcription (Smeal *et al.*, 1994). Another coactivator that interacts with the amino-terminal activation domain of *c-jun* is Jun activation domain binding protein (JABt) (Claret et al., 1996). JABt interacts with *c-jun* and *JunD* but not with *JunB* and this interaction is not affected by *c-jun* phosphorylation. Protein-proteine interactions are also important for the normal functiong of AP-1. For example, *c-fos* and Myocyte-Enhancer Factor 2 (MEF2) interact and induce *c-jun* expression. *Fra-1* and *c-jun* are the main AP-1 components, induced by the positive regulators of tumorigenesis, the oncogenic *ras* and *ras*-mediated cell transformation is suppressed in fibroblasts that lack *c-jun* (Chang *et al.*, 2001; Mechta *et al.*, 1997; Johnson *et al.*, 1996).

1.6.3 AP-1 and cancer

AP-1 is an important factor in regulation of cell growth and survival. The *fos* and *jun* components of the AP-1 proteins were first identified as the viral oncoproteins *v-fos* and *v-jun* in the Finkel–Biskis–Jinkins osteosarcoma virus and avian sarcoma virus 17, respectively (Vogt 2002). The overexpression of AP-1 proteins or its upregulation by oncogenic *ras* correlates with cell transformation. It was found that several of the AP-1 protein, *c-fos, fosB* and *c-jun* took part in cell transformation in culture (Jochum *et al.*, 2001). *C-fos* overexpression was associated with the transformation of chondroblasts and osteoblast in the formation of osteosarcoma (Wang *et al.*, 1991; Grigoriadis *et al.*, 1993). It has been reported that reducing c-Jun/AP-1 activity using a dominant-negative *c-jun* (TAM67) in basal keratinocytes or inactivation of *c-jun* in the liver interferes with the development of chemically induced papillomas and liver tumours respectively (Young *et al.*, 1999; Eferl *et al.*, 2003). Similarly, Overexpression of *Fra1* and *Fra2* in transgenic mice resulted in the development of lung tumours and epithelial tumours respectively (Jochum *et al.*, 2000). AP-1 proteins are reported to be involved in

Table 1.3 AP-1-dependent genes (Modified and reproduced from Ozanne *et al.*, 2000)

UPREGULATED GENES	**DOWNREGULATED GENES**
MMP10	FIBRONECTIN
MMP2	COLLAGEN TYPE 1
MMP9	MGC-24
UROKINASE	TCS-1
CATHEPSIN L, B, D	FIBRILLIN-1
PAI-1	B-ACTIN
CD44	TUBULIN BETA-5
TGF *B*	PKC-Z BINDING PROTEIN
ARF-1	PROTOCADHERIN 43
RHO KINASE A	FRIZZLED RELATED PROTEIN
COX-2	FISP-12
HISTONE DACETYLASE (HAD)4	FEZ
METASTASES-ASSOCIATED	PP2A B SUBUNIT
(MTA)1	CASPASE 11
FRA1	GAS-5
FRA2	TRANSCRIPTION FACTOR S-11
C-JUN	STAT 6
*JUN*D	*C-FOS*
VEGF	

tumorigenesis as well as tumour suppression by their effects on pro-oncogenic and anti-oncogenic genes with effects on cell proliferation, transformation, invasion, angiogenesis and apoptosis (**Table 1.3**).

1.6.3.1 Cell proliferation and AP-1

AP-1 has been reported to promote cell proliferation but also has anti-proliferative activities with *jun* proteins as the major players with *fos* proteins having little effect (Shaulian *et al.*, 2001). Jun amino-terminal kinases (JNKs) cause activation of *c-jun* protein, which in turn induces the transcription genes such as cyclin D1, responsible for cell proliferation or p53, tumour suppressor and negative regulator of growth (Schreiber *et al.*, 1999; Behrens *et al.*, 1999). The significance of *c-jun* in cell growth is outlined by studies in which c-*jun*-deficient fibroblasts had a marked proliferative defect *in vitro* and *c-jun*-deficient hepatocytes failed to proliferate during liver regeneration *in vivo* (Behrens *et al.*, 2002; Schreiber *et al.*, 1999; Wisdom *et al.*, 1999). The other components of the *Jun* family, *JunB* and *JunD* are considered as negative regulators of cell proliferation. It is reported that fibroblasts from mice overexpressing *JunB* showed reduced proliferation and *JunD*-deficient immortalized fibroblasts showed increased cell proliferation (Passegue *et al.*, 2000; Weitzman *et al.*, 2000). However, *JunD* may have both positive and negative effects on cell-cycle progression as primary *JunD*-deficient fibroblasts also show reduced proliferation. An antagonistic effect of overexpression of *JunB* on *c-jun*-mediated induction of cyclin D1 was observed in fibroblasts (Passegue *et al.*, 2000). Thus, in highly proliferative tumours *c-jun*-containing complexes may be the main regulators of AP-1 DNA-binding activity and *JunB* and *JunD* may be selectively downregulated.

1.6.3.2 Cell invasion and AP-1

The AP-1 complex is reported to induce a number of genes involved in angiogenesis and invasive growth of cancer cells (**Table1.3**). For example, *c-fos* and *Fra1* regulate matrix metalloproteinases (MMPs), and proteases responsible for angiogenesis and cell invasion (Kustikova *et al.*, 1998; Hu *et al.*, 1994). *C-fos* has also been reported to target the gene that encodes the angiogenic factor vascular endothelial growth factor D (VegfD) (Marconcini *et al.*, 1999). Both *c-fos* and *c-jun* can induce epithelial–mesenchymal transition (EMT), associated with loss of cell polarity and invasiveness in mammary epithelial cells with implications on tumour metastasis (Reichmann *et al.*, 1992; Fialka *et al.*, 1996). Further evidence for importance of *c-fos* in tumorigenesis

comes from a study in which the progression of chemically induced papillomas to invasive squamous-cell carcinomas was impaired in *c-fos*-deficient mice *in vivo* (Saez *et al.*, 1995).

1.6.4 AP-1 in tumour suppression

Some of the components of AP-1 such as *JunB* and *JunD* are thought to have anti-oncogenic effects and may suppress tumorigenesis (Deng *et al.*, 1993) The anti-oncogenic activity of *JunB* was confirmed recently *in vivo* using JunB-deficient mice that carry a *JunB* transgene. The transgene rescued the embryonic lethality of JunB-deficient foetuses, but its expression was epigenetically silenced in cells of the myeloid lineage. This resulted in progressive myeloid leukaemia, with increased proliferation of granulocytic progenitor cells (Passegue *et al.*, 2001). *JunD* is thought to act as negative regulator of cell growth but its anti-oncogenic activity is less clear.

1.6.5 AP-1 and apoptosis

The activity of AP-1 components is essential for the proliferation and differentiation of many cell types. However, certain AP-1 components may also be involved in apoptosis (Kerr *et al.*, 1972). Early *in vitro* studies have indicated that increased AP-1 activity can lead to apoptosis in human tumour cells (Shaulian *et al.*, 2002). Conversely, oncogenic AP-1 has been reported to antagonize apoptosis in liver tumours (Eferl *et al.*, 2003). It has been suggested that *c-jun*, which is essential for transformation by *ras* is also involved in induction of apoptosis (Johnson *et al.*, 1996; Colotta *et al.*, 1992). High levels of *c-fos* expression were also observed in mouse in tissue in which apoptosis is part of normal development (Smeyne *et al.*, 1993). The involvement of AP-1 components in apoptosis may be the result of differential regulation of pro-apoptotic and anti-apoptotic target genes by AP-1 (**Table 1.3**). *C-jun* is reported to regulate the expression of pro-apoptotic while in T cells, both *c-jun* and *c-fos* regulate the gene encoding Fas ligand (FasL) (Kasibhatla *et al.*, 1998; Whitfield *et al.*, 2001). Another pro-apoptotic gene that is repressed by *c-jun* in tumours is *p53*. It has been reported that liver tumours with upregulated p53, which lack c-Jun, are prone to apoptosis (Eferl *et al.*, 2003). The other members of the *Bcl* family, Bcl3 is induced by *c-jun* in T cells and inactivation of JunB increases the expression of anti-apoptotic *Bcl2* and *Bcl-xl* in myeloid cells leading to reduced apoptosis (Rebollo *et al.*, 2000 ; Passegue *et al.*, 2001). The differential regulation of pro-apoptotic and anti-apoptotic genes indicates that AP-1 can promote apoptosis in some tumour types, whereas it induces survival in others.

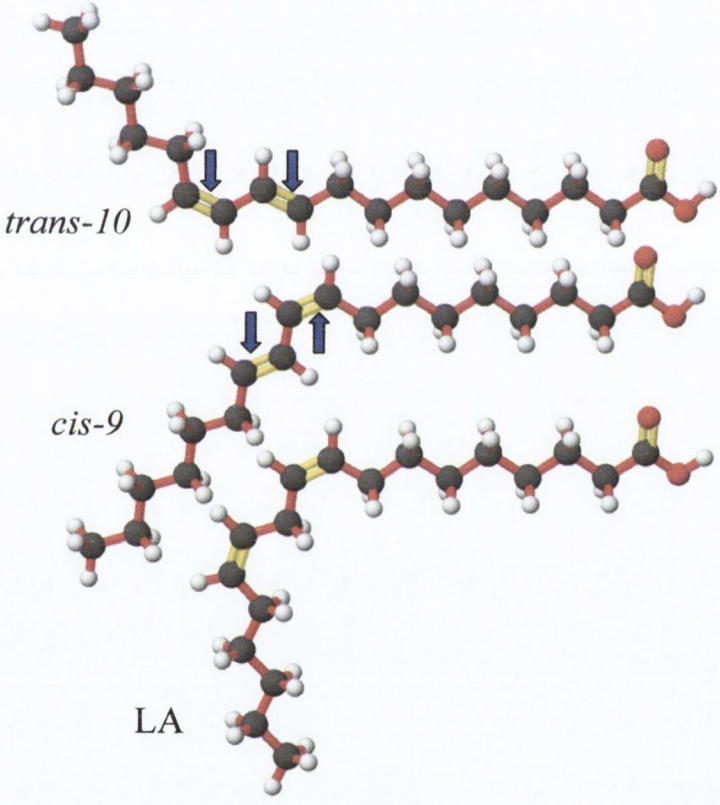


Fig. 1.10 Structures of *t-10, c-12*-CLA (top), *c-9, t-11*-CLA (center), and ordinary linoleic acid, *c-9, c-12*-octadecadienoic acid (bottom) (Reproduced from Steinhart1996)

1.7 CONJUGATED LINOLEIC ACID (CLA)

CLA was discovered in 1950s but antimutagenic effects of CLA were identified in late 1970s, at the University of Wisconsin's Food Research Institute. Michael Pariza was investigating mutagens in cooked beef. He discovered a fraction from grilled and raw beef that consistently modulated mutagenesis in the Ames (*Salmonella*) test and frequently showed marked antimutagenic activity. The active material was identified as CLA, and subsequent work by Pariza and his associates and others began to reveal its astonishing range of biological effects (Steinhart 1996). Investigators in the 1950s found that microorganisms in the rumen of ruminant animals produce CLA from polyunsaturated fat. The *c-9,t-11* isomer was shown to be the first intermediate product in the biohydrogenation of linoleic acid by the anaerobic rumen bacterium *Butyrivibrio fibrisolvens.* The reaction is catalyzed by the enzyme linoleate isomerase, which converts the cis-12 bond of free linoleic acid to a trans-11 bond. The normal intestinal flora of rats can also convert linoleic acid to the *c-9,t-11* isomer, but the reaction does not take place in animals lacking the requisite bacteria. One reason why ruminants produce more *c-9,t-11*-CLA than nonruminants is that hydrolysis of fat within the rumen provides more unesterified linoleic acid than is available to bacteria in nonruminants. CLA is found in natural sources, fat of milk, and meat of ruminant animals. CLA is the predominant naturally occurring CLA isomer in human diet (Griinary *et al.*, 2000, Bauman *et al.*, 2000). A mixture of conjugated linoleic acid isomers is also produced during food processing, by thermal isomerization and by some industrial processes by partial hydrogenation.

CLA refers to a group of positional and geometric isomers of the omega-6 essential fatty acid, linoleic acid (*cis-9, cis-12*, octadecadienoic acid). Linoleic acid (LA) is 18-carbon fatty acid with two double bonds. CLA is formed when reactions shift the location of one or both double bonds of LA (**Fig. 1.10**). LA is a single molecule and dozen different CLA isomers are possible depending on which double bonds are relocated and resultant isomeric reconjugations. The most commonly occurring CLA isomers found in diet is cis-9, trans-11, octadecadienioc acid (*cis-9,t-11* CLA). Research has been conducted on mixtures of CLA isomers, *c-9,t-11* CLA and *t-10,cis-12* CLA, octadecdienoic acid isomers. These two isomers are present in equal amounts in synthesized CLA (Kritchevsky 2000). CLA concentration of dairy products range from 2.9-8.92 mg CLA/gm fat, *c-9,t-11* CLA and *t-10,cis-12* CLA make up 73-93 % of total CLA (MacDonald 2000). CLA has been implicated in obesity, insulin resistance and as anticarcinogenic agent in animal and human studies but evidence is inconclusive.

1.7.1 CLA and cancer

Epidemiological evidence also suggests an association between intake of dietary fat and incidence and mortality of colorectal and prostate cancer (Giovannuci *et al.*, 1997; Willett *et al.*, 1989; Erickson 1998). The increased intake of LA is associated with high incidence of colorectal and prostate cancer (Erickson 1998; Zock *et al.*, 1998). The first test of CLA as an anticarcinogen in animals was carried out using the 7,12-dimethylbenz(a)anthracene (DMBA) mouse epidermal papilloma model. It was shown that treatment of the mouse skin with CLA, 5 min before DMBA treatment reduced the number of mice with papillomas as well as the number of papilloma-bearing mice (Pariza and Hargraves, 1985). Ha *et al.*, (1990) reported anticancer effects of CLA in a study in mice. Mice were given either 0.1 ml olive oil or 0.1 ml CLA plus olive oil or linoleic acid plus olive oil. All mice were given orally 2 mg benzo(a)pyrene in 0.2 ml olive oil once weekly for 4 weeks. The results showed that CLA significantly reduced tumour incidence. Then Ip *et al.* (1991) showed that dietary CLA could inhibit chemically induced mammary tumours in rats independent of other dietary fat or type of carcinogen. The effects of CLA were also investigated in other cancers. Liew *et al.* (1995) investigated the effect of CLA on IQ-induced colon carcinogenesis in male F344 rats. The carcinogen was administered at weeks 3 and 4 orally (100 mg/kg body weight) and CLA was given on alternate days orally at 5 g CLA/kg in the diet. The number of aberrant crypt foci per rat were 4·3 (SEM 2·4) in the IQ-fed controls; and 1·1 (SEM 1·3) in rats given IQ + CLA. The number of aberrant crypts in the three groups were 14·2 (SEM 11·6), and 4·0 (SEM 4·6) respectively. Belury *et al.* (1996) studied effects of increasing levels of dietary CLA on phorbol ester-promotion of skin tumours in mice. Tumour incidence decreased significantly with increasing levels of dietary CLA, from 6·71 in controls to 5·92, 4·83, and 4·67 in mice fed on 50, 10 or 15 g CLA/kg respectively. Many studies have demonstrated in *vitro* anticancer activity for CLA. It inhibited proliferation of human hepatoma cell lines (Yeoung *et al.*, 2000). CLA has also been shown to have an inhibitory effect on lung adenoma cell lines and glioblastoma cell lines (Igarashi *et al.*, *2001*). It was shown to inhibit proliferation of estrogen receptor-positive MCF-7 breast cancer cells, but not estrogen receptor-negative MDA-MB-2BI cells (Schonberg *et al.*, 1995; Durgam *et al.*, 1997). CLA has demonstrated anticancer and antimetastatic activity in prostate (Cesano *et al.*, 1998). SCID mice injected with human prostatic carcinoma cells and fed 1 % CLA for 14 weeks, had smaller local tumours and lower lung metastasis compared to controls (Liew *et al.*, 1995). Most of the animal studies have concentrated on mammary cancer. In female rats fed a diet containing 1 % CLA between early post-weaning and a

period analogous to puberty (21-42 days of age), tumour formation as a result of methylnitrosourea (MNU) administration at 56 days of age was substantially reduced. If CLA was withdrawn at any point after MNU administration during the period when breast tissue was maturing, tumour inhibition appeared to be lost (IP *et al.*, 1995). In a study in SCID mice, continuous CLA administration (1 % of diet), beginning two weeks prior to inoculation of human breast adenocarcinoma cells and continuing throughout the study time period, inhibited local tumour growth and tumour metastasis to lungs, blood and bone marrow (Visonneau *et al.*, 1997). In murine mammary cancer model, CLA is protective in mice fed a diet of 20 % fat, supplemented with no CLA or 0.1 %, 0.5 %, 1 % CLA.. Latency, metastasis and pulmonary tumour burdon of transplatable murine mammary tumours were measured. Tumour latency in mice receiving CLA diet was significantly increased, metastasis reduced and tumour burdon reduced (Hubbard *et al.*, 2000). Experimental studies have shown that in contrast to LA, CLA is an effective inhibitory agent of human colorectal, mammary and prostate cancer in vitro and *in vivo* (Park *et al.*, 2000; Rose *et al.*, 1993; Cesano *et al.*, 1998; Shultz *et al.*, 1992, 1997).

1.7.2 Anticancer mechanisms of CLA

The precise mechanisms through which CLA inhibits tumorigenesis are not yet clear though many researchers have proposed a number of mechanisms. *In vitro* research has suggested that CLA'S anticancer activity might partially be the result CLA-inducing lipid peroxidation but evidence is inconclusive (Igarashi *et al.*, 2001, Cesano *et al.*, 1998, IP *et al.*, 1996). Others e suggest that degree of CLA's activity might be the result of modifying eicosanoid production. Feeding CLA to mice resulted in a decrease in arachidonic acid production (Banni *et al.*, 1999, Belury *et al.*, 1997). Dietary CLA resulted in dose-dependent trend towards a reduction in the release of leukotriene B4 (LTB4) and reduction in serum PGE 2 levels (Sugano *et al.*, 1998). *In vivo* evidence suggests an ability of CLA to induce apoptosis may play a role in its antitumour properties (IP *et al.*, 2000). Another suggested mechanism is the modulation of membrane phospholipids by CLA. LA and other polyunsaturated fatty acids are readily incorporated into the cell membrane phospholipid, thereby modulating cell signalling responses and metabolism (Palombo *et al.*, 1996, 1999). The replicating cancer cells have increased requirement for lipids for metabolic energy and dietary intervention with fatty acids with anticarcinogenic properties presents a novel practical and relatively safe approach to reduce colon cancer proliferation. Further evidence for the effects of CLA on cell signalling comes from the reports that CLA feeding to mice for 14 weeks, significantly

reduced DAG levels (Parks 2001). CLA feeding is reported to decrease the amount of LA metabolites in mammary tissues (Banni *et al.*, 1999).

Increased expression of COX-2 and overproduction of prostaglandins (PG) have been reported in the development and progression of colorectal cancer. COX-2 inhibitors have been shown to be beneficial in colon cancer prevention (Oshima *et al.*, 1996, Prescott *et al.*, 2000). Reduced arachidonic acid, a substrate for COX and lipoxygenase, would result in reduced eicosanoid synthesis. CLA feeding in rats have resulted in the reduction of PGE 2 and 6-keto-PGF1 alpha levels in colonic mucosa (Xu 1999). *In vitro* studies have shown CLA inhibits PGE2 synthesis from arachidonic acid in murine keratocytes (Liu and Belury 1998). Eicosanoid receptors have been shown to control release of cyclic AMP, 1,2 DAG and inositol-1,4,5-triphosphate involved in cell proliferation, differentiation and apoptosis (Marks *et al.*, 2000). Colorectal tumours overexpressing COX-2 are resistant to apoptosis (Tsujii and Dubois 1995).Thus, CLA feeding might inhibit colon cancer by inducing apoptosis through mechanism involving inhibition of eicosanoid synthesis (Park 2001). Another reported mechanism for anticancer effects of CLA feeding is the reduced formation of heterocyclic amine-induced crypt foci in colon of Fisher 344 rats (Liew *et al.*, 1995; Xu 1999). COX-2 plays an important role in the development of colorectal carcinoma (Gupta *et al.*, 1998). Sulindac, a non-steroidal anti-inflammatory with COX-2 inhibitory effects causes regression of precancerous colonic lesions in animals (Moorghen *et al.*, 1988) and humans (Takayama *et al.*, 1998). CLA lowered both PGE-2 and thromboxane-2 (TXB-2) levels in colonic mucosa of DMH-injected rats (Park 2001). In vitro mouse keratinocytes cultures, CLA reduced cellular AA content and arachidonic acid-derived PGE 2 synthesis induced by TPA (Liu and Belury 1998).

1.7.2.1 CLA, Peroxisome proliferator-activated receptors (PPARs) and colon cancer

Peroxisome proliferators are a group of diverse chemicals which cause an increase in the number and size of hepatic peroxisomes, a subcellular organelle involved in lipid metabolism. A wide variety of chemicals such as hypolipidemic agents herbicides, polyunsaturated fatty acids can cause peroxisome proliferation. A nuclear hormone receptor was discovered that was activated by these chemicals (Issemann and Green 1990), hence the name peroxisome proliferator-activated receptor (PPAR). Fatty acids are the endogenous activators of PPAR.

Three subtypes of PPAR (α, β and γ) have been identified (Lemberger *et al.*, 1996). Activation of PPARα is expressed in hepatic tissue and involved in hypolipidemia and liver

tumours. PPARβ is expressed ubiquitously and with unclear function. PPARγ is essential for adipocyte and macrophage differentiation. PPARs respond to fatty acids and their metabolites. Each subtype has preferences for certain branches of the linoleic and arachidonic acid, although there is overlap among the receptor subtypes. PPARα and PPARγ play key roles in regulating fatty acid metabolism, albeit in seemingly opposite direction (Kersten *et al.*, 2000). PPARγ has shown to be beneficially involved in different types of cancer including CRC (Sarraf *et al.*, 1998). CLAs are activators of the PPAR family of nuclear receptors and the biological effects of CLAs are similar to that of potent PPARγ ligands, the thiazolidinediones, including beneficial effects cancer (Belury 2000). PPAR have also been linked to the pathogenesis of colorectal cancer and upregulation of PPARγ has been reported in a rat model with azoxymethane induced colorectal cancers (DuBois *et al.*, 1998; Girnun et al., 2002). Upto four loss-of-function mutations of PPARγ have been characterized of in colorectal cancer patients suggesting a possible role of PPARγ as a tumour-suppressor gene (Sarraf *et al.*, 1999). Another member of the PPAR, β/δ is also upregulated in human colonic adenocarcinomas and its upregulation is associated with inactivation of *APC* in vitro (He *et al.*, 1999; Gupta *et al.*, 2000).

1.7.3 Miscellaneous effects of CLA

1.7.3.1 CLA and obesity

CLA feeding has been shown to have beneficial effects in several animal models of obesity. Feeding dietary CLA (1 % of diet) to AKR/J mice for five weeks resulted in 50 % reduction in adipose tissue weight compared with mice in the group not receiving CLA. Total body weight was similar between two groups, suggesting an increase in lean mass as well as decrease in fat mass with CLA (West *et al.*, 2000). Mice fed 0.5 % of CLA for twelve weeks in another study, resulted in significant reduction in fat and increase body protein (DeLany *et al.*, 1999). Reduction in adipose tissue by CLA feeding has also been shown to occur in mice fed high-fat diets (45% of energy intake as dietary fat (West *et al.*, 2000; DeLany *et al.*, 2000; West *et al.*, 1998). In another study, growing female rats fed diets containing 0.5 % CLA experienced a modest reduction in adipose tissue mass; however, reduction was far less compared to other studies (Azain *et al.*, 2000). CLA feeding at 2.7 gm/day during a six-month period in a placebo-controlled, randomized, double blind study in humans, showed no changes in the body composition in obese subjects (Atkinson *et al.*, 1999; Zambell *et al.*, 2000). By contrast, another study reported positive influence of CLA on body composition (Blankson *et al.*, 2000). Studies have suggested that CLA might influence body composition

in part by increasing lipolysis and beta-oxidation of fatty acids and decreasing deposition of fatty acids in adipose tissue (Park *et al.*, 1997).

1.7.3.2 CLA and diabetes

Dietary CLA have been reported to increase insulin levels (West *et al.*, 2000; DeLany *et al.*, 2000, Tsigos *et al.*, 1999). In ARK/J mice CLA, feeding has been reported to cause a two-fold increase in plasma insulin levels. Female pigs fed on CLA resulted in 33 % increase fasting insulin levels (Stangl *et al.*, 1999). *In vivo* human research among women (20-40 years), confined to metabolic suite for 94 days, 3gm/day CLA resulted in an insignificant increase in mean insulin levels (Medina *et al.*, 2000).

1.7.3.3 CLA and cardiovascular health

There is suggestion that CLA might benefit cardiovascular health (Raloff *et al.*, 2001), though there is not enough evidence to support this hypothesis. Diets of New Zealand white rabbits fed on 0.1 % -0.2 %, cholesterol Supplemented with 0.1 % CLA inhibited atherogenesis (Kritchevsky *et al.*, 2000). In a study in rabbits fed 0.1 % cholesterol and 0.5%, CLA reduced atherosclerosis and lipid levels (Lee *et al.*, 1994). In humans with BMI 25-35 $kg/m^{2,}$ significant reduction in LDL, HDL and total cholesterol was found in groups receiving CLA, but was not clinically significant (Blankson *et al.*, 2000).

1.8 Objectives

Secondary bile acids, the most predominant of which is deoxycholic acid has been implicated in the pathogenesis of CRC. On the other hand, ursodeoxycholic acid has been reported to have protective effects in animal studies and clinical studies in humans. The first aim was to investigate the effects of bile acids on activation and translocation of different isoenzymes of protein kinase C. Secondly, to investigate the effects of DCA and UDCA on transcription factors, nuclear factor kappa B and activator protein-1, involved in CRC.

Thirdly, to investigate the effects of conjugated linoleic acid on PKC and NF-κB expression in CRC. As shown by the data above these CLA have protective effects in CRC but precise mechanisms are not clear. We aimed to examine the effects of CLA on the signalling molecules, compared with linoleic acid in CRC.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II

Materials and Methods

2.1 MATERIALS

2.1.1 Cell lines

HCT116, a cell line derived from adenocarcinoma patient with Lynch's syndrome, SW480 Cells, colorectal adenocarcinoma cell line and MCF7 cells, human breast adenocarcinoma cell line were obtained from American Type Culture Collection (ATTC, Rocksville, MD). The human T cell leukaemic HUT 78 cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA).

2.1.2 Cell culture reagents

RPMI1640 medium, McCoy's 5a medium, L-15, medium, foetal calf serum, penicillin, streptomycin, L-glutamine, Hank's balanced salt solution (HBSS) and trypsin were obtained from GIBCOBRL (Life Technologies Renfrewshire, Paisley, Scotland).

2.1.3 Chemicals

The phorbol 12-myristate 13-acetate (PMA), bile acids, sodium deoxycholic acid (DCA) and ursodeoxycholic acid (UDCA) and Oleic acid (OA) were obtained from Sigma Chemical Co. (St. Louis, MO). C2-ceramide and dihydroceramide were purchased from Clontech (Palo Alto, Ca). Conjugated Linoleic Acids (CLA), *c9,t11-CLA t10,c12-CLA* and Linoleic acid (LA), were purchased from Cayman Chemicals (Michigan, USA). Bovine serum albumin, ammonium persulphate, acrylamide:bisacrylamide (29:1), Nonidet P40 (NP40), PMA, leupeptin, 2-mercaptoethanol, and ampicillin, EDTA, IL-lβ and PMSF were obtained from Sigma (Poole, Dorset, UK and St. Louis, MO., USA). Agarose was purchased from GIBCOBRL (Life Technologies, Paisley, Scotland). Sodium chloride, magnesium chloride, calcium chloride, ethanol, methanol, Tween-20, acrylamide, bisacrylamide, chloroform, glycerol, acetone, acetic acid, dimethylsulphoxide, TEMED and Tris (hydroxymethyl) aminomethane (Tris) were obtained from BDH Ltd (Poole, UK). Ethylether was obtained from Lennox Laboratories Ltd., (Dublin, Ireland). Ethanol was obtained from Merck KgaA, (Darmstadt, Germany).

2.1.4 Plasmids and PKC inhibitors

The plasmids PKC ε-EGFP and pEGFP were kindly provided by Dr. Naoaki Saito (Kobe University, Kobe Japan). PKC β1-EGFP PKC δ-EGFP and PKC ζ-EGFP plasmids were

purchased from Clontech (Palo Alto, Ca). PKC inhibitors, Calphostin C, Indolocarbazole (Gö6976), Bisindolylmaleimide were from Calbiochem Novabiochem Corp., (La lolla, CA).

2.1.5 Oligonucleotides and antibodies

NF-κB consensus oligonucleotide was obtained from Promega (Promega Corp., Madison, WI). polyclonal antibodies, anti-I kappa B-alpha (IκB-α) and anti-p65, anti-p50 (sc-114X), anti-RelA (anti-p65 (sc-l09X) and anti-c-Rel (sc-70X) and anti-Fos and anti-Jun (c-jun, junD, junB, c-fos, fos-B, fra-1, fra-2) for gel supershift assays were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2.2 CELL CULTURES

2.2.1 Maintenance of cell cultures

All cell lines were grown in medium supplemented with 10% foetal calf serum (FCS), 100 Units/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine. The cells were used between passages 5-20 and maintained at 37° C in a humidified incubator containing 5 % CO2. Media was renewed every third day and cells split every fifth day. Cells were grown to 70-80% confluence depending on the experiment. The medium was stored at 4° C and used within two weeks, as glutamine can become enzymatically converted by serum enzymes. A stock solution of penicillin/streptomycin and L-glutamine was prepared and stored in aliquots at -20°C [Appendix A]. Aliquots of FCS were prepared, by heat inactivating complement at 56° C for 60 minutes.

2.2.2 Storing cells

Stocks of viable cells were kept by cryopreservation. Sterile FCS containing 10 % dimethylsulphoxise (DMSO) was used for freezing the cells, with aliquots of 1X 10^6 cells/ml in sterile cryoresistant tubes (Sarsted, Numebrech, Germany) at -70° C and then transferred to liquid nitrogen. Cells were subcultured after trypsinization. For resuscitation, frozen cells were thawed and resuspended in 10 ml medium. Cells were then centrifuged at 1500 rpm for 5 minutes, washed twice with HBSS and then resuspended in tissue culture flasks containing medium in a CO2 incubator at 37° C.

2.2.3 Cell counting

Ethidium bromide (EB) and Acridine orange (AO), fluorescence staining was used for counting and determining the viability of the cells. Cells were diluted (1/10) with EB/OA solution for counting at Neubauer haemocystometer. The cells were counted and amount of

cells to be seeded was calculated. The cells were then seeded at 5 X 10^5 cells/ml and grown to 70-80 % confluence.

2.3 TRANSIENT TRANSFECTION

2.3.1 Transformation of bacterial cells

Competent *E. Coli* DH5α cells were thawed on ice for approximately 30 minutes. Competent *E. Coli* DH5α cells were transformed by incubating 100 µl of the cells with 2-5 µl of (5ng/ µl) the plasmid DNA. The competent cells (100 µl) were also incubated without DNA as a control. The competent cells with plasmid DNA were incubated on ice for 30 minutes, followed by heat shocking the cells for 1-2 minutes at 42°C. Cells were cooled on ice for 1-2 minutes before being transferred to 1 ml warm broth (LB) medium [Appendix A]. Cells were allowed to recover for 2 hours with gentle shaking at 37°C. Transformed cells were then plated onto L-agar containing ampicillin or kanamycin and left for 12-24 hours at 37°C. Single colony purification was then performed by taking a single colony and growing this on a new plate overnight at 37°C. Pure colonies from the plates were again incubated on new plates for further 12-24 hours at 37°C. LB broth (5 ml) was inoculated with pure colonies, and cells were grown for 8 hours at 37°C under ampicillin/kanamycin antibiotic selection to make starter culture. Starter culture (100 µl) was inoculated with 40 ml of LB medium with antibiotics selection and grown overnight at 37°C. Stock solutions were made by adding 930 µl aliquots of growing culture into sterile cryotubes containing 70 µl DMSO and stored at -70°C. Plasmid DNA was isolated from this secondary culture using Gene Elute Endotoxin-free Plasmid Midiprep Kit (Sigma Chemical Co. St. Louis, MO).

2.3.2 Preparation and purification of plasmid DNA from E. coli DH5α (Midiprep DNA Peparation)

DNA for transfection purposes was isolated using the Gene Elute Endotoxin-free Plasmid Midiprep Kit. The procedure was as follows; 20 ml of overnight bacterial culture was pelleted in an Oak Ridge tube by centrifuging at 3,000-5,000 g (4000 RCF) on Beckman centrifuge for 10 minutes. All the media supernatant was removed. The bacterial pellet was completely resuspended with 1.2 ml of Resuspension Solution by pipetting up and down until homogenous. The RNase A Solution was added to the Resuspension Solution Prior to first time use. Resuspended cells were lysed by adding 1.2 ml of Lysis Solution and immediately mixed by gentle inversion. The mixture was incubated at room temperature until clear and viscous.

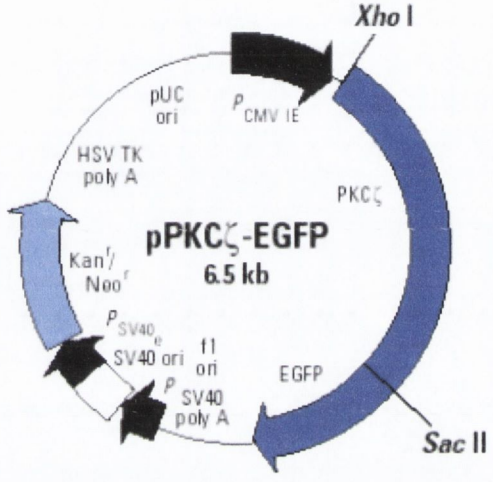
Neutralization Solution (0.8 ml) of was added to the lysate, and mixed thoroughly by gentle inversion. Cell debris was pelleted by centrifuging at 15000 g for 15 minutes at 4° C. The cleared lysate was then transferred in to 15 ml conical tube. The steps were repeated to remove debris further. Endotoxin Removal Solution (300 µl) was added to the lysate. It was mixed thoroughly by inversion for 1 minute. The tube was chilled on ice for 7 minutes and mixed a few times during the ice incubation. The clearblue solution was heated in a 37°C water bath for 5 minutes until turned cloudy. The tube was then centrifuged at 4000 g in a swing bucket rotor for 5 minutes at room temperature. The clear upper phase containing the plasmid was transferred into a fresh 15 ml conical tube. The blue lower phase was discarded. All the steps were repeated for further removal of the endotoxins. The DNA Binding Solution (0.8 ml) was added to the endotoxin-free lysate and mixed thoroughly by inversion or vortexing. The lysate was loaded into a GenElute Midiprep binding column seated in a collection tube. The tube was centrifuged in a swinging bucket rotor at 3,000-5,000 g for 1-2 minutes. The flow-through liquid was discarded. Optional Wash Solution (2.0 ml) was added to the column and centrifuged in a swinging bucket rotor at 3,000-5,000 g for 2 minutes. The flow-through liquid was discarded. The column was then washed with 3 ml of diluted Wash Solution and centrifuged in a swinging bucket rotor at 3,0005,000 g for 5 minutes. The column was transferred to a fresh collection tube and 500 µl of endotoxin free water added to the column. The mixture was centrifuged in a swinging bucket rotor at 3,000-5,000 g for 4 minutes. The flow-through elute containing the DNA was collected and stored at -20°C. Recovery and purity of the plasmid DNA was determined by spectrophotometric analysis and the size and quality of DNA was determined by agarose gel electrophoresis.

2.3.3 Restriction endonuclease digestion for PKC ζ

Restriction endonucleases are bacterial enzymes, which hydrolyze (cut) DNA into defined and reproducible fragments. In bacteria, they form part of restriction-modification defence mechanism against foreign DNA for example viruses. Bacterial DNA is protected from these enzymes by process of methylation. The identification of restriction enzymes in 1960s and 1970s was the key discovery, which allowed the cloning of DNA to become a reality. In the experiment restriction enzymes, NdeI was made to cut and analyse PKC ζ DNA and to compare and contrast the fragments patterns after agarose gel electrophoresis. The microfuge tubes were labelled and DNA. After adding 1 µl NdeI in one tube with 2.5 µl Restriction enzymes Buffer (10x) and ddH2O to each tube, the solution was mixed and centrifuge to bottom of tubes and incubate for 4 hours at 37°C. Agarose gel analysis was performed as

described below. BP ladder was also included (Fig 2.1-2.5). As shown below NdeI cuts PKC ζ in 2, at 235 and 5547 bases. This is illustrated in Fig 2.5.

Digest of pPKC zeta-EGFP: 6455 bases (circular)



Map of pPKCζ-EGFP Vector. All restriction sites are unique

```
 Bgl I                  NdeI                          AatII
  |                      |
CTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACG
GAACCGTCATGTAGTTCACATAGTATACGGTTCATGCGGGGGATAACTGCAGTTACTGC
       220       230       240       250       260
```

2.3.4 Agarose gel electrophoresis

Agarose gel was prepared by adding 0.32 gm of agarose to 40 ml of 1 X TAE buffer. The mixture was then heated in the microwave to dissolve agarose. This 0.8 % agarose gel solution was cooled and poured in to gel box. The loading gel buffer was added to the sample DNA and DNA of known concentration, mixed and loaded on to the gel. In case of PKC ζ, restriction digest along with BP ladder were also loaded. The gel was run at 90V, 100 mA for 90 minutes. The gel was then stained with Ethidium bromide for 30 minutes and examined under ultraviolet light and photographed (Figures 2.1-2.5).

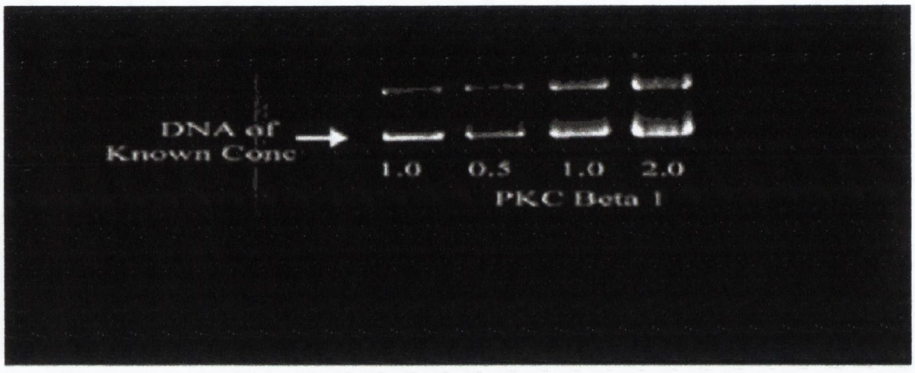


Fig 2.1 Agarose gel, showing PKC β1-EGFP plasmid DNA against a DNA of known concentration.Quality and concentration of PKC β1plasmid DNA was assessed for transfection experiments.

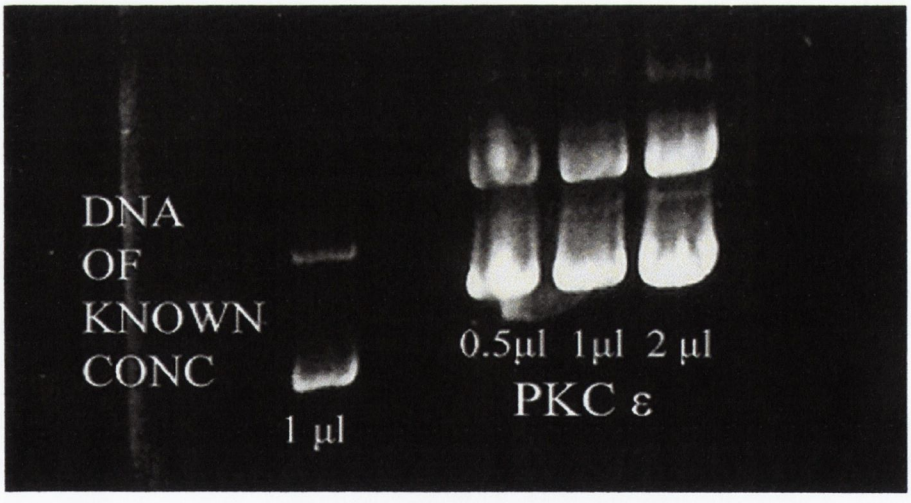


Fig. 2.2 PKC epsilon-EGFP plasmid DNA Agarose gel

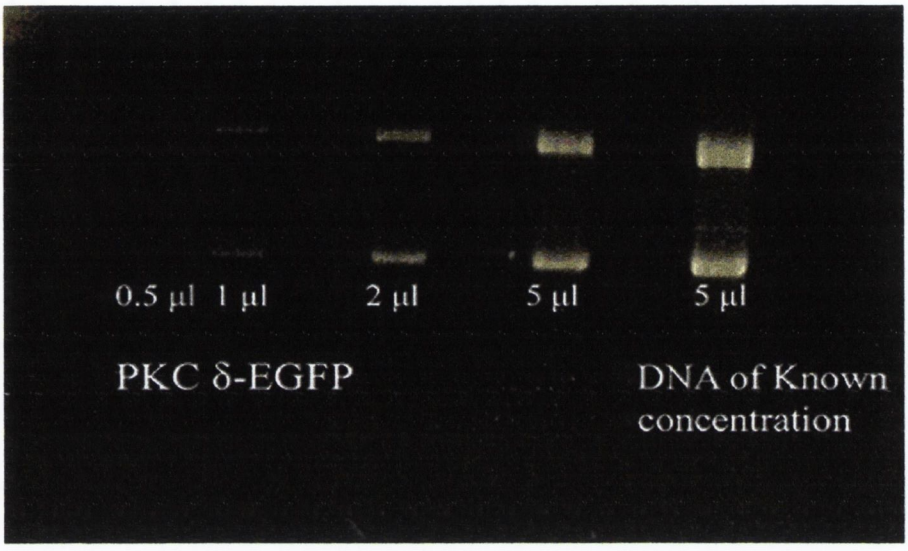


Fig. 2.3 PKC Delta-EGFP plasmid DNA Agarose gel

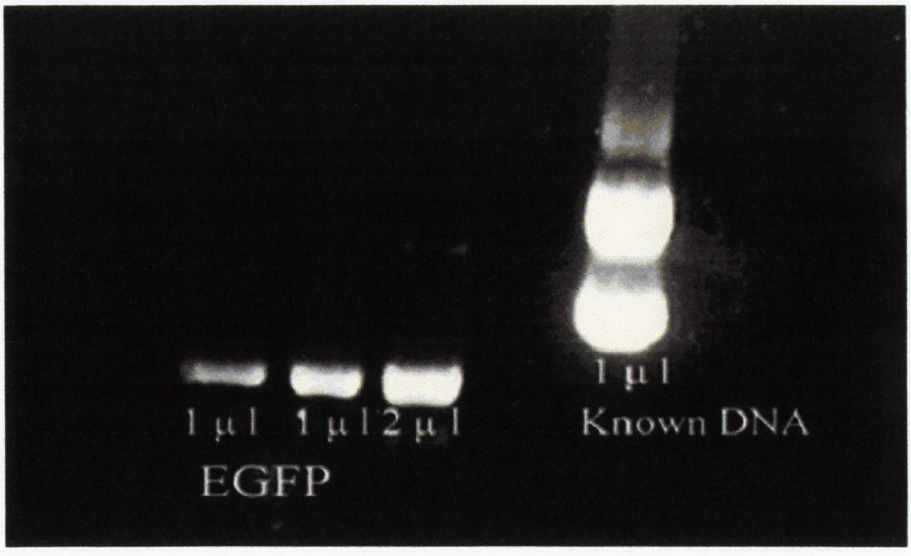


Fig. 2.4 EGFP plasmid DNA Agarose gel

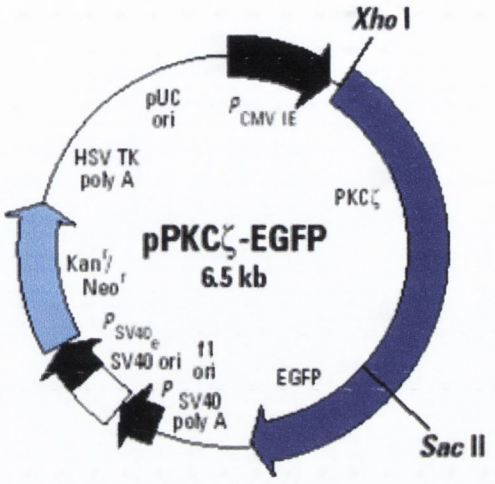


Fig. 2.5A Map of pPKCz-EGFP Vector. All restriction sites are unique.

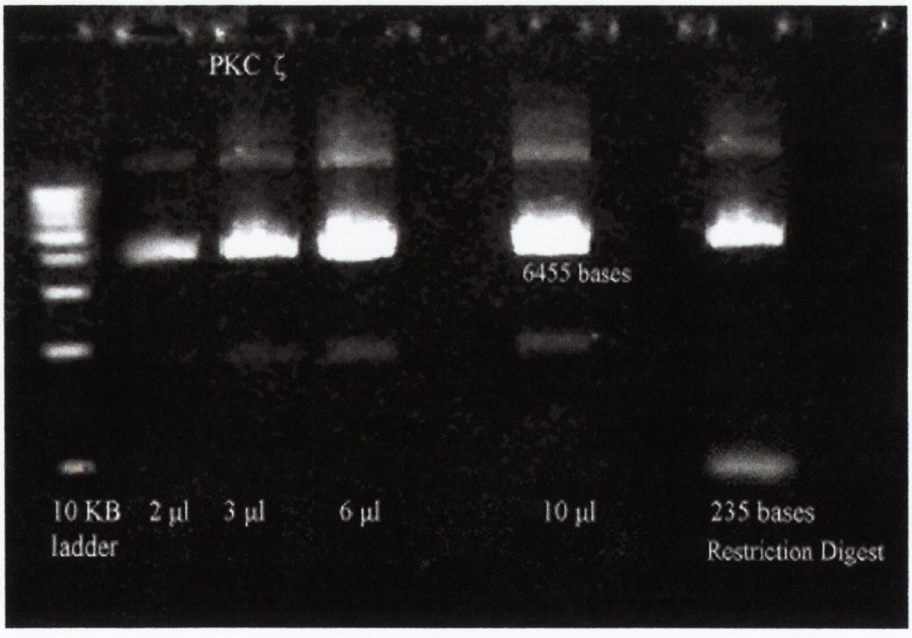


Fig. 2.5B PKC ζ restriction digest and agarose gel analysis. PKC ζ DNA is 6455 bases. Restriction digest with Ndel cuts PKC ζ DNA in to 5547 and 235 base as shown.

2.3.5 Spectrophotometric analyses of PKC isoforms

A cubet, 78 µl of sterile water and 2 µl of the sample PKC DNA and the other with only 80 µl of sterile water were loaded in to spectrophotometer and read at 260 nm and 280 nm. The ratios of absorbance determined at 260 nm to 280 nm (A_{260}/A_{280}) was 1.7 to 1.9 for the DNA of all the PKC isoforms, used in our experiments. Similarly, concentrations were also determined and appropriate concentrations of PKC isoenzyme DNA and EGFP DNA were calculated for efficient transfections.

2.3.6 Transfection of the PKC-EGFP fusion constructs to the cultured HCT116 cells

The HCT116, SW480 cells or MCF7 cells were seeded in 8 well Permanox glass chamber slides (Nunc, Naperville, IL) or 6 well plate at a density of 1 x 10^4 or 2 x 10^5 respectively. The cell cultures were 60-90% confluent on the day of transfection. The cells were transfected with 1-5 µg of plasmid construct carrying DNA (PKC β1-EGFP, PKC ε-EGFP, PKC δ-EGFP, PKC ζ-EGFP or EGFP only), using GenePORTER transfection reagent (Gene therapy Systems Inc., San Diego, CA) according to the manufacturer's instructions. GenePORTER Reagent is the formulation of neutral lipid dioleoyl phosphatidylethanolamine (DOPE) and a proprietary cationic lipid derived from direct hydrophilic conjugation used in human gene therapy trials. It has high transfection efficiency, low cytotoxicity, and exceptional stability. Briefly, the DNA (1-5 µg) was diluted with serum-free medium using half of the transfection volume (125 µl for 8 well and 500 µl for 6 well plates) and the GenePORTER reagent was diluted with serum-free medium using the other half of the transfection volume. The diluted DNA was added to the diluted GenePORTER reagent and mixed rapidly and incubated at room temperature for 30 minutes. The culture medium was aspirated from the cells and the DNA-GenePORTER mixture was added to the cells and incubated at 37°C for 4 hours. Four hours post transfection, 250 µl (500 µl for 8 well plates) of the McCoy's medium containing 20 % FCS was added and incubated overnight under 5-10 % CO2 at 37°C in humidified incubator. The cells were examined for transfection the next day fresh growth media was added. Photographs of the cells were taken to verify transfection efficiency comparing with EGFP only (Fig. 2.6). In all experiments, EGFP transfection was performed for control.

2.3.7 Cell activation and analyses of EGFP-tagged PKC

Transfected HCT116 cells expressing PKC-β_1-EGFP, PKC ε-EGFP, PKC δ-EGFP were treated with 150-300 µM DCA, 1 µM PMA, and 150-300 µM UDCA alone for 2 hours or

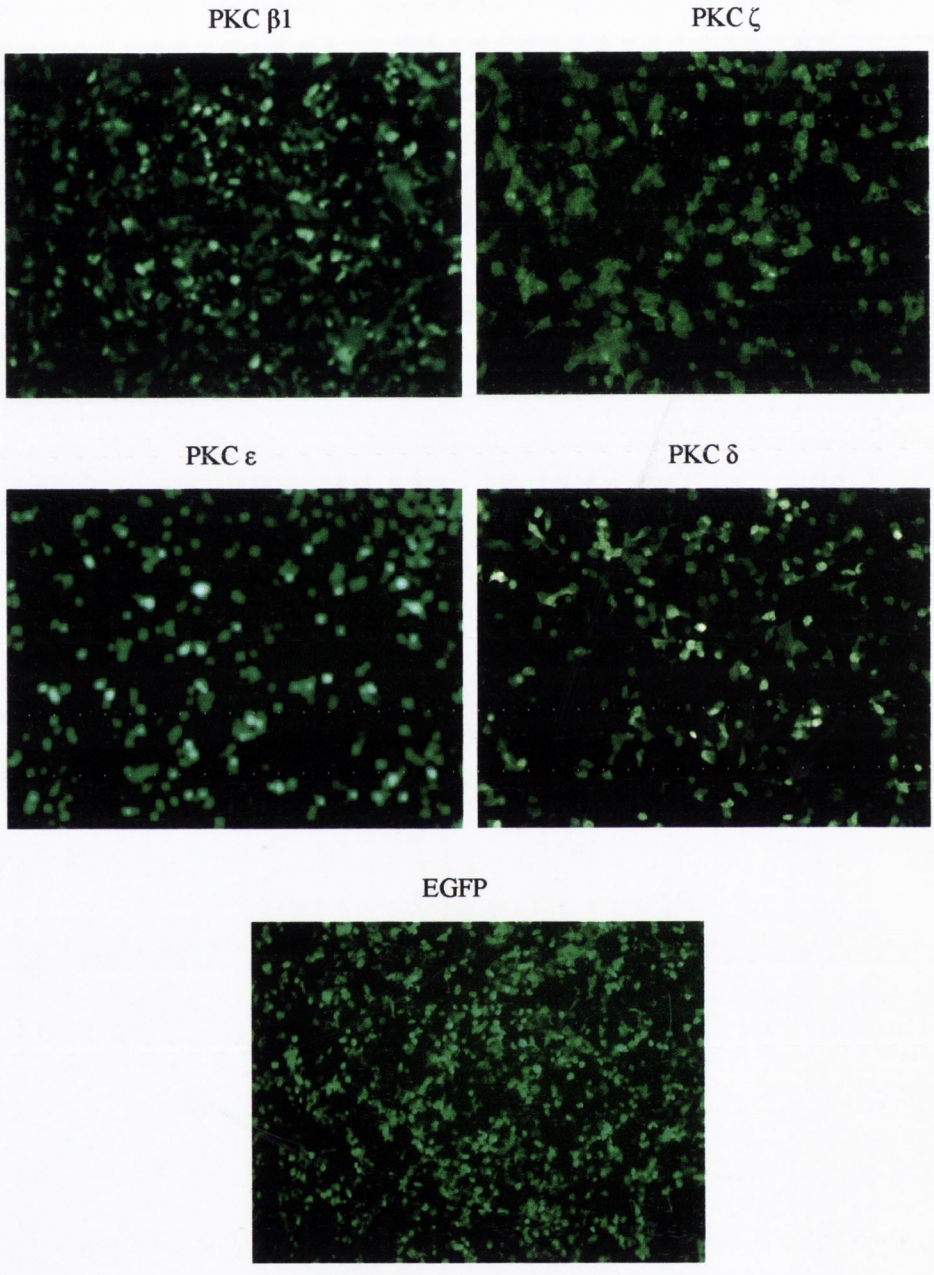


Figure 2.6 Transfection Efficiency of PKC β1, ζ, ε, δ-EGFP compared with Enhanced Green Fluorescent Protein (EGFP) alone.

pretreated with UDCA for 2 hours and then stimulated with DCA or PMA. Both DCA and UDCA were freshly prepared and dissolved in DMSO to standardize the effects of these two bile acids. As the atypical PKC ζ do not respond to phorbol esters, C2-ceramide was used as control for experiments with PKC ζ., because ceremide is know to activate this PKC isoenzyme. HCT116 cells transfected with PKC ζ-EGFP were treated with C2-ceramide and dihydroceramide at doses 25- 200 μM. It was observed that 300 μM was the optimal concentration of the bile acids to observe changes in all the PKC isoenzymes. During pretreatment of the cells, UDCA was either left in or taken out before activation with DCA or PMA for different experiments. SW480 cells and MCF7 cells were transfected with PKC β1-EGFP and similarly treated. Fast rate acquisition photo microscopy using Photometrics KAF-0400 cooled digital CCD camera was employed to observe the effects of treatments.

For CLA experiments, HCT116 cells were pretreated with *c9,t11-CLA or t10,c12-CLA*, LA or OA at 100-200 μM concentrations for 24 hours for acute treatment and 50 μM for 14 days for chronic. Cells were grown in 8 well permanox glass chambers for acute treatment and in small flasks for chronic treatment. Cells were cultured in serum free media for six hours before CLA treatment to minimise the interference of FBS with CLA. These treated cells were then transfected as above and activated.

For NF-κB experiments, HCT116 cells were either pre-treated with 300 μM UDCA for 2 hours and the stimulated with 300 μM DCA or 20ng/ml IL-1β for 2 hours or treated alone with UDCA, DCA or IL-1β.

For AP-1 experiments, the cells were either pre-treated with 300 μM UDCA for 2 hours and then activated with 300 μM DCA, 20ng/ml IL-1β or 20 nM PMA for 4-6 hours or treated alone with DCA, UDCA, IL-1β or PMA for 4-6 hours.

2.3.8 Effects of PKC inhibitors on PKC-β1-EGFP translocation

HCT116 cells, transfected with PKC β1-EGFP were treated with Calphostin C, at doses 100-600 nM, Go6976, 1-6 μM Bisindolylmaleimide, 1-6 μM for half an hour and then stimulated with either PMA or DCA to elucidate the mechanisms involved in PKC translocation and its relevance to cell shape change.

2.4 IMMUNOLUORESCENCE PROTOCOL

2.4.1 F-Actin and nuclear staining

After establishing a consistent pattern of effects of treatment by real time photomicroscopy, the cells were prepared for subsequent examination. The cells were washed with sterile

filtered PBS twice and fixed with 400 µl of 4 % paraformaldehyde per well for 20 minutes at room temperature. The cells were stained with tetramethylrhodamine isothiocyanate (TRITC) conjugate of phalloidin (diluted 1/200 with PBS), for 20 minute at room temperature to visualize PKC translocation to F-actin. The cells were washed gently with PBS and then nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI) for 5 minutes at room temperature. After a wash with PBS, excess liquid was removed from the slides. A drop of fluorescent mounting media (DAKO) was added to the slide, coverslip applied gently to avoid trapping of air bubbles. The slides were left to dry at room temperature in dark for subsequent examination by fluorescent microscopy. Slides were later examined using Nikon TE 300 inverted microscope equipped with Leica DC 100 color digital camera. Photomicrographs were taken using oil immersion lens at 100 X magnification.

The TE300 is a multi-port inverted optical microscope with infinity optics and has fluorescence capacity for FITC and Rhodamine excitation-emission wavelengths, differential interference contrast (DIC) optics and a temperature regulated stage. The objectives include 10, 20 and 40X CF infinity plan fluor dry objectives. High-resolution immersion objectives include a CF1 plan fluor 40X oil (N.A. 1.30), a DF1 plan apo 60X oil (N. A. 1.40) and a Cf1 plan apo 100X oil (N.A. 1.40). It has fluorescence cubes cube A: UV - Excitation BP 340 - 380, Emission LP 425,Cube I3: Blue light - Excitation BP 450 - 490, Emission LP 515 Cube N2.1: Green light - Excitation BP 515 - 560, Emission LP 590. Different filters were used for FITC (green), TRITC (red), phalloidin (red), Dapi (blue) and GFP (green) anlyses.

2.4.2 Immunofluorescent staining for endogenous PKC isoenzymes

HCT116 cells were cultured on 8 well Permanox chamber slides (Nun, Naperville, IL) for 24 hours to 60-70% confluence, treated with 150-300 µM DCA, 100 nM PMA, 150-300 µM UDCA and 50 µM C2-ceramide alone for 2 hours or pretreated with UDCA for 2 hours and then exposed to DCA or PMA or C2-ceramide. The cells were washed with warm PBS and fixed by gentle immersion in acetone at $-20^{o}C$ for 10 minutes. Permealisation of fixed cells was carried out using 0.1 % Triton X-100/PBS for 30 minutes. This was followed by a blocking with 40 µl of normal goat serum. PKC α, β, ε, δ, ζ-rabbit polyclonal antibody kit (R&D, CA) were used to determine their subcellular localisation. The cells were incubated with primary antibodies 1 hour at room temperature. The slides were washed and incubated with Alexa-Fluor 488 conjugated anti-rabbit secondary antibody, mounted under coverslips with fluorescence-

preserving mounting medium (DAKO) and photographed using Nikon TE 300 inverted microscope equipped with Leica DC 100 color digital camera.

2.4.3 Immunofluorescent staining for NF-κB

HCT116 cells were cultured on 8 well Permanox glass chamber slides (Nunc, Naperville, IL) for 24 hours to 60-70% confluence. The cells were then treated with 300 μM DCA, 20 nM PMA, 20 ng/ml IL-1β or 300 μM UDCA alone for 2 hours or first pretreated with UDCA. The slides were gently washed with sterile phosphate-buffered saline (PBS), fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton X-100 in PBS for 10 min. The slides were incubated with primary antibody (anti-RelA) for 1 hour at room temperature, and then washed three times in 0.1 % Tween 20 in PBS, followed by 30 minutes incubation with TRITC or FITC-conjugated secondary antibody. Coverslips were mounted and images were acquired on Nikon TE 300 inverted microscope equipped with Leica DC-100 colour digital camera.

2.5 PREPARATION OF CELL PROTEIN

2.5.1 Preparation of whole cell extracts

Confluent cells in 6 well plates (2 ml volume) were treated, as described in previous sections and whole cell extracts were prepared. Treatment was terminated by washing cells with ice-cold PBS, pH 7.4 [Appendix A], and cells were collected by scraping and centrifugation, and subsequent steps were carried out on ice. Cells were lysed in 0.5 ml PBS containing 1 μl PMSF (0.1 mM), 3 μl leupeptin (2 mg/ml) and 0.5 ml 1% NP40 [Appendix A], vortexed every minute for 5 minutes to deposit cell debris. The supernatants were removed and stored at -20°C.

2.5.2 Protein estimation

The protein concentrations were determined by the dye-binding methods of Bradford or Lowry as described below, in order to standardize the amount of the protein in each lysate [Appendix A]. Bovine serum albumin (BSA) served as the protein standard.

2.5.3 Preparation of subcellular fractionation

Subcellular fractions of PKC β1-EGFP transfected and treated cells were prepared with protocols by Chakravarti (Chakravarti *et al.*, 1989), with some modifications as described. Cells were washed in ice cold PBS and resuspended in 0.5-1 ml of hypotonic lysis buffer ($NaHCO_3$ 1mM, $MgCL_2$ 5 mM, 5 μl of .1mM PMSF and 15 μl of 10mg/ml leupeptin (all

reagents from Sigma). Samples were vortexed for 2 minutes and sonicated at 15 seconds interval on power 3 for total of I minute at 4°C and spun at 600g for 5 minutes at 4°C to remove nuclei and unlysed cells. Then supernatant was centrifuged at 100000g in ultracentrifuge for 30 minutes at 4°C. The resulting supernatant was designted as cytosolic fraction. The pellet was then washed in ice cold PBS, centrifuged at 420 g for 10 minutes, washed and resuspended in buffer B (20 mM Tris-HCL, PH7.5, containing 1 % (w/v) Nonidet P-40, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1 mM PMAF and 10mg/ml leupeptin) and centrifuged at 420g. The resuspended pallet was vortexed at 5 minutes interval for 30 minutes at 4°C and ultracentrifuge at 100,000 g for 30 minutes. The supernatant was designated as the detergent-soluble membrane fraction. Protein concentrations were determined by Lowery reagent method (Lowery *et al.*, 1951), before polyacrylamide gel electrophoresis (PAGE).

2.5.4 Lowry reagent method for determination of protein concentration

Protein concentrations of the subcellular fractionation were determined by method described by Lowery. Briefly, 25 mg of Bovine serum albumin (BSA) was diluted with 25 ml of PBS. Tubes were labeled and 0-100 % BSA as protein standard. In a separate set of tubes, 10 µl of the protein and 90 µl of PBS were added to each tube. To all tubes 900 µl of Reagent C (25 ml Reagent A and 250 µl of Reagent B) [Appendix A], was added. This mixture was quickly vortexed and left at room temperature for 10 minutes. Then 90 µl of Reagent D [Appendix A], was added to all the tubes and incubated for 45 minutes. Photospectrometric reading was performed at 660 nm. A standard curve was constructed using BSA solution at 0, 25, 50, 75 or 100 mg per ml of the solution. The sample concentration was determined within this range and test solution falling outside the range were diluted 1:2, 1:5, 1:10.

2.6 POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

2.6.1 Preparation of protein samples and molecular weight markers

After protein estimation, 50 µg of sample protein were aliquoted. This volume was diluted 1:5 with ice-cold acetone to concentrate the protein sample, vortexed and allowed to incubate at -20°C for at least 30 minutes. The samples were then centrifuged at 15,000 g for 2 min and the supernatant was discarded. Excess acetone was allowed to evaporate at room temperature for 15 min. Samples containing 50 µg were then suspended in 20 µl of 1 X sample buffer [Appendix A]. Samples were vortexed and boiled for 5-10 min and

centrifuged to precipitate any insoluble solids. The molecular weight markers were prepared in a similar fashion.

2.6.2 Polyacrylamide gel electrophoresis (PAGE)

Equal amounts of proteins were separated on 10% non-reducing polyacrylamide gel. Both resolving and stacking gels were prepared in the order indicated in Table 2.1 [Appendix A for composition of solutions]. The APS and TEMED were added last with gentle swirling of the mixture. Samples containing 50 μg and standards were loaded into the wells of the gel, the electrodes were connected, and the gels were run at 25 mA. At the end of electrophoresis, the proteins on the gel were electrotransferred to PVDF membrane (Gelman Sciences Inc., Ann Arbor, MI, USA) by semi-dry transfer. Following transfer, the PVDF membrane was processed for immunoblotting. The lane with the molecular weight marker was stained with Commaasie Blue [Appendix A], followed by destaining with 50% methanol.

2.6.3 Immunoblot detection

PVDF membranes were incubated with a freshly prepared PBS containing 5 % nonfat skimmed milk for 1 hour with gentle shaking (Orbital Shaker S03, Stuart Scientific, UK) at room temperature to block non-specific proteins (Blotto- Tween solution) [Appendix A]. The membranes were washed twice with PBS and incubated with specific primary antibodies (PKC β1, p65 or IκB-α.) in sealed plastic bags in a dilution 1:500 to 1:1000 in Blotto-Tween for 1 hour with shaking at room temperature followed by washing 10 minutes each time, with PBS-Tween. Blots were then incubated with the relevant secondary antibody (antimouse antibody, Swine anti-rabbit for polyclonal antibodies) horseraddish peroxidase conjugate in a dilution 1:1000 to 1:2000 in PBS-Tween for 1 hour at room temperature with gentle shaking. Blots were washed again (x3), 10 min each time, with PBS-Tween. The immunoblots were developed using enhanced chemiluminescence method (ECL).

Table 2.1: Gel composition for PAGE electrophoresis

Component	Resolving gel (10%)	Stacking gel (10%)
Distilled water	8.23 ml[a]	5.55 ml
Resolving buffer	5.0 ml	--
(1.5 M Tis , pH 8.8)		
Stacking buffer	--	3.05 ml
(1 M Tris, pH 6.8)		
Acrylamide/Bisacrylamide	6.66 ml	1.33 ml
10% APS	100 µl	50 µl
TEMED[b]	10 µl	10 µl

10% APS: 0.1g/1 ml
a: Volume (20 ml) is sufficient for 2 gels b: N,N,N' ,N' -tetra-methylenediamine

2.6.4 Enhanced chemiluminescence (ECL)

Proteins in immunoblots were detected by enhanced chemiluminescence (ECL) method. The blot membranes were incubated for 1 minute in a solution of iodophenol (400 µM), luminol (1.25 mM) and hydrogen peroxide (0.01 % v/v) in 0.I M Tris-HCI (pH 8.8) [Appendix 1]. The membranes were then placed between two acetate sheets, wiped out to exclude air bubbles, and any residual developing buffer solution. Blots were exposed to Kodak X-OMAT S film for 10-30 seconds. The films were then developed using automatic developer (CURIX 60, AGFA, Type 9462/100/140, Agfa Gevaert AG Miinchen, Germany).

2.7 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

2.7.1 Cell fractionation and nuclear extract preparation

The nuclear extracts were prepared as follows. The cells were grown in 6 well plates to 80-90 percent confluence. They were treated as described above and treatments were terminated by removal of the medium and washing the cells twice with ice-cold PBS. All subsequent steps were carried out on ice using ice-cold buffers. Cells were removed with a cell scraper, and transferred to centrifuge tubes on ice. They were pelleted by centrifugation at 1400 rpm for 5 min and washed once in (1 ml) buffer A [Appendix A], and centrifuged at 10,000 rpm for 10 minutes. The pellet of cells was then resuspended in 20 µl buffer A (containing 0.1 % (v/v) Nonidet NP40 for 10 minutes on ice and lysed cells were centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. The nuclear pellet was extracted with 15 µl buffer C [Appendix A] for 15 minutes on ice. After incubation, the nuclei were centrifuged at 10,000 rpm for 10 minutes and the supernatant was diluted with 4 volumes of buffer D

[Appendix A]. The nuclear extracts were also prepared from HuT 78 cells, constitutively expressing NF-κB, and used as positive controls for EMSA assays. The protein estimation in the nuclear extracts was performed using Bradford method described above and then used for Gel shift assays or stored at -70°C.

2.7.2 Labelling of consensus oligonucleotides for EMSA

The 5'-end-labelling of the 22 base pair oligonucleotide containing the NF-κB consensus sequence (5'-AGT TGA GGG GAC TIT CCC AGG C-3')(3'-TCA ACT CCC CTG AAA GGG TCC G-5') was performed. The reaction mixture was assembled in a sterile microcentrifuge tube in a final volume of 50 µl with sterile water, Table 2.2. The reaction mixture was incubated in a pre-warmed perspex box at 37°C for 10 minutes and the reaction was terminated by addition 2 µl of 0.5M EDTA. To this mixture, 50 µl of phenol:chloroform solution (1 part TE-saturated phenol and 1 part choroform:isoamyl alcohol (24: 1 ratio) was added to extract the DNA, and vortexed for 1 minute and centrifuged at 13,000 g for 2 minutes. The upper aqueous layer was transferred to a fresh tube and 2 µl of 5M NaCl was added. The tube was vortexed and 100 µl of ethanol was added followed by incubation at -70°C for 30 minutes to allow ethanol precipitation of the DNA. The mixture was centrifuged at 13,000 g for 5 minutes, the supernatant was carefully removed and the pellet was dried in vacuum dryer. Finally, the pellet was resuspended in 50 µl of TE buffer [Appendix A].

Table 2.2: Constituents of consensus oligoneucleotide for labelling reaction

Component	Amount
5'-ends of DNA probe (1-50pmol)	5.7 µl
T4 polynuclotide kinase (10-20 U)	2.5 µl
10X Kinase buffer	5.0 µl
[^{32}-p]A TP (3000 Ci/mmol, 10mCi/ml, 50pmol)	15 µl
Sterile water	21. 8 µl
	Final volume=50 µl

To calculate the activity of the labelled oligonucleotide, 1 µl of this solution was counted in 5 ml Ecosint and counted using a Wallac 1409 DSA Liquid Scintillation Counter. The labelled oligonucleotide (10,000 cpm) was used per reaction for EMSA.

2.7.3 Preparation of gels for EMSA

The nuclear extracts proteins were separated on 5% polyacrylamide non-reducing gels using the ATTO gel system (ATTO, Japan). The glass plates were washed and cleaned thoroughly to remove any residue, which may interfere with running of the gel and allowed to air dry. The composition of the gel is shown in Table 2.3. Both DTT and TEMED were added last to the gel with gentle swirling of the gel solution. The gel mixture was poured gently into a 1 mm thick gel mould, and allowed to polymerize at room temperature for 30 minutes.

2.7.4 Preparation of DNA-protein binding reaction

The binding reaction for detection of NF-κB activity was prepared by electrophoresis. Nuclear extract proteins (4 µg) were incubated with 10000 cpm of the ^{32}P-labelled NF-κB. The assay was performed in 20 µl binding reaction in the presence of binding buffer [Appendix A] and 2 µg of poly(dI-dC) as non specific competitor [Table 2.4].

Table 2.3: Gel formulation for EMSA

Component	Amount
Distilled water	19.4 ml
Acrylamide Mix (40%)[a]	3.125 ml
10X TBE buffer	2.5 ml
Ammonium persulphate	0.05 g
TEMED[b]	15 µl
DTT[c]	5 µl

a: Accugel (29:1) b: N,N,N,N',N'-tetra-methylenediamine c: Dithiothreitol

The reaction mixture was incubated at room temperature for 30 minutes. The binding reaction was terminated by addition of one tenth volume of gel loading dye [Appendix A] and samples loaded onto the gel for electrophoresis.

Table 2.4: DNA-protein binding reaction

Component	Amount
Nuclear extract	2-4 μg
10X binding reaction buffer	1 μl
[^{32}-p] labelled DNA probe	1 μl
Poly (dI-dC)	2 μl
Sterile a final volume of water up to	20 μl

2.7.5 Electrophoresis of DNA-protein complexes

DNA-protein complexes were separated on 5 % polyacrylamide gels that had been pre-run in 0.5X TBE buffer [Appendix A] for 30 minutes at 80V. The gels were run in 0.5X TBE buffer at 150V until the bromophenol blue dye was three quarters of the way down the gel. The power supply (Consort EUSS microcomputer electrophoresis power supply) was disconnected from the gel box, the gels were removed from the plates, transferred to the filter paper and wrapped with cling film. The gels were then dried on an automatic dryer and exposed to Kodak X-OMA T S film for 24-48 hours at -70°C with intensifying screens. The autoradiograph films were developed using CURIX 60, AGFA, Type 9462/100/140 (Agfa-Gevaert AG Miinchen, Germany).

2.7.6 Supershift and competition assays

The supershift assays or competition assays were performed to identify the specificity of DNA-protein complexes for NF-κB and AP-1. For competition assays, l00-fold molar excess of unlabelled NF-κB/AP-1 oligonulcelotide was added to 4 μg of nuclear extract 30 minutes prior to addition of the labelled probe. In supershift assays, rabbit polyclonal antibodies (400 ng) against various *Fos* and *Jun* (*cjun, junD, junB, cfos, fosB, Fra1, Fra2*) or NF-κB/Rel subunits (anti-p50, anti-ReIA, and anti-c-Rel), was incubated at room temperature with nuclear extracts for 30 minutes before the binding reaction.

CHAPTER III

EFFECTS OF BILE ACIDS ON PROTEIN KINASE C TRANSLOCATION IN COLON CANCER CELLS

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3.1 INTRODUCTION

The development of colorectal cancer is a multistage process involving environmental factors and the development of multiple sequential gene mutations. Early mutations in the adenoma-carcinoma sequence affect the tumour suppressor gene, adenomatous polyposis coli (APC) and activating mutation of the oncogene *K-ras*. Late mutations involve *p53* and deleted in colorectal cancer gene. An alternative pathway of tumorigenesis for a subset of colorectal tumours has been proposed, characterized by the presence of microsatellite instability (MSI). MSI is observed in almost all adenocarcinomas from patients with hereditary non-polyposis colorectal cancer (Jass *et al.*, 2002; Leslie *et al.*, 2002) .The events occurring proximal to the development of mutations are, however poorly understood. An understanding of the mechanisms that explain the initiation and early evolution of colorectal cancer will facilitate the development of new approaches for effective intervention and prevention of this common malignancy.

There is considerable suggestive evidence from epidemiological studies that a high fat content in Western diet predisposes to colorectal cancer (Armstrong *et al.*, 1975). One possible mechanism for the role of a high fat diet relates to stimulation and abundance of tumour promoting bile acids (Imary *et al.*, 1992; Bayerdorffer *et al.*, 1993). A high fat diet stimulates greater secretion of bile acids into the intestine to facilitate absorption and digestion of fats (Hofmann *et al.*, 1999). High serum as well as faecal levels of bile acids is found in patients with adenomas and colorectal carcinomas (Bayerdorffer *et al.*, 1993; Reddy *et al.*, 1977). It has been suggested that bile acids act as tumour promoters by disturbing the balance between proliferation, differentiation and apoptosis in colonic epithelial cells (Hague *et al.*, 1995) Deoxycholic acid (DCA) is the most predominant secondary bile acid implicated in the colonic tumorigenesis Carcinogen-treated rats, fed a diet supplemented with DCA developed more colonic tumours than those treated with carcinogen alone (Hori *et al.*, 1998; Narisawa *et al.*, 1974). Furthermore, surgically altering the bile acid flow in rat colon, hence changing the faecal bile acid excretion also altered the incidence of tumours (Morvay *et al.*, 1989).

Several mechanisms have been suggested by which bile acids influence colon cancer. DCA is known to induce hyperproliferation in colon cells (Ochsenkuhn *et el.*, 1999). Evidence suggests that DCA exerts its tumour promoter effect through intracellular signalling pathways including PKC and mitogen-activated protein kinase (MAPK) (Qiao *et al.*, 2000; Huang *et al.*, 1992). Growth and inflammation regulatory transcription factors AP-1 and NF-κB are regulated by PKC and MAPK activity (Qiao *et al.*, 2000). Impairment of mitochondrial function and lipid peroxidation leading to the generation of free radicals (Hino *et al.*, 2001) and suppression of p53 by DCA (Qiao *et al.*, 2001) has also been suggested. Several growth regulatory genes such as 7-alpha hydroxylase (Stravitz *et al.*, 1995), cyclooxygenase 11 (Zhang *et al.*, 1998) and c-myc (Hallstrom *et al.*, 1991) are regulated by bile acids through PKC.

Protein kinase C (PKC) is a family of 12 Serine-threonine kinase isoenzymes, which are subclassified as classical PKC (cPKCs), novel PKC (nPKCs), or atypical PKCs (aPKCs). All PKC isoenzymes possess a catalytic and a regulatory domain. Depending on the class of PKC isoenzyme, the regulatory domain can include a calcium binding and DAG-phorbol ester-binding site. Activation of classical PKC isoenzymes requires interaction with phospholipids (Lee *et al.*, 1991). Inactive PKC isoenzymes are located predominantly in the cytosol. Once activated, PKC isoenzymes translocate to a variety of intracellular sites including cell membrane, nucleus and membrane associated cytoskeleton (Hug *et al.*, 1993). A number of cytoskeleton proteins including vinculin, vimentin, talin and filamentous actin (F-actin) target PKC translocation and may serve as PKC substrates and regulate membrane and cytoskeleton interactions as an integral part of PKC signaling. Furthermore, changes in localization of PKC are associated with cell differentiation and transformation, indicating that determinants of subcellular localization of PKC can modify cell growth, differentiation and transformation (Janken *et al.*, 1996) as well as cell motility (Volkov *et al.*, 1998). It is notable that prolonged activation of PKC may lead to down regulation of the cellular content of the enzyme.

Many studies have characterized the role of PKC in colonic cancer. A number of studies have shown decreased PKC β, δ, and ζ in human and rodent colonic tumour relative to normal mucosa (Kahl -Rainer *et al.*, 1994; Doi *et al.*, 1994). It has also been shown that PKC ε acts as an oncogene in rat colonic epithelial cells and over expression of PKC ε is associated with increases cell growth rates (Perletti *et al.*, 1996). Studies have shown PKC β1 was most effectively activated by secondary bile acids, suggesting possible function of specific isoenzymes of PKC in colorectal carcinogenesis and tumour growth

control (Pongracz *et al.*, 1995). PKC has also been shown to play a requisite role in the Wnt/APC/β-catenin proliferative signalling pathway, known to be involved in colonic carcinogens (Cook *et al.*, 1996). The use of GFP fusion proteins has transformed our ability to examine the translocation events of PKC in real time and has identified many pathways of PKC translocation not previously seen in simple translocation experiments. Ursodiol, the 7β- epimer of chenodeoxycholic acid (CDCA) has been used in patients with primary sclerosing cholangitis because it substantially improves biochemical indices of liver function (Trauner *et al.*, 1999). Experimental evidence in animal models suggests that ursodiol administration inhibits colonic carcinogenesis (Earnest *et al.*, 1995; Narisawa *et al.*, 1998, 2002). In humans, patients with ulcerative colitis (UC) and primary sclerosing cholangitis (PSC), who have taken UDCA, were significantly less likely to develop colonic dysplasia than who did not use UDCA suggesting a potential chemoprotective effect of this molecule (Tung *et al.*, 2001; Pardi *et al.*, 2003).

3.2 OBJECTIVES

1 To investigate the role of the bile acid, DCA in mediating endogenous PKC isoenzymes translocation events in colonic tumour cells

2 To investigate the role of the bile acid, DCA in mediating GFP tagged-PKC isoenzymes translocation events in colonic tumour cells

3 To investigate the role of UDCA in mediating endogenous PKC isoenzymes translocation events in colonic tumour cells

4 To investigate the role of UDCA in mediating GFP tagged-PKC isoenzymes translocation events in colonic tumour cells

3.3 RESULTS

The following sections describe the results of immunofluorescence performed on HCT116 cells for endogenous PKC isoenzymes treated as described in methods and materials and also the analyses of the EGFP-tagged PKC isoenzymes, transfected and treated as described in materials and methods. Briefly for endogenous PKC isoenzymes HCT116 cells were treated with DCA, UDCA or PMA alone or pretreated with UDCA first, followed by stimulation with either DCA or PMA. The cells were fixed with either acetone or paraformaldehyde and incubated with anti-PKC antibodies and secondary Alexa fluor conjugated antibodies. The cells were also transfected with various PKC isoenzymes fused with EGFP. Real time photomicrsocopy was performed after

treatment to confirm the effects and then fixed and prepared for immunofluorescent microscopy.

3.3.1 Effects UDCA treatment on endogenous PKC α translocation in HCT116 cells (Fig. 3.1)

Both PMA 100 nM and DCA 300 μM caused translocation of PKC α to the cell membrane. UDCA did not cause PKC α translocation. DCA and PMA-induced PKC α were inhibited by pre-treatment of the cells by 300 μM UDCA. Endogenous PKC β1 was not seen in these cells.

3.3.2 Effects UDCA treatment on endogenous PKC δ translocation in HCT116 cells (Fig. 3.2)

PMA and DCA caused translocation of endogenous PKC δ to the nucleus and to the membrane. This was inhibited by pre-treatment of the cells by 300 μM UDCA. UDCA did not induce PKC δ translocation.

3.3.3 Effects UDCA treatment on endogenous PKC ε translocation in HCT116 cells (Fig. 3.3)

Both PMA and DCA caused translocation of PKC ε to the cell membrane. UDCA did not cause PKC ε translocation. UDCA also inhibited PMA and DCA-induced translocation of endogenous PKC ε.

3.3.4 Effects UDCA treatment on endogenous PKC ζ translocation in HCT116 cells (Fig. 3.4)

C2-ceramide at 50 μM caused translocation of the PKC ζ isoenzyme to the nucleus. Neither UDCA nor DCA induced PKC ζ translocation and UDCA pre-treatment did not prevent C2-ceramide-induced PKC ζ translocation.

3.3.5 Effects UDCA treatment on PKC β1-EGFP translocation in HCT116 cells (Fig. 3.5A)

DCA or PMA treatment of PKC β1-EGFP transfected HCT116 cells resulted in translocation of PKC β_I from cytosol to the membrane. UDCA did not cause PKC β_I translocation and pre-treatment of HCT116 cells with UDCA, inhibited DCA-induced membrane translocation of the PKC β1. Pre-treatment with UDCA did not prevent 1 μM PMA-induced membrane translocation of the PKC β1 in either cell lines. There was no

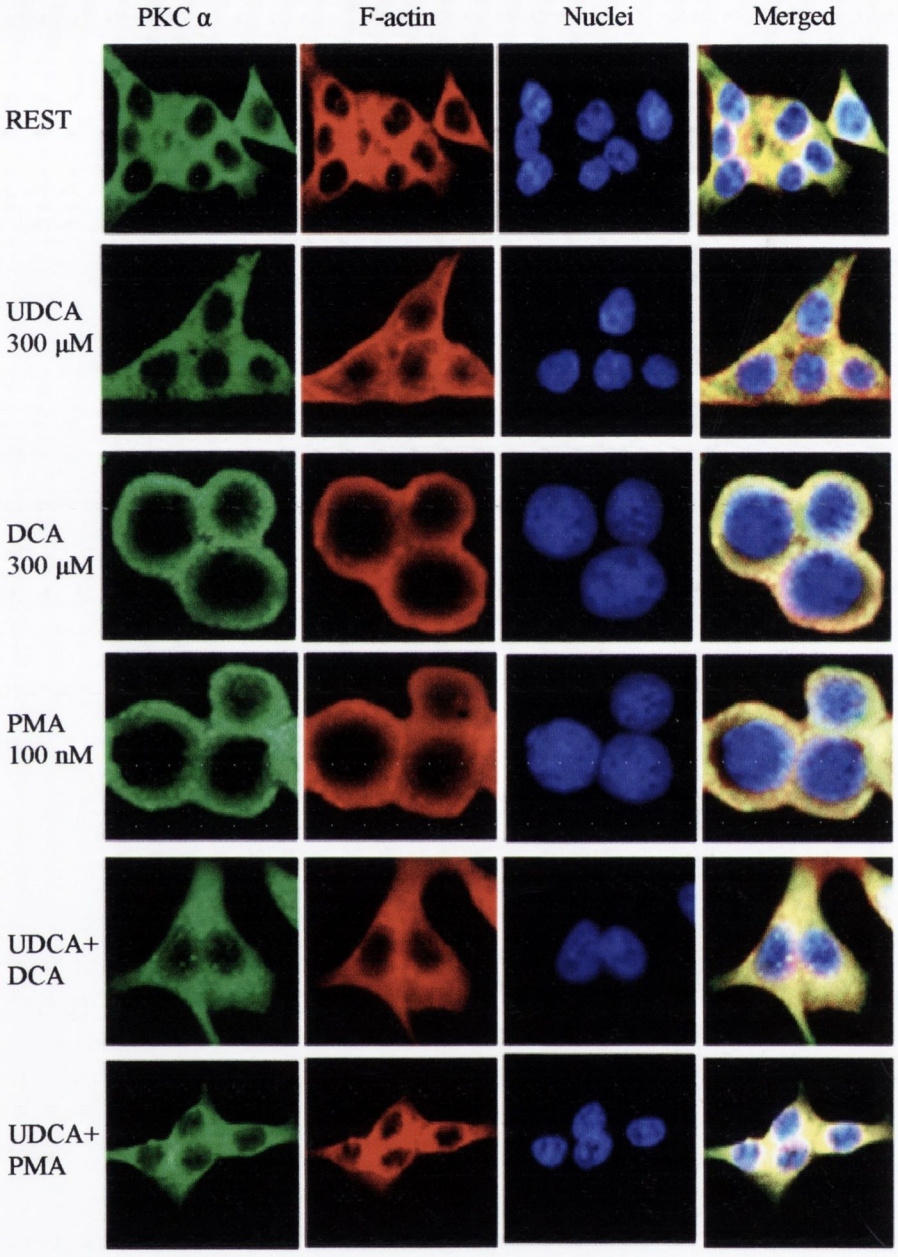

Fig. 3.1 Immunoflourescent staining for endogenous PKC isoenzymes α in HCT116 cells, treated with Ursodeoxycholic acid (UDCA), Deoxycholic acid (DCA) or Phorbol myristate acetate (PMA) for 2 hours, or pre-treated with UDCA for 2 hours before stimulation with DCA or PMA. The cells were fixed and incubated with primary anti-PKC α antibody (R&D, Berkely CA) for 1 hour, followed by Alexa fluor 488 conjugated secondary antibody for 1 hour . Untreated cells are labelled Rest. F-actin is stained using TRICT conjugate of Phalloidin and nuclei with DAPI.

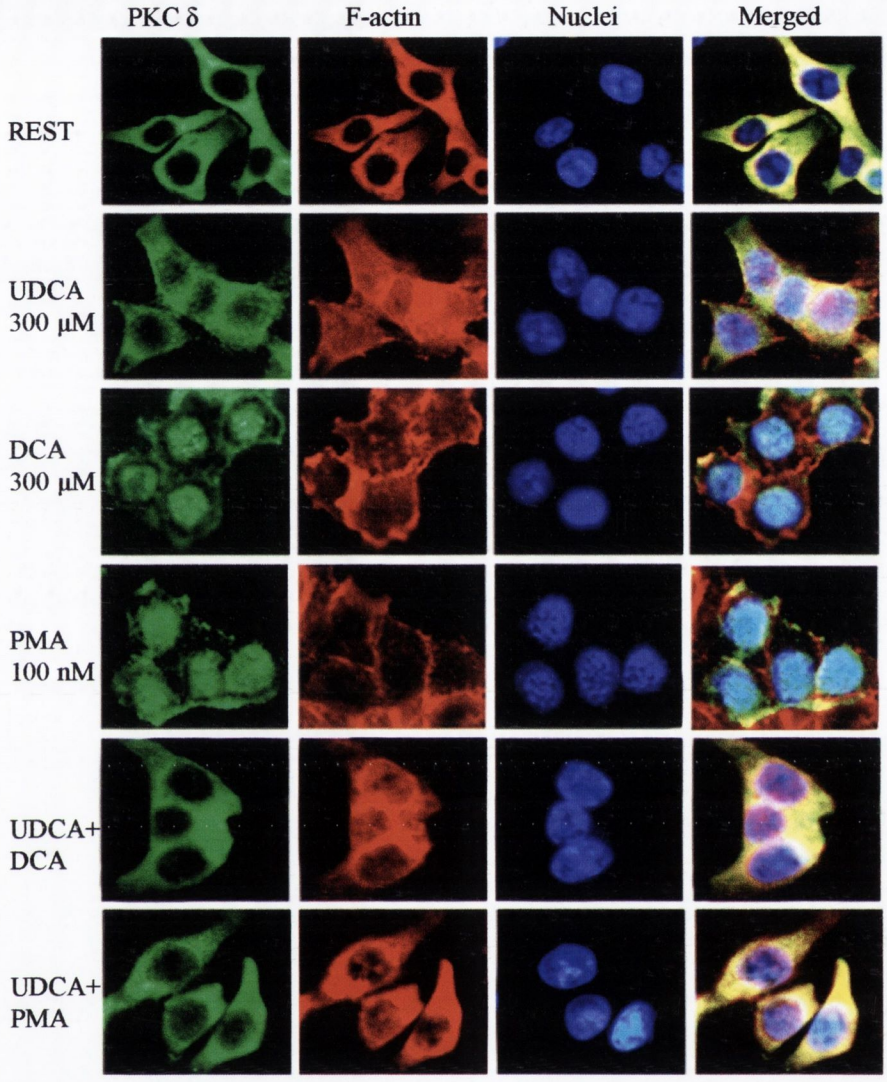

Fig. 3.2 Immunoflourescent staining of endogenous PKC isoenzymes δ in HCT116 Cells. The cells were treated with UDCA, DCA or PMA for 2 hours, or pre-treated with UDCA and then with DCA or PMA. The cells were incubated with primary anti-PKC α antibody (R&D, Berkely CA) for 1 hour followed by Alexa fluor 488 conjugated secondary antibody for 1 hour. Untreated cells are labelled Rest. F-actin is stained using TRICT conjugate of Phalloidin and nuclei with DAPI.

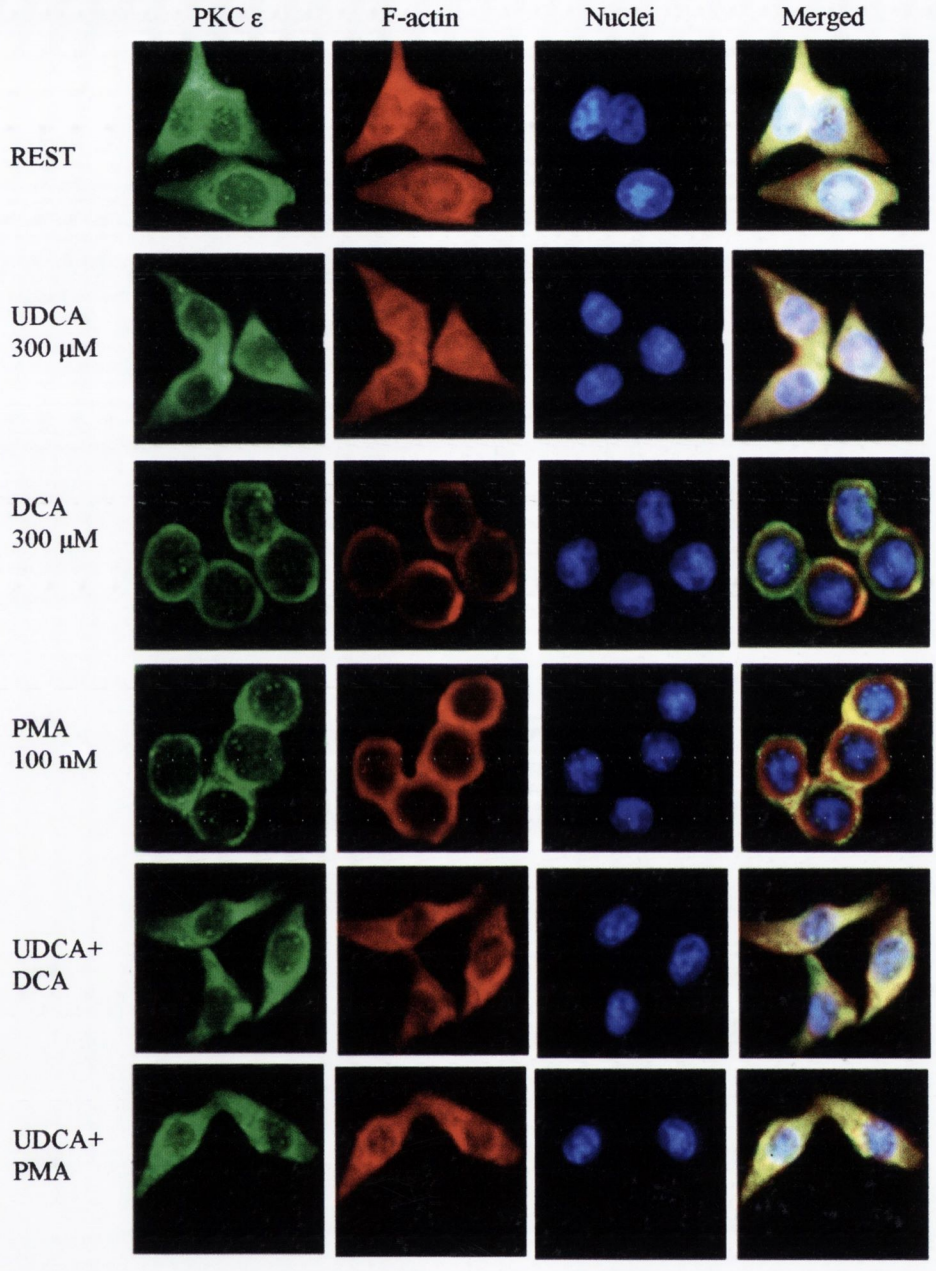

Fig. 3.3 Immunoflourescent staining of endogenous PKC isoenzymes ε in HCT116 Cells. The cells were treated with UDCA, DCA or PMA for 2 hours, or pre-treated with UDCA and then with DCA or PMA. The cells were incubated with primary antibody, followed by secondary antibodies. Untreated cells are labelled Rest. F-Actin is stained using TRICT conjugate of Phalloidin and nuclei with DAPI. Merged photos show co-localization of PKC isoenzymes to the F-actin, seen as yellow.

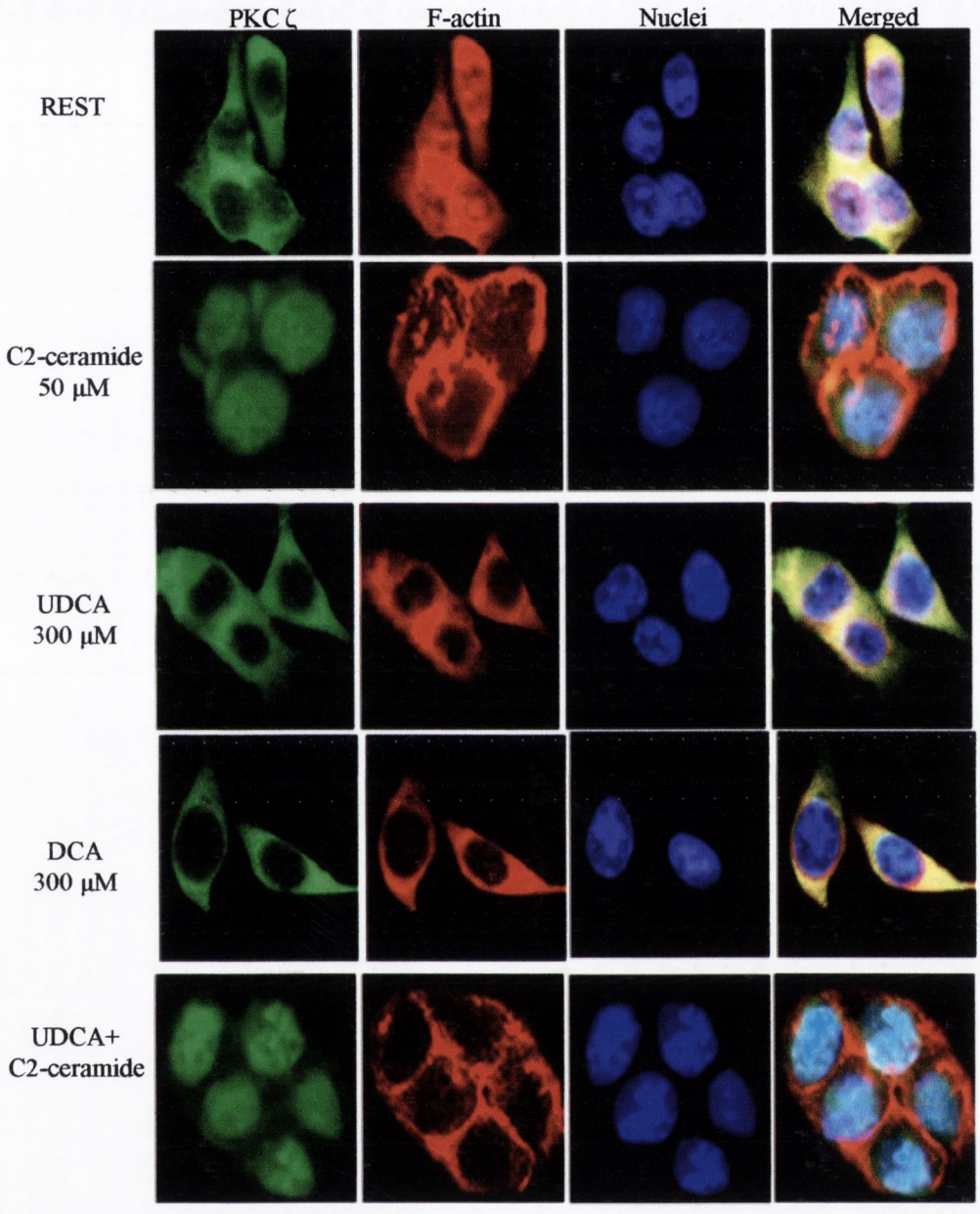

Fig. 3.4 Immunoflourescent staining of endogenous PKC isoenzymes ζ in HCT116 Cells. The cells were treated with UDCA, DCA or PMA for 2 hours, or pre-treated with UDCA for 2 hours followed by stimulation with DCA or C2-ceramide. The cells were incubated with primary secondary antibodies as described above. Untreated cells are labelled Rest. F-actin is stained using TRICT conjugate of Phalloidin and nuclei with DAPI.

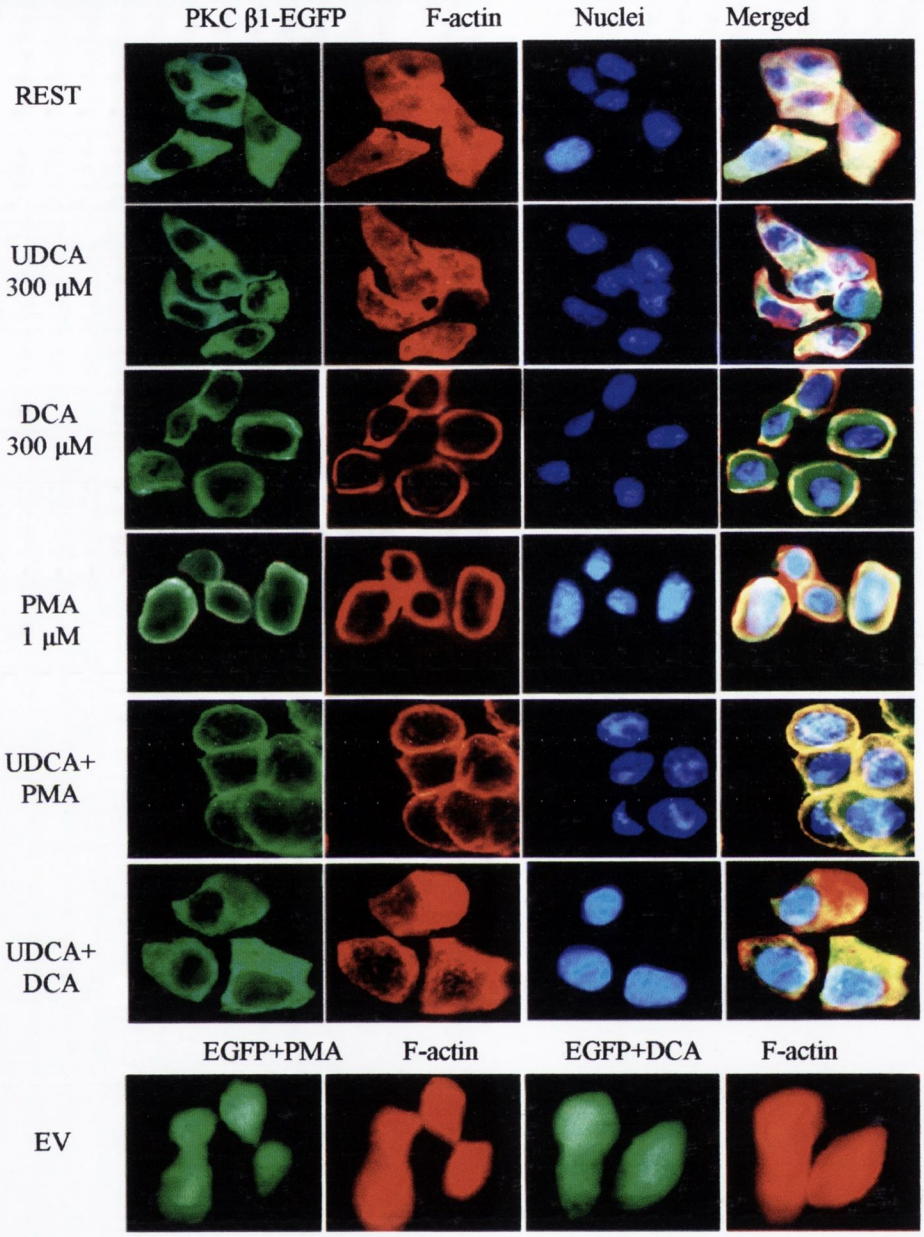

Fig 3.5A Analyses of PKC β1-EGFP in HCT116 treated with UDCA, DCA or PMA for 2 hours, or pre-treated with UDCA for 2hours the with DCA or PMA. Untreated cells are labelled as Rest. Empty Vector (EV), indicate EGFP only transfected cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI. UDCA+DCA, denotes pre-treatment of the cells with 300 μM UDCA, followed by activation with DCA.

change in fluorescence pattern in EGFP-only transfected cells whether treated with PMA or DCA. One important observation was localisation of PKC β1 to the membrane corresponded to F-actin distribution pattern in cells treated by DCA or PMA, not seen in untreated, UDCA treated or EGFP-only cells. These results suggest that PKC β1 activation by DCA was inhibited by UDCA pre-treatment.

To confirm these data, Western blot analyses was performed on subcellular fractionations of HCT116 cells transfected with PKC β1 and treated as above. In untreated cells and cells treated with UDCA, PKC β_1 was found predominantly in the cytosolic fraction of the HCT116 cells (**Figure 3.5B**). After DCA and PMA stimulation, PKC β1 was found predominantly in membrane fraction of the cells consistent with observation made by immunofluorescence. Pre-treatment of cells with UDCA, inhibited the membrane translocation of PKC β1 caused by DCA. This data indicates that UDCA inhibits membrane translocation and hence activation of PKC β1 caused by DCA.

3.3.6 Effects of UDCA treatment on PKC β1-EGF translocation in SW480 cells (Fig. 3.6)

DCA or PMA treatment of PKC β1-EGFP transfected SW480 cells resulted in translocation of PKC β1 from cytosol to the membrane. UDCA did not cause PKC β1 translocation and pre-treatment of SW480 cells with UDCA, inhibited DCA-induced membrane translocation of the PKC β_1. Pre-treatment with UDCA did not prevent 1 μM PMA-induced membrane translocation of the PKC β1 in either cell lines. PMA or DCA did not change fluorescence pattern in EGFP-only transfected cells. PKC β_1 membrane localization corresponded to F-actin distribution pattern in cells treated by DCA or PMA but not in untreated UDCA treated or EGFP-only cells. These results suggest that PKC β_1 activation by DCA was inhibited by UDCA pre-treatment.

3.3.7 Effects UDCA treatment on PKCδ-EGFP translocation in HCT116 cells (Fig. 3.7)

The cells transfected with PKC δ-EGFP were treated as described earlier. PMA and DCA treatment resulted in translocation of PKC δ-EGFP to the cell membrane as well as nuclear membrane, though the effect of DCA was much slower, occurring in 30 minutes to 1 hour compared with PMA, which caused PKC δ-EGFP translocation in a few minutes. Pre-treatment of cells with UDCA inhibited the PKC δ-GFP translocation

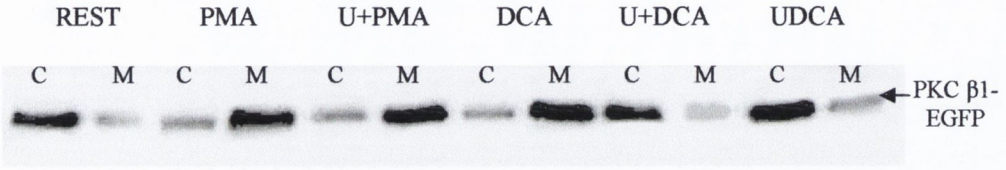

Fig 3.5B Subcellular localization of PKC β1-EGFP in HCT-116. The cells were transfected with PKC β1-EGFP and treated with 300 μM UDCA, DCA or 1 μM PMA for 2 hours, or pre-treated with UDCA for 2 hours followed by DCA or PMA. Equal amounts of subcellular fractionation proteins were separated on a 10% SDS-polyacrymide gel, electrotransferred onto PVDF membrane. Membranes were incubated with PKC β1-specific antibody followed by incubation with secondary anti-mouse HRP-conjugated antibody. Protein bands were visualized by enhanced chemiluminescence (ECL).C, cytosol M, membrane

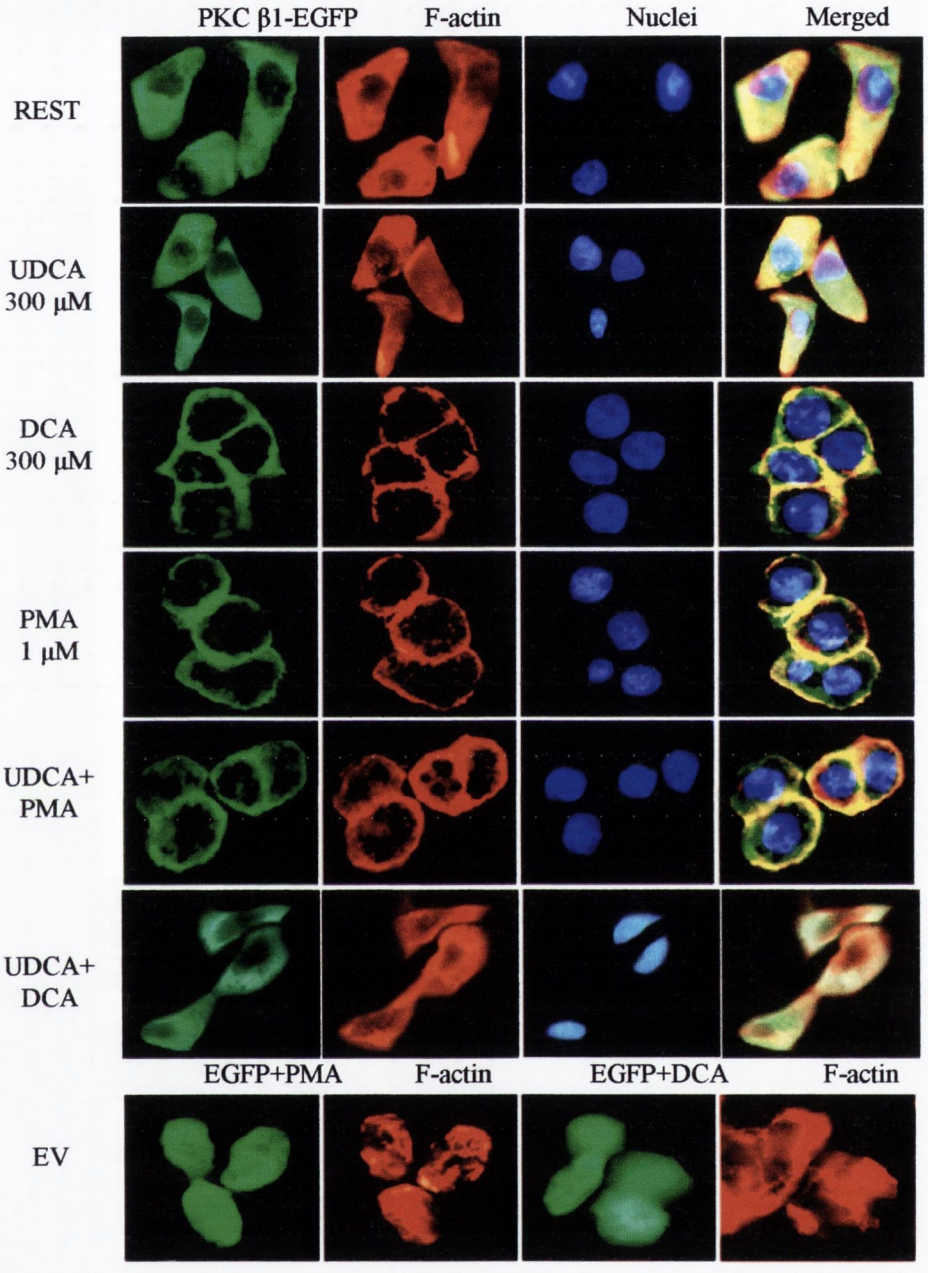

Fig. 3.6 SW480 cells transfected with PKC β1-EGFP or EGFP for 24 hours, treated with UDCA, DCA or PMA for 2 hours, with and without pre-treatment with UDCA. Untreated cells are labelled as Rest. Empty Vector (EV) indicates EGFP only transfected cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI.

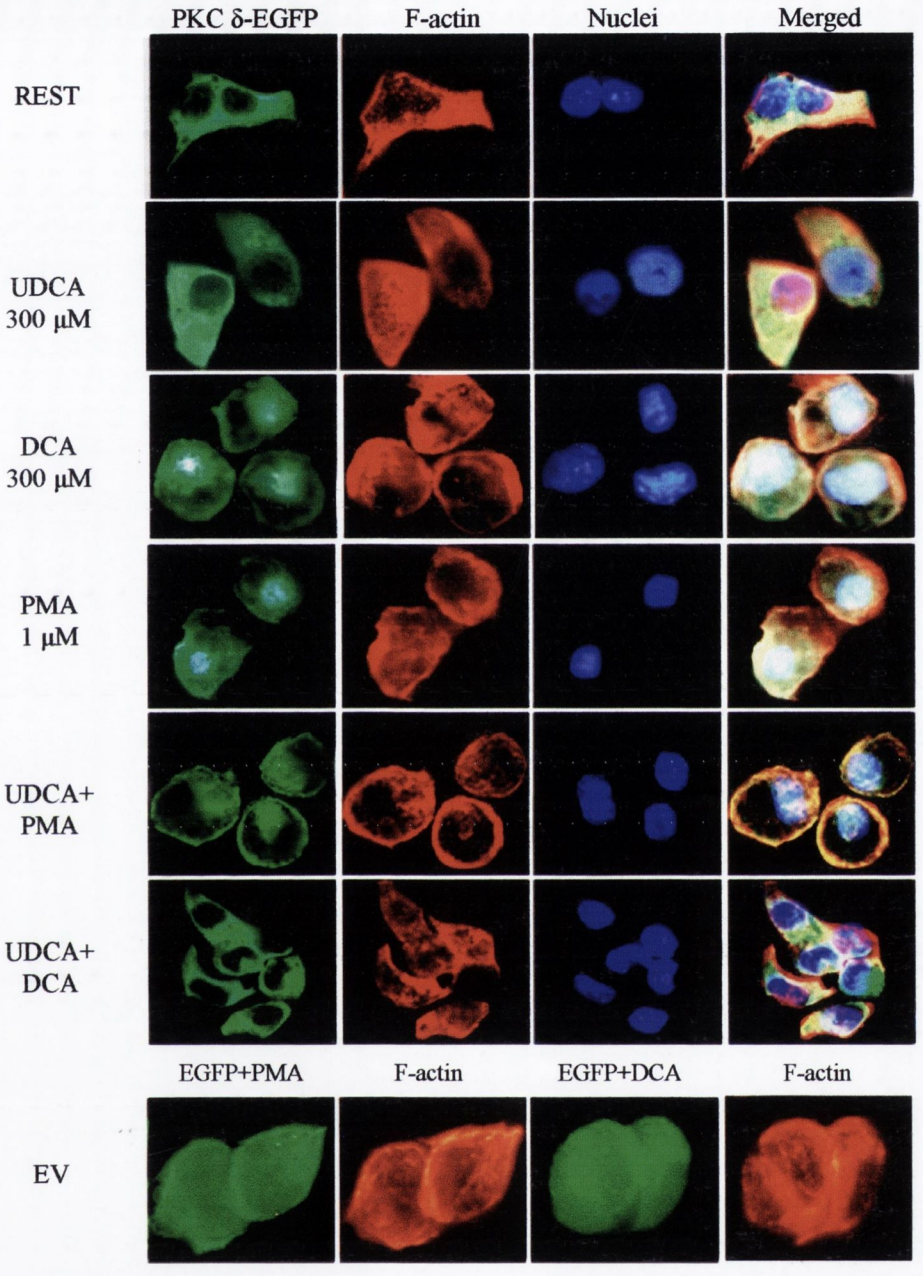

Fig 3.7 HCT116 cells transfected with EGFP or PKC δ-EGFP, were then treated with UDCA, DCA or PMA alone for 2 hours, or pre-treated with UDCA and then stimulated with DCA or PMA. Untreated cells are labelled as Rest. Empty Vector (EV) indicates EGFP only transfected cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI.

caused by of DCA but not by PMA. The distribution of PKC δ-GFP did not correspond with F-actin pattern distribution.

3.3.8 Effects UDCA treatment on PKCε-EGFP translocation in HCT116 cells (Fig. 3.8)

Treatment of PKC ε transfected cells with DCA or PMA resulted in translocation of PKC ε-EGFP from cytosol to the membrane. Pre-treatment of HCT 116 cells with UDCA and subsequent stimulation with DCA did not result in membrane translocation of PKC ε-EGFP reflecting inhibition of PKC ε-EGFP activation. Prior treatment with UDCA did not prevent PMA-induced membrane translocation of the PKC ε-EGFP. The distribution of PKC ε also appeared to correspond with the F-actin distribution pattern. Similar to PKC β1, DCA-induced activation of PKC ε was blocked by UDCA.

3.3.9 Effects UDCA treatment on PKC ζ-EGFP translocation in HCT116 cells (Fig. 3.9)

Atypical PKC ζ-GFP transfected cells were treated with C2-ceramide, PMA, DCA or UDCA, with or without pre-treatment with UDCA. C2-ceramide at doses 25-150 μM had no effect on PKC ζ-GFP, but at 200 μM caused translocation of PKC ζ-GFP cell membrane and also to the nucleus. DCA, UDCA or PMA treatment, on the other had no effect on PKC ζ-GFP at the doses described. Pre-treatment of cells with UDCA did not inhibit C2-ceramide PKC δ-EGFP -induced PKC ζ-GFP translocation.

3.3.10 Effects UDCA treatment on PKC β_1-EGFP translocation in MCF7 cells (Fig. 3.10)

To elucidate the specificity of results to colonic cancer cells, a breast cancer cell line, MCF7 were transfected with PKC β_1-EGFP and then treated with UDCA, DCA or PMA described above. However, PMA treatment, caused membrane translocation of the PKC β_1-EGFP in these cell lines, UDCA and DCA treatment had no effect on PKC β_1 distribution. These data indicate that the effect of these bile acids may be specific to colon cancer cells, not observed in breast cancer cells, MCF7.

3.3.11 Effects of PKC inhibitors on PKC β1-EGFP translocation and cell shape change in HCT116 cells (Fig. 3.11-3.13)

It was observed that the activation of PKC isoenzymes was associated with the cell shape change to rounded phenotypes. This effect was universal and occurred in all the

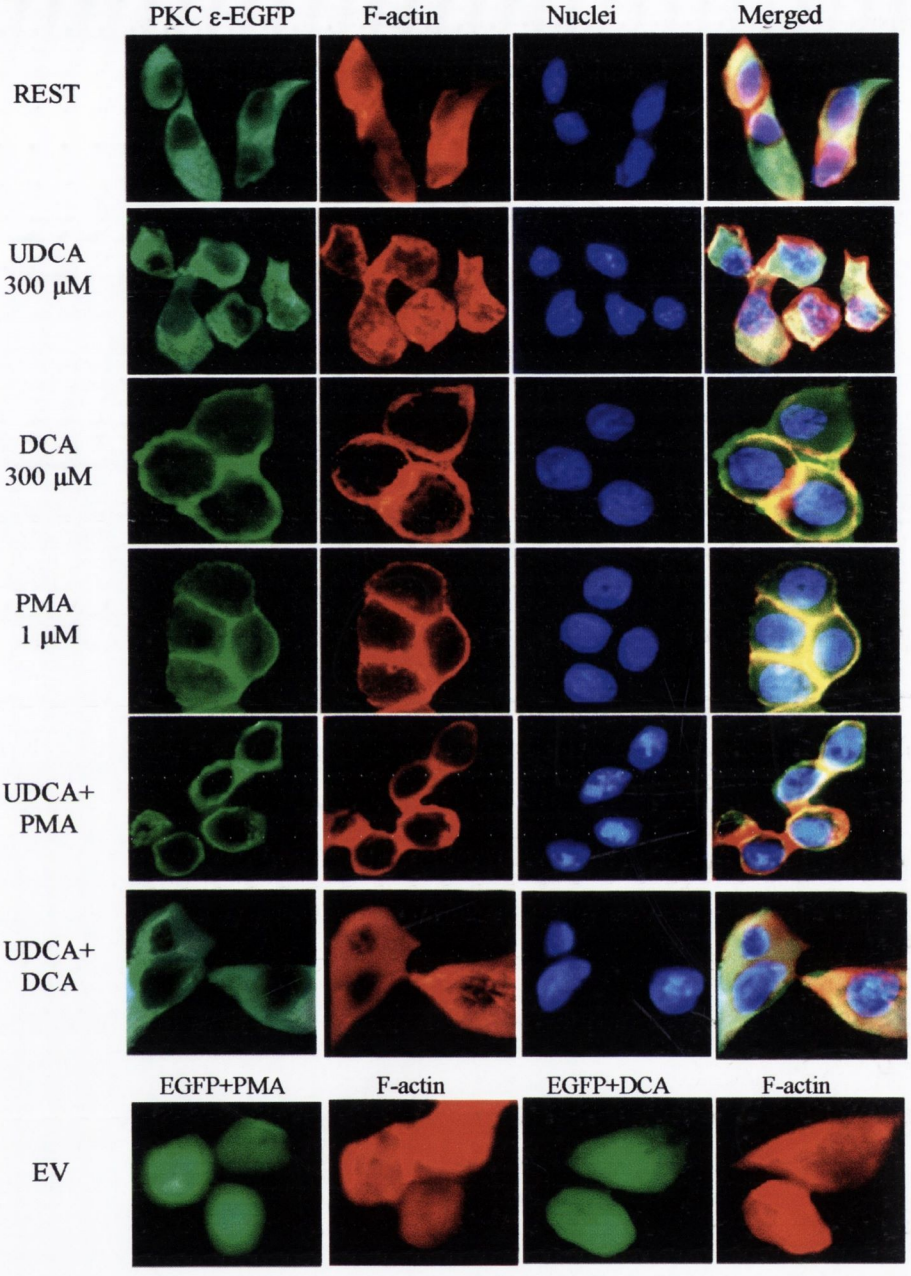

Fig. 3.8 HCT116 cells transfected with EGFP or PKC ε-EGFP, either pre-treated with UDCA for 2 hours followed by stimulation with DCA, PMA or treated with DCA, PMA or UDCA alone. Untreated cells are labelled as Rest. Empty Vector (EV), are the EGFP only transfected cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI.

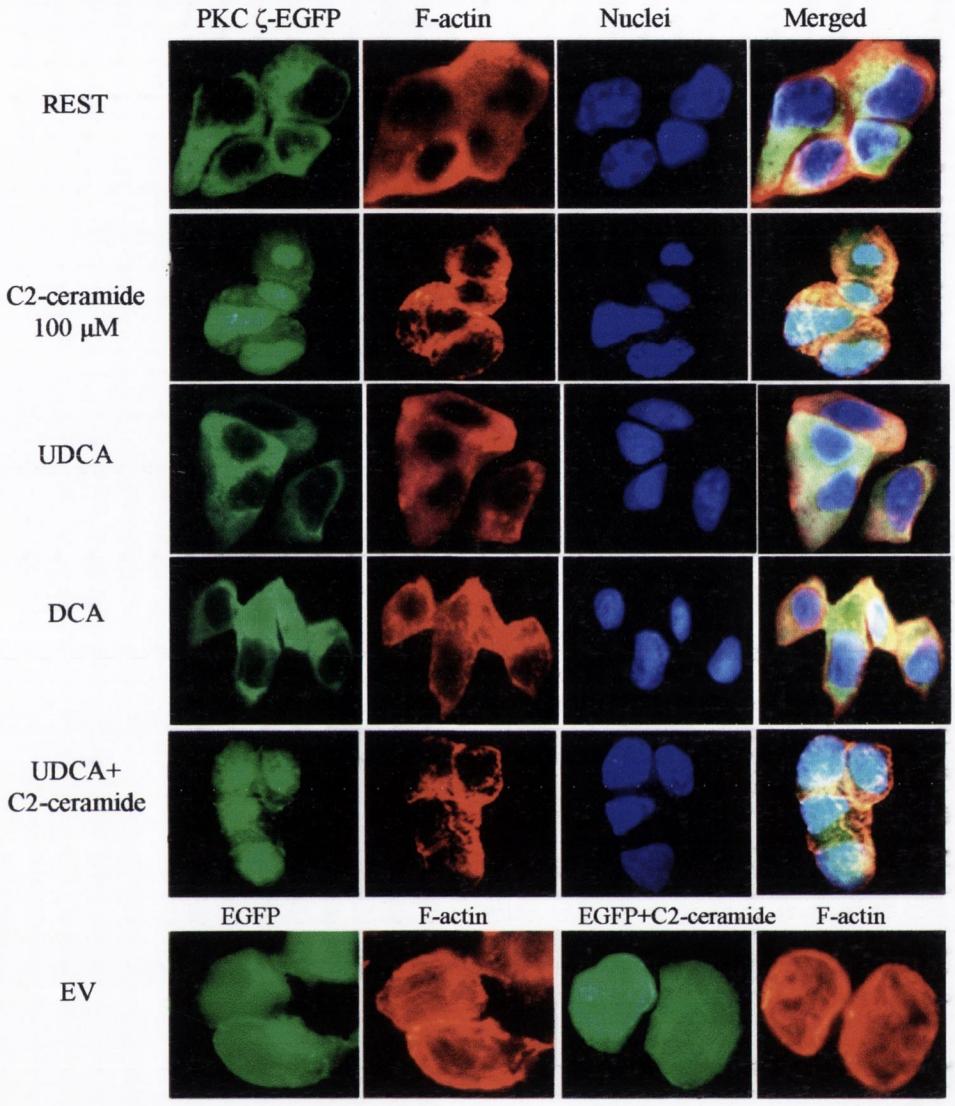

Fig. 3.9 Effect of DCA, UDCA and C2-ceramide on transfected PKC ζ-EGFP in HCT116 cells. The cells transfected with PKC ζ-EGFP or EGFP were treated with UDCA, DCA or C2-ceramide, for 2 hours or pre-treated with UDCA and then activated with DCA or C2-ceramide. Dihydroceramide was used as negative control (data not shown). Untreated cells are labelled as Rest. Empty Vector (EV) indicates the EGFP only transfected cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI.

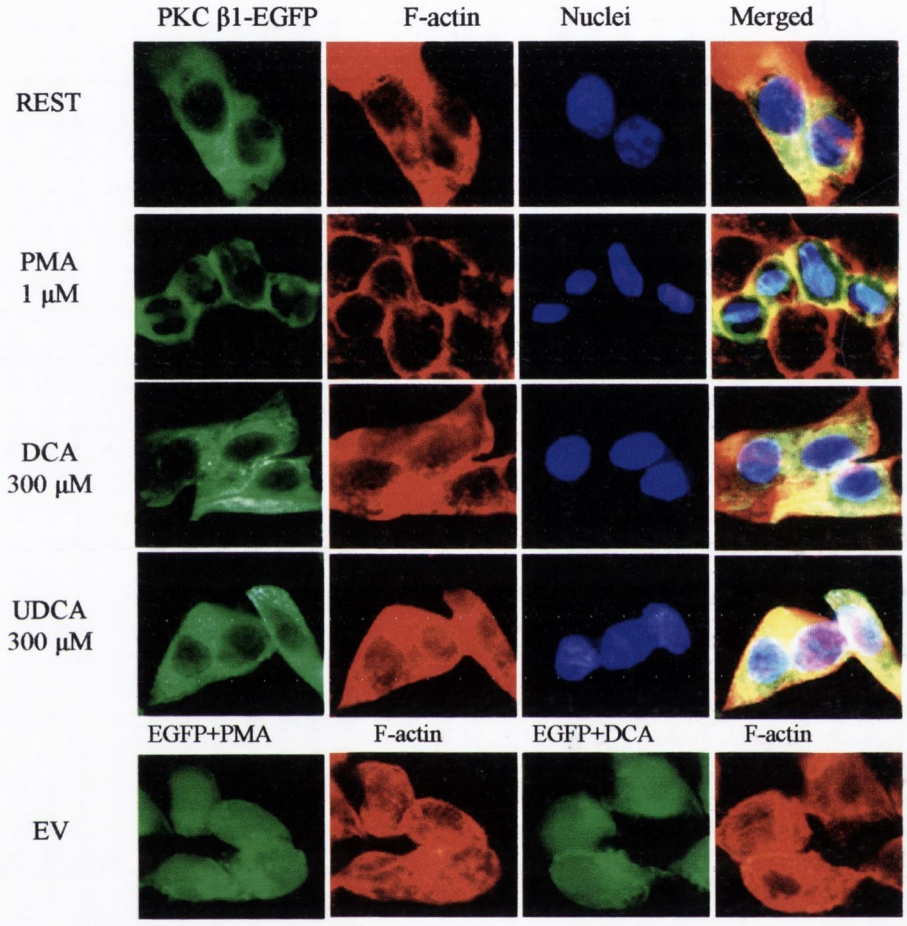

Fig. 3.10 MCF7 cells, a breast cancer cell line, were transfected with EGFP or PKC β1-EGFP for 24 hours. The cells were either treated with UDCA, DCA, or PMA or pre-treated with UDCA before stimulation with DCA or PMA. Untreated cells are labelled as Rest. Empty Vector (EV) indicates EGFP only cells. F-actin is stained with TRICT conjugate of Phalloidin and nuclei with DAPI.

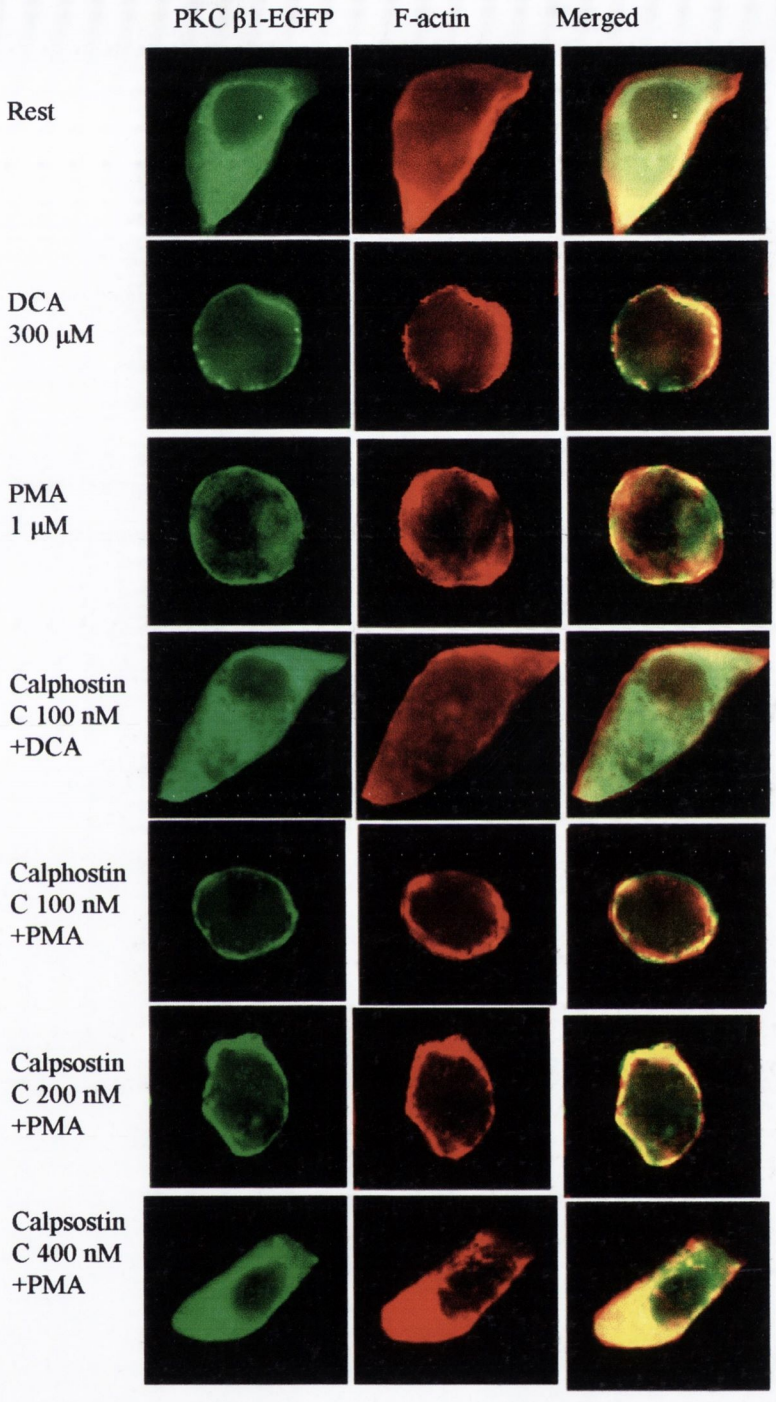

Fig 3.11 Effects of PKC inhibitor, Calphostin C and PKC β1 translocation and cell shape. PKC β1-EGFP transfected HCT116 cells were pretreated with increasing doses of Calphstin C for 30 minutes and then treated with either DCA or PMA. Untreated cells are labelled as Rest. F-actin is stained with TRICT conjugate of Phalloidin.

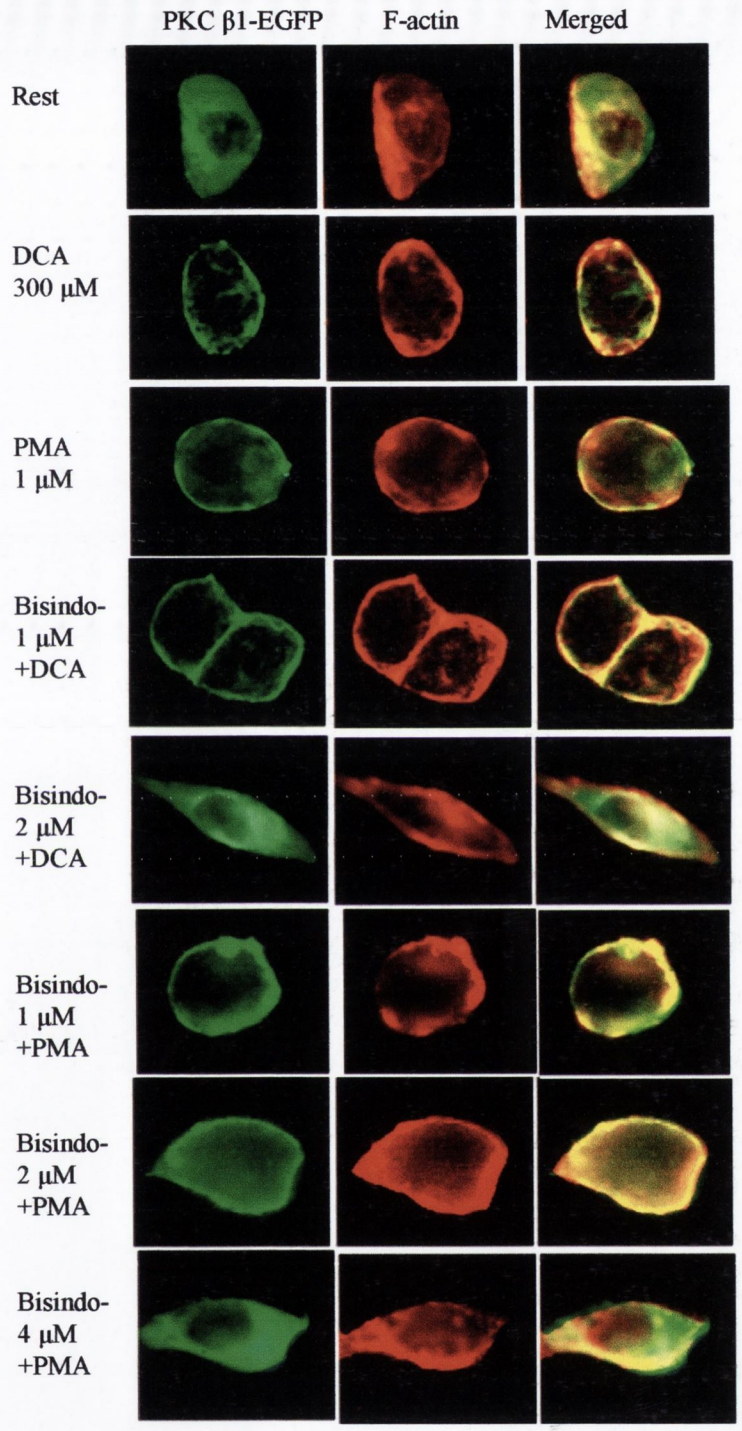

Fig 3.12 Effects of PKC inhibitor Bisindolylmaleimide (Bisindo) on PKC β1 translocation and cell shape change. PKC β1-EGFP transfected HCT116 cells were pretrearted for 30 minutes with different doses of Bisindolylmaleimide and then with either DCA or PMA. Untreated cells are labelled as Rest. F-actin is stained with TRICT conjugate of Phalloidin.

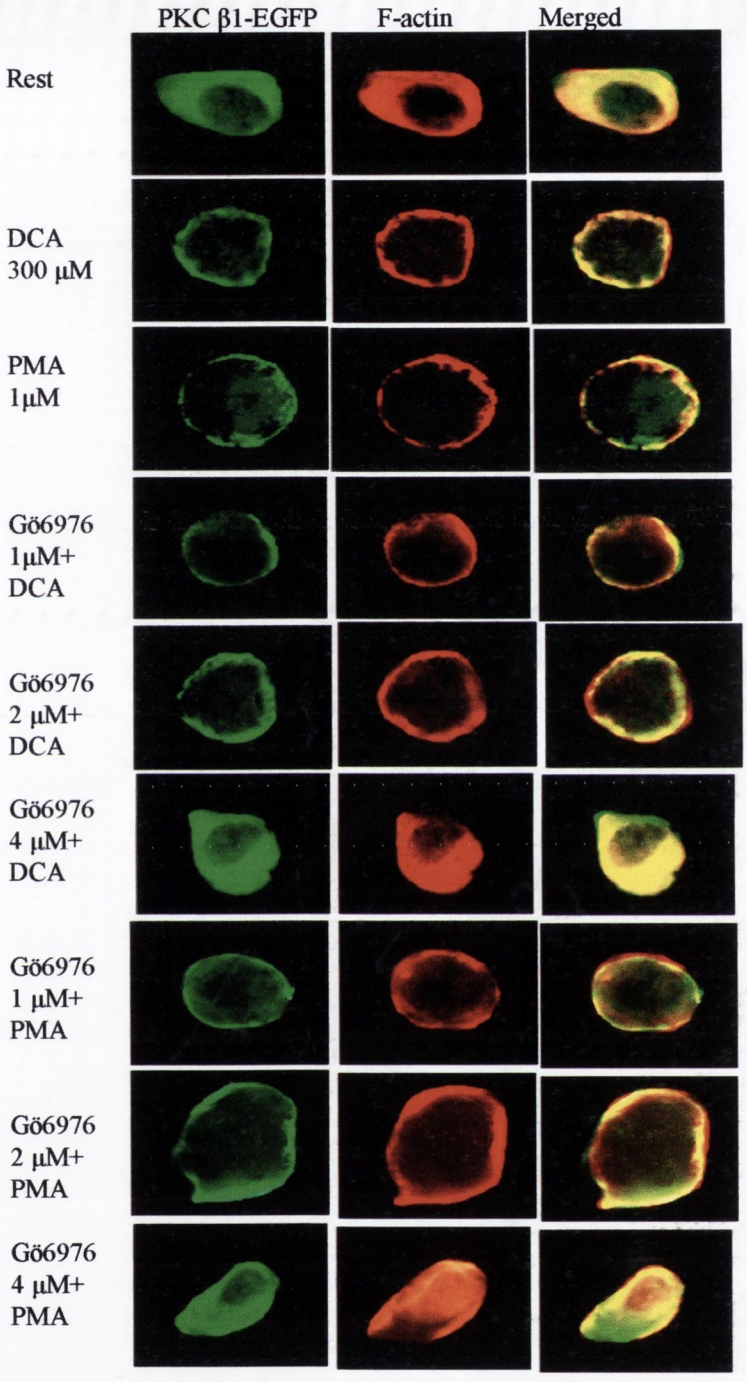

Fig 3.13 Effects of PKC inhibitor Gö6976 on PKC β1 translocation and cell shape change. PKC β1-EGFP transfected HCT116 cells were pretreated with different doses of Gö6976 for 30 minutes and then activated with either DCA or PMA. Untreated cells are labelled as Rest. F-actin is stained with TRICT conjugate of Phalloidin.

cells where translocation of the PKC isoenzymes was observed. Further association of PKC isoenzymes PKC β1 and PKC ε to the F-actin was also seen in activated cells. PKC δ and ζ localization to membrane associated F-Actin was diffuse and less clear. To investigate the role of PKC in cell shape change and F-Actin localization, PKC β1-EGFP transfected HCT116 cells were first treated with different doses of the PKC inhibitors Calphostin C (**Fig 3.11**), Bisindolylmaleimide (**Fig 3.12**) or Indolocarbazole (Gö6976) (**Fig 3.13**) for 30 minutes and then activated with either DCA or PMA. DCA-induced shape change and PKC β1 translocation to membrane associated F-ctin was inhibited by concentrations of PKC inhibitors predicted to inhibit PKC activation, however higher doses of these inhibitors were required to prevent PMA-induced PKC β1 translocation. These data may reflect the higher potency of PMA in mediating PKC activation. The effect of these PKC inhibitors on cell shape and inhibition of rounded phenotypes are summarized in **Table 3.1**.

3.4 DISCUSSION

UDCA improves the biochemical parameters of cholestasis in various cholestatic disorders including primary biliary cirrhosis and primary sclerosing cholangits (Trauner *et al.*, 1999). There is some evidence that UDCA may be protective in human colorectal cancer associated with inflammatory bowel disease (IBD), though most of the mechanistic evidence of chemopreventive role of UDCA in colorectal carcinoma has come from animal models. It has been shown that UDCA treatment reduces hepatic carcinogenesis in mice fed a diet supplemented with UDCA and given diethylnitrosamine (DEN) (Oyama et *al.*, 2002). Rats treated with AOM and intrarectal N-methylnitrosourea and fed a diet supplemented with UDCA had a significant reduction in Aberrant crypt formation (ACF) formation and a lower incidence of colon tumours (Momen *et al.*, 2002, Narisawa *et al.*, 2002). In another animal model of colonic cancer study, feeding rats with UDCA significantly reduced the proportion of faecal deoxycholic acid (DCA) concentration (Batta *et al.*, 1998). It has been suggested that UDCA may prevent colonic tumours and polyps by reducing the colonic concentration of DCA thus inhibiting its ability to mediate potentially toxic effects

In this study, we examined comparative effects of DCA and UDCA in human colonic cancer cells. Our findings may explain some of the differences between these agents in terms of potential role in carcinogenesis. Secondary bile acids have been shown to activate PKC and evidence of DCA as tumour promoting agent comes from animal as

Table 3.1 Effect of PKC Inhibitors on PKC β1 translocation and its relationship to cell shape change

	PMA-induced PKCβ1 translocation	Change in Cell Shape	DCA-induced PKCβ1 translocation	Change in Cell Shape
Calphostin C				
100nM	–	–	+	+
200nM	–	–	+	+
400nM	+	+	+	+
600nM	+	+	+	+
Go6976				
1μM	–	–	–	–
2μM	–	–	–	–
4μM	+	+	+	+
6μM	+	+	+	+
Bisindolylmaleimide				
1μM	–	–	–	–
2μM	–	–	+	+
4μM	+	+	+	+
6μM	+	+	+	+

–, translocation of PKC β1 and change in cell shape not inhibited, +, translocation of PKC β1 and change in cell shape inhibited. Inhibition of PKC β1 translocation by PKC inhibitors corresponds with inhibition of cell shape change.

well as human studies. PKC isoforms are the major receptor for the tumour-promoting phorbol esters and are therefore thought to play an important role in signal transduction and carcinogenesis (Clarke *et al.*, 2000). Diacyglycerol (DAG), produced by the receptor-coupled hydrolysis of membrane phospholipids, activates PKC (Nashizuka 1984). Phorbol esters bind to the DAG binding site of the regulatory domains of the all but atypical PKC and activate their catalytic activity (Nishizuka 1988). DCA has previously been reported to induce PKC activation using Western blot analysis. In the current study, we first examined the effects of these two bile acids on endogenous PKC isoenzymes and then the effects on PKC translocation were investigated using GFP tagged PKC isoenzymes. Specifically, we also examined the effects of UDCA in blocking these translocation events. In untreated cells, DCA induced activation of the different PKC isoenzymes and their translocation to subcellular sites. It caused translocation of all the endogenous PKC isoenzymes used in the study and the trasfected PKC isoenzymes. UDCA on the other hand caused neither endogenous nor GFP-tagged PKC translocation; rather it inhibited the translocation of the endogenous PKC α, ε, δ and PKC ζ induced by DCA, PMA or c2-ceramide. It also inhibited the translocation of GFP-tagged PKC β1 and PKC ε and PKC δ caused by DCA but not by PMA. The potency of DCA on PKC activation was found to be equivalent to the tumour-promoting agent, PMA. As higher doses of PMA were required for the adequate translocation of various PKC isoenzymes compared with endogenous PKC, this may explain the failure of UDCA to prevent PMA-induced PKC-GFP translocation. It is not clear why higher PMA concentration was required for GFP-tagged PKC isoenzymes.

A second observation was the finding that PKC β1 and PKC ε localize to the F-actin in activated cells. Activated cells showed significant shape change with appearance of rounded phenotypes of the cells. This phenomenon was universal and all activated cells showed cell shape change and F-Actin localization of the PKC isoenzymes. The conformational change in membrane associated F-actin was associated with localization of the different PKC isoenzymes to the F-actin and the rounded phenotypes of the cells. To investigate the role of activated PKC in acquisition of the shape change of the cells, experiments using PKC inhibitors were conducted. Various PKC inhibitors, some with specificity for conventional PKC and others with non-specific and broad effects on PKC were employed. The use of PKC inhibitors prevented the change in cell shape, which corresponded with inhibition of activation of the PKC isoenzymes. Colocalization of the PKC isoenzymes to the membrane associated cytosketal structures such as F-actin was

also affected. Cytoskeletal structures including F-actin play an important role in cell shape and migration as well as their adherence to matrix through connections with surface molecules. These conformational changes and remodelling of F-actin might have important implications in cancer cell growth and migration. In addition, shape change would be predicted to have a significant impact on tight junction function. It has been suggested that agents causing PKC translocation and activation play a critical role in cell adhesion and can cause increase in tight junction leakiness. This increase in tight junction permeability results in enhanced cellular flow of solutes between the epithelial cells with potential implications for cell adhesion and differentiation (Mullen *et al.*, 1996). PKC ε in particular has been shown to play a significant role in cell adhesion and has been immunolocalized to the junctional region in gastrointestinal epithelia (Haller *et al.*, 1998, Saxon *et al.*, 1994), hence it might play a role the cell shape modification, observed in the study. The effect of bile acids on PKC activation was specific to colorectal cancer as these agents had no effect on PKC in breast cancer cells in our study. UDCA might mediate anticancer effects directly through prevention of PKC activation or alternatively by altering the outcome of downstream processes such as cell shape change by modulating F-actin function and this could have implications for the spread and metastasis of the colorectal cancer. It has been shown that DCA causes migration of the cancer cells (Milovic *et al.*, 2001) and UDCA by inhibiting the cytosketal alterations induced by DCA might play a significant role in the progression of the cancer. Though it is known for a number of years that secondary bile acids and in particular DCA cause activation of PKC, the mechanisms by which DCA activates PKC is unknown. A number of hypotheses have been put forward by different investigators for the effects of DCA on PKC activation. The calcium and the DAG are implicated in the activation of the PKC and conventional PKC calcium and DAG binding sites while atypical PKC isoenzymes possess none of the sites. It has been reported that secondary bile acids increase intracellular calcium, which may be one of the mechanisms by which DCA could activate conventional PKC, though it may not entirely explain the activation of novel PKC δ and PKC ε by DCA. On the other hand, neither DCA nor UDCA activated atypical PKC ζ, which lacks DAG-Phorbol binding and calcium binding site, suggesting that bile acids might target signalling pathways similar to PMA. The effects on phospholipids in the cell membrane, downstream cytoplasmic events ad even nuclear receptors may be targets for DCA but further investigations are required to elucidate these mechanisms.

Several mechanisms have been suggested previously, by which UDCA might prevent colorectal cancer. It has been suggested that UDCA dilutes more toxic bile acids in enterohepatic circulation and colonic mucosa (Rodriguez *et al.*, 1995). It has also been suggested that UDCA supplementation blocks two separate putative neoplastic pathways i.e., alteration in protein kinase C expression and induction of phospholipase A2 expression and significantly attenuated levels of phospholipase A2 were found in colonic mucosa of UDCA treated animals (Ikegami *et al.*, 1998). While other studies suggest that, multiple other processes that check tumorigenesis might be involved. These include modulation of arachidonic acid metabolism (Lukivskoya *et al.*, 2001). This is the first study, which specifically examines and explores the molecular mechanisms of chemoprotective effect of UDCA in human colorectal cancer and specially its relation to toxic bile acid, DCA and all three classes of protein kinase C. We suggest that UDCA acts as chemoprotective agent in colorectal carcinogenesis by inhibiting the toxic and tumorigenic effects of DCA in human colonic mucosa, mediated though modification of PKC signal transduction.

CHAPTER IV

EFFECTS OF BILE ACIDS ON NUCLEAR FACTOR KAPPA B AND ACTIVATOR PROTEIN-1 DNA BINDING IN COLON CANCER CELLS

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EFFECTS OF BILE ACIDS ON NUCLEAR FACTOR KAPPA B AND ACTIVATOR PROTEIN-1 DNA-BINDING IN COLON CANCER CELLS

4.1 INTRODUCTION

There is plethora of evidence from epidemiological studies that high fat diet predisposes to colon cancer (Armstrong *et al.*, 1975). It has been suggested that diets high in fat content cause increased secretion of bile acids in the intestine (Rafter *et al.*, 1987; Lapre *et al.*, 1992). Secondary bile acids released in the colon due to bacterial metabolism are cytotoxic and cause crypt proliferation, which may lead to the development of colorectal carcinoma (Stadler *et al.*, 1988). In this regard bile acids may act as tumour promoters by disturbing the balance between cell proliferation, differentiation and apoptosis in the colonic epithelial cells (Milovic *et al.*, 2002). High serum and faecal levels of secondary bile acids are found in patients with colorectal adenomas and carcinomas (Reddy *et al.*, 1977; Bayerdorffer *et al.*, 1993). Carcinogen-treated rats, fed a diet supplemented with deoxycholic acid (DCA) developed more colonic tumours than those treated with carcinogen alone (Hori *et al.*, 1998; Narisawa *et al.*, 1974). Ursodeoxycholic acid (UDCA), on the other hand, is thought to have chemprotective effects in colon cancer in animal models of colon carcinogenesis (Wali *et al.*, 1995; Narisawa *et al.*, 2002). Furthermore, there is evidence from clinical studies in humans of protective effect of UDCA in colorectal cancer (Tung *et al.*, 2001). A number of mechanisms have been proposed by which bile acids influence colon carcinogenesis. Bile acids are thought to induce hyperproliferation in colon mucosal cells (Ochsenkuhn *et al.*, 1999; Barone *et al.*, 2002). Others suggest that bile acids may exert their cytotoxic effects through activation of intracellular signalling pathways such as Protein Kinase C (PKC) and Map Kinases (MAPK) (Qiao *et al.*, 2000; Huang *et al.*, 1992). Bile acids are reported to cause DNA damage in colon cells leading to genetic mutations (Venturi *et al.*, 1997; Kandell *et al.*, 1991). In addition bile acids induce transcription factors, Nuclear Factor Kappa B (NF-κB) and Activator Protein 1 (AP-1), which may play an role in the pathogenesis of colon cancer (Glinghammer *et al.*, 2002; Hirano *et al.*, 1996; Matheson *et al.*, 1996).

NF-κB is heterodimeric transcription factor consisting of p50, p52 and RelA, Rel B and cRel subunits. Most commonly encountered dimmer of NF-kB in mammalian cells is p65/p50 (Ghosh *et al.*, 1998; Miyamoto *et al.*, 1995; Siebenlist *et al.*, 1994). NF-κB

resides in the cytoplasm in an inactive form bound to the inhibitory molecule IκB. Once a stimulus is received, a number of proteins such as IκB kinases (IKKs), Protein Kinse C (PKC) and MAP kinases are released and cause phosphorylation and degradation of IκB (Martin *et al.*, 2001). Released NF-κB translocates to the nucleus where it binds to specific DNA site and subsequently induces genes involved in cell transformation, proloferation, invasion and angiogenesis (Baldwin 2001; Mayo *et al.*, 1997; Bharti *et al.*, 2002). Aberrant and sustained activation of NF-κB has been reported in human cancer including colon cancer (Rayet and Gelinas 19991; Lind *et al.*, 2001). NF-κB activation has been reported to induce resistance to apoptosis and confer survival to transforming cells (Gilmore *et al.*, 1996; Gilmore *et al.*, 2001). AP-1 is another heterodimeric transcription factor composed of *fos* and *jun* family of genes. Ap-1 is a protooncogene and like NF-κB activates transcription of proinflammatory genes as well as genes involved in cell proliferation and transformation (Matheson *et al.,* 1996; Angel and Karin 1991). It has been suggested that bile acids activate AP-1 through PKC signalling pathways (Qiao *et al.*, 2000).

Protein kinase C (PKC) is a family of serine-threonine kinase isoenzyms, which are subclassified as classical PKC (cPKCs), novel PKC (nPKCs), or atypical PKcs (aPKCs) depending on cofactor requirements. Inactive PKC isoenzymes are located predominantly in the cytosol. Once activated, PKC isoenzymes translocate to a variety of intracellular sites including cell membrane, nucleus and membrane associated cytoskeleton (Nishizuka 1988). Bile acids are reported to activate PKC in colon cells and hence may impact indirectly on transcription factors (Huang *et al.*, 1992).

4.2 OBJECTIVES

1 To investigate the differential effects of DCA and UDCA on transcription factor NF-κB

2 To investigate the effects of DCA and UDCA on AP-1

3 To investigate the role of PKC in NF- κB activation

4 To investigate the role of PKC in AP-1 activation

4.3 RESULTS

The effects of DCA and UDCA on transcription factors NF-κB and AP-1 and their interaction with PKC are described. HCT116 cells were treated with DCA, UDCA, IL-1β, PMA alone or pretreated with UDCA before activation with DCA, IL-1β or PMA. Nuclear extracts and total cell extracts of the cells were prepared for gel shift assays for NF-κB or AP-1 and Western blot analyses for IκB-α or p65 levels. The cells were also transfected with PKC β1 and immunofluorescence performed for p65.

4.3.1 Effects of UDCA on NF-κB DNA binding (Fig 4.1)

Exposure of the cells to bile acids have distinct effects on NF-κB DNA binding activity. Gel Shift assay performed on nuclear extracts of IL-1β, DCA and UDCA treated HCT116 shows that both DCA and IL-1β induce NF-κB DNA binding activity. UDCA does not cause NF-κB DNA binding. The two bile acids have distinct effects. The Human T cell leukaemia HuT 78 cell line constitutively expressing NF-κB was used as positive control (C).

4.3.2 Effects UDCA on DCA and IL-1β-induced NF-κB binding (Fig 4.2a)

This gel shift assay on nuclear extracts of the cells, either treated with DCA, UDCA, IL-1β alone or pretreated with UDCA and then stimulated with DCA or IL-1β shows that UDCA had no effect on NF-κB DNA but inhibited DCA and IL-1β-induced NF-κB DNA binding in UDCA pretreated cells. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

4.3.3 Effect of bile acids on IκB-α levels (Fig 4.2b)

NF-κB is found in the cytosol of the unstimulated cells bound to an inhibitory molecule 1κB-α. On activation IκB-α is phophorylated and degraded and NF-κB is released, translocates to nucleus where it binds to DNA elements to cause induction. Western blot was performed on the total cell extracts of the treated cells to observe the effects on IκBα levels. This result shows that DCA and IL-1β but not UDCA reduce IκB-α levels. Reduction in levels of IκB-α in cells pretreated with UDCA and the activated with DCA and IL-1β was not observed. Reduced IκB-α levels correspond to the NF-κB DNA binding as shown in Gel shift assay.

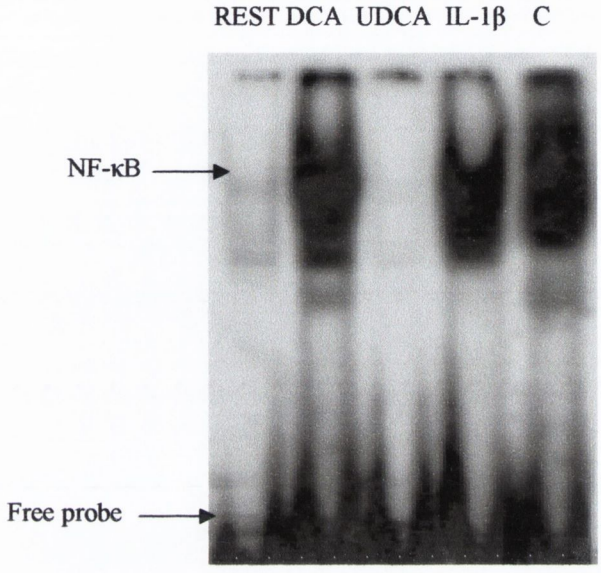


Fig 4.1 HCT116 cells were treated with 300 μM DCA, UDCA or 20ng/ml IL-1β for 2 h. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

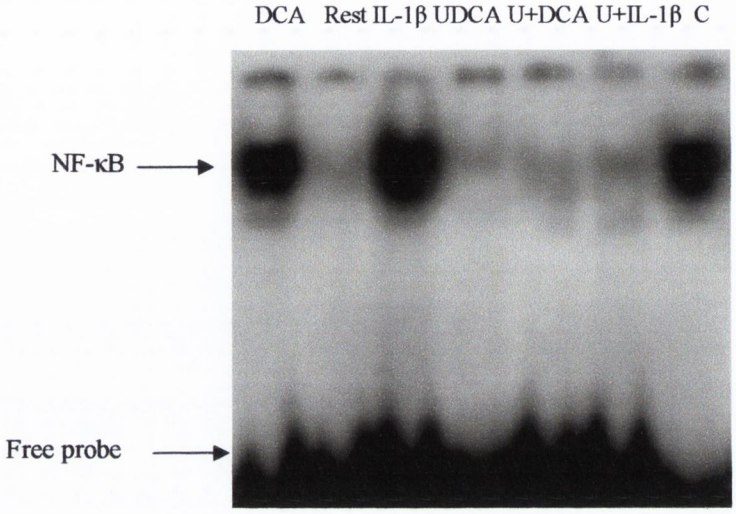


Fig. 4.2a HCT116 cells were treated with 300 μM DCA, UDCA or 20 ng/ml IL-1β for 2 h either alone or pre-treated with UDCA. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

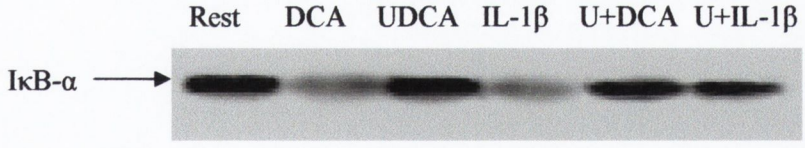


Fig. 4.2b HCT 116 cells were treated with 300 μM DCA, UDCA or 20 ng/ml IL-1β for 2 h either alone or pretreated with UDCA and then activated with DCA or IL-1β. Western blot analyses were performed on total cell extracts for IκB-α levels.

4.3.4 Effect of bile acids on p65 levels (Fig 4.2c)

These data were further investigated by Western blot analysis, performed on the nuclear extracts of treated cells for p65 component of NF-κB. It shows that both NF-κB and IL-1β induce p65. UDCA does not induce p65, by contrast it inhibits DCA and IL-1β-induced p65 in the cells pretreated with UDCA. These data demonstrate UDCA by contrast with DCA does not activate NF-κB but also inhibits DCA-induced NF-κB activity.

4.3.5 Supershift assay for NF-κB (Fig 4.2d, 2e)

In order to identify the components of the NF-κB DNA-complex induced by DCA supershift assay was performed. A panel of antibodies directed against various NF-κB subunits (p50, p65, and c-Rel) were preincubated with nuclear extracts from HCT 116 cells treated with DCA **(Fig 4.2d)** and IL-1β **(Fig 4.2e).** Antibodies to p65 and p50 recognized this NF-κB-DNA complex, while anti-c-Rel had no effect in the supershift assay. Moreover, competition assays with a 100-fold molar excess of unlabelled NF-κB oligonucleotide confirmed the specificity of NF-κB DNA-complex induced by DCA and IL-1β.

4.3.6 Immunofluorescence for NF-κB (Fig 4.3)

To provide further evidence for the presence of activated NF-κB caused by the DCA IL-1β and abrogation of this effect by UDCA pretreatment , immunofluorescence was performed on the cells treated with DCA, IL-1β, UDCA alone or cells pretreated with UDCA first and then stimulated with DCA or IL-1β. After incubating the cells with p65 primary antibody, FITC conjugated secondary antibody was used to visualize p65. Immunofluorescence shows nuclear translocation of p65 in DCA and IL-1β treated cells. p65 remains in the cytosol in UDCA treated cells as in untreated cells. Pretreatment with UDCA followed by DCA and IL-1β activation clearly shows p65 in the cytosol rather than the nucleus, hence inhibition of p65 induction by UDCA.

4.3.7 Effect of bile acids on PKC β1 and NF-κB (Fig 4.4)

HCT 116 cells were transfected with PKC β1-EGFP and the treated with DCA, UDCA or IL-1β alone or pretreated with UDCA followed by stimulation with either DCA or IL-1β. Real time microscopy and fluorescent microscopy was performed to observe the effects on PKC β1 translocation. Cells were then fixed and stained with TRITC labelled

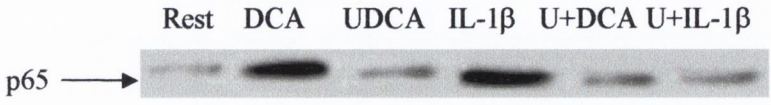


Fig. 4.2c HCT116 cells were treated with 300 μM DCA, UDCA or 20 ng/ml IL-1β for 2 h either alone or pretreated with UDCA. Western blot analyses wer performed on nuclear extracts of the cells for p65 levels.

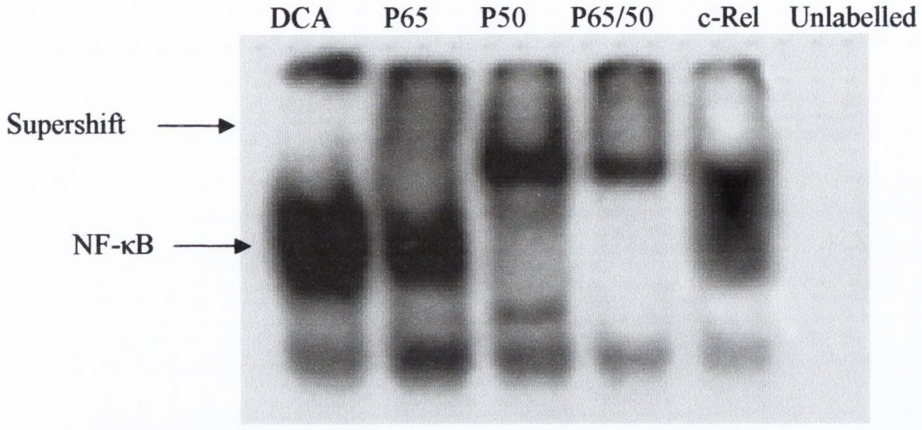


Fig. 4.2 d

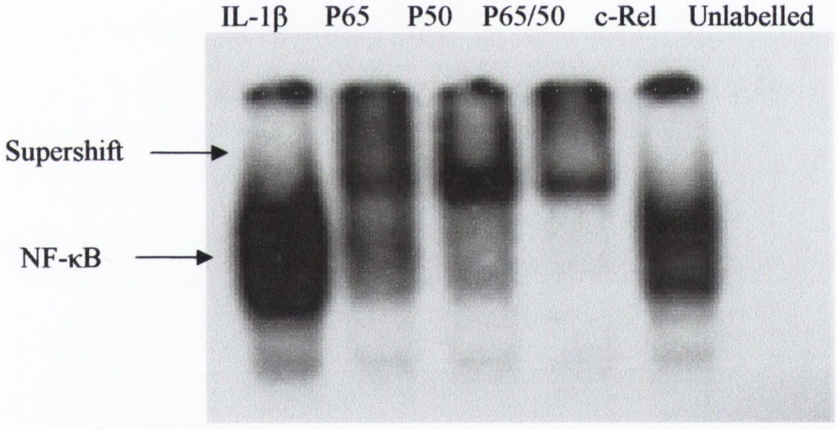


Fig. 4.2 e

Fig. 4.2d,e Supershift and competition assays were carried out on nuclear extracts from HCT116 cells stimulated with 300 μM DCA (Fig. 4.2d) and 20 ng/ml IL-1β (Fig. 4.2e). A panel of antibodies directed against various NF-κB subunits (p50, p65, c-Rel), 450 ng were preincubated with nuclear extracts from treated HCT116 cells for 30 minutes..

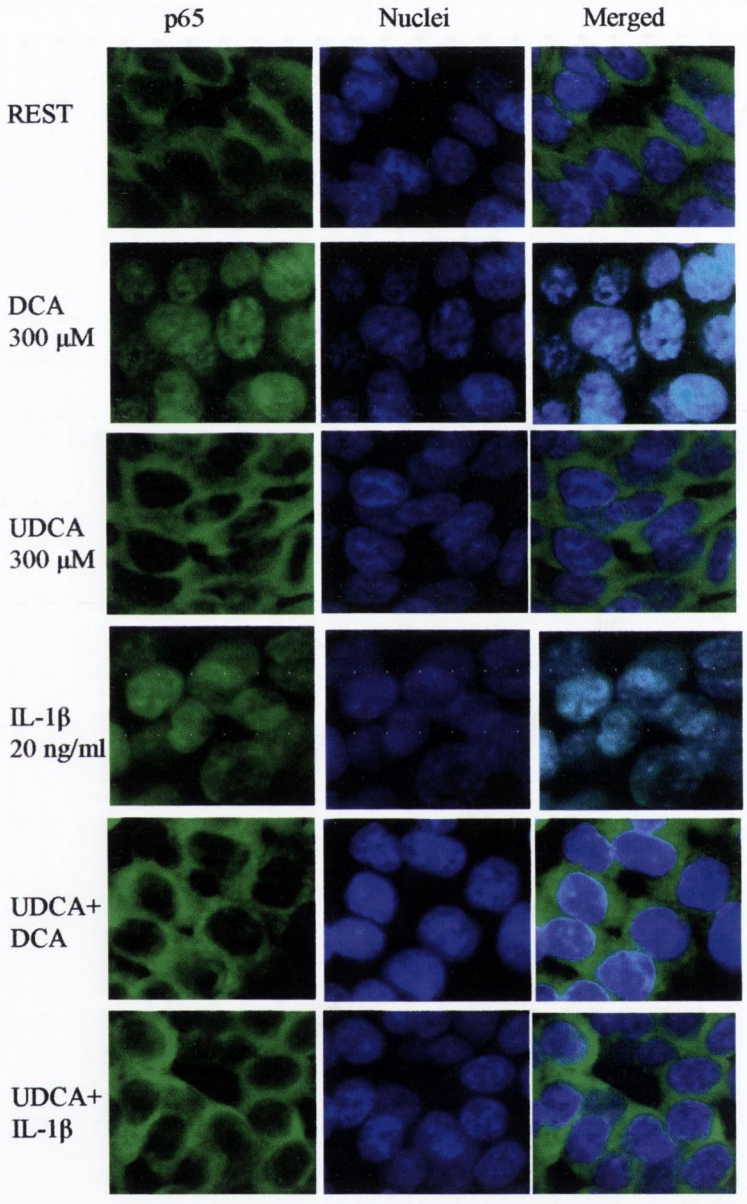


Fig. 4.3 Immunofluorescence for p65. HCT116 cells treated with DCA, IL-1β, UDCA alone or cells pretreated with UDCA first and then stimulated with DCA or IL-1β. Cells were the incubated with p65 for 1 -2 hours followed by FITC conjugated secondary antibody for 1 hour.

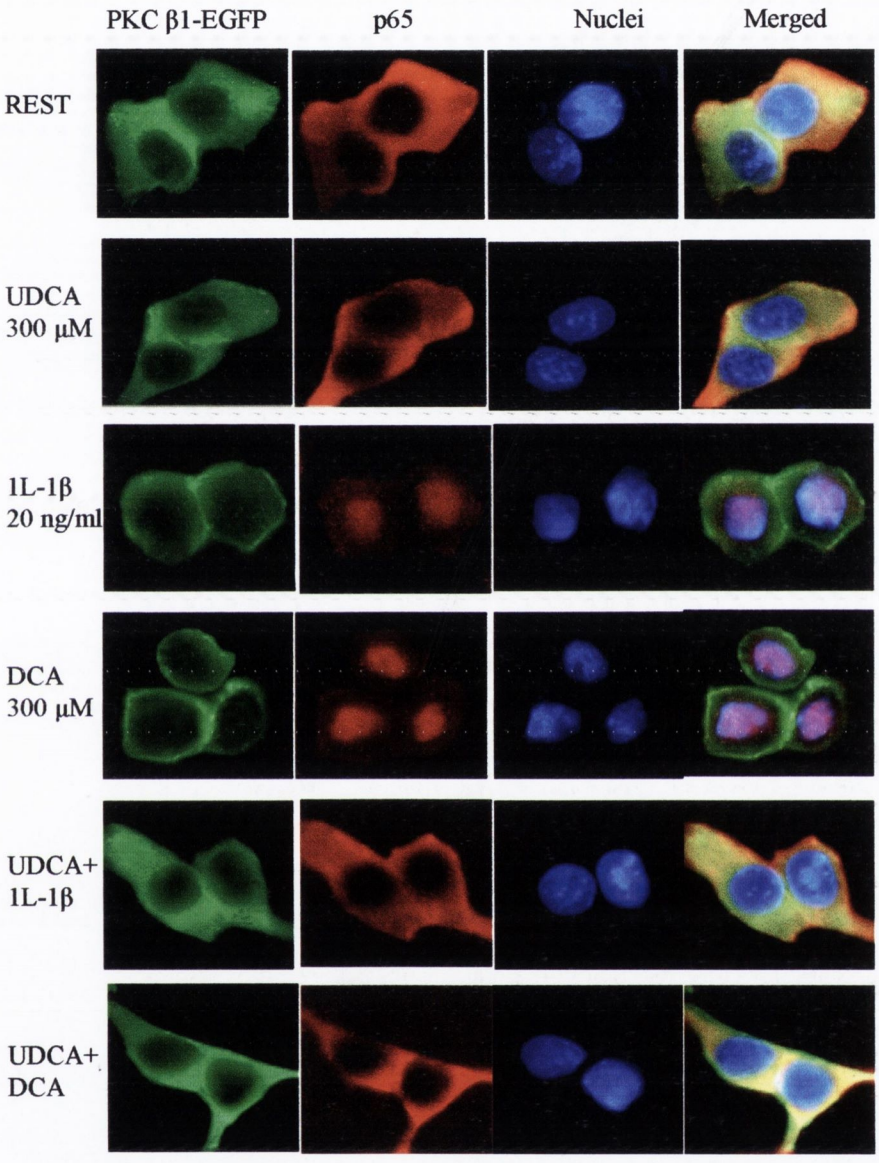


Fig. 4.4 Interaction between PKC β1and NF-κB. HCT116 cells were transfected with PKC β1-EGFP and the treated with DCA, UDCA or IL-1β alone or pretreated with UDCA. Fluorescent microscopy was performed on the cells, fixed and stained with TRITC labelled p65 antibody.

p65 antibody. Results show both DCA and IL-1β induce PKC β1 translocation to the cell membrane and NF-κB translocation to the nucleus. Pretreatment of the cells with UDCA inhibited PKC β1 membrane and NF-κB nucleus translocation.

4.3.8 Effects of UDCA on DCA, IL-1β and PMA-induced AP-1 binding (Fig 4.5a, 4.5b)

Gel shift assay was performed on the nuclear extracts of the cells treated with DCA, UDCA, IL-1β or PMA alone or cells pretreated with UDCA and the treated with DCA, IL-1β or PMA for AP-1 DNA binding. Result shows that DCA, IL-1β and PMA induce AP-1 DNA binding (Fig. 4.5a). UDCA has no effect on AP-1 DNA binding but also inhibits DCA, IL-1β and PMA-induced AP-1 DNA binding. To identify the components of the AP-1 complex in stimulated by DCA, supershift assays were performed. HCT116 cells treated with 300 μM DCA for 4-6 hours and nuclear extracts were prepared. Antibodies against Fos and Jun proteins were added to detect AP-1 components. The results show that JunD, Fra-1, and c-Fos created supershift bands. No supershift was created by the other antibodies. This indicates that JunD, c-Fos and Fra-1 are the components involved in the DCA-induced AP-1 DNA binding in HCT 116 cells.

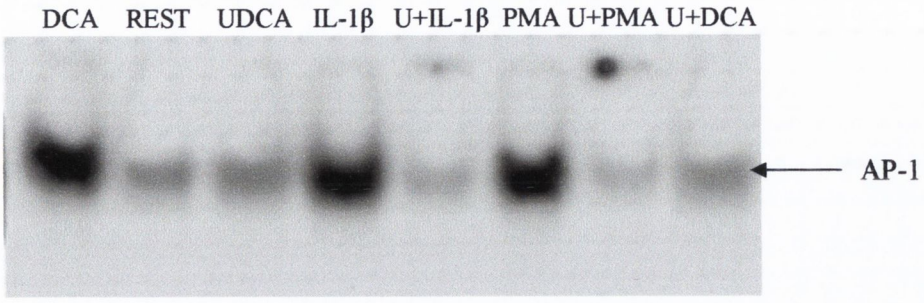
4.3.9 Role of PKC signalling pathway on NF-κB and AP-1 activation by the bile acids (Fig 4.6 a, b, c)

Gel shift assay was performed on the nuclear extracts of the cells pretreated with PKC inhibitors and activated with either DCA or IL-1β. Results show that both PKC inhibitors Calphostin C and Bisindolylmaleimide prevented NF-κB DNA binding caused by DCA at standard doses but fail to inhibit NF-κB DNA binding by IL-1β **(Fig 4.6a)**. Western blot analyses on the total cell extracts showed that inhibition of NF-κB DNA binding was associated with inhibition of IκB-α degradation in DCA treated cells **(Fig 6.4b)**. This may indicate that DCA and IL-1β activate NF-κB by different mechanisms and PKC in not the target for IL-1β-induced NF-κB activation.

On the other hand both DCA and PMA-induced AP-1 DNA binding was blocked by pre-treatment of the cells with PKC inhibitors, indicating AP-1 activation by both of these agents through PKC signalling pathway **(Fig 4.6c)**.

4.4 DISCUSSION

Transcription factors NF-κB and AP-1 are emerging as important targets in the pathogenesis of colon cancer. NF-κB is one of the most important regulators of



Fg. 4.5a Gel shift assays for AP-1 DNA binding on nuclear extracts of HCT116 cells, treated with 300 μM DCA, UDCA, 20 ng/ml IL-1β or 20 nM PMA, alone for 4-6 hours or pretreated with UDCA for 2 hours and then treated with DCA, IL-1β or PMA.

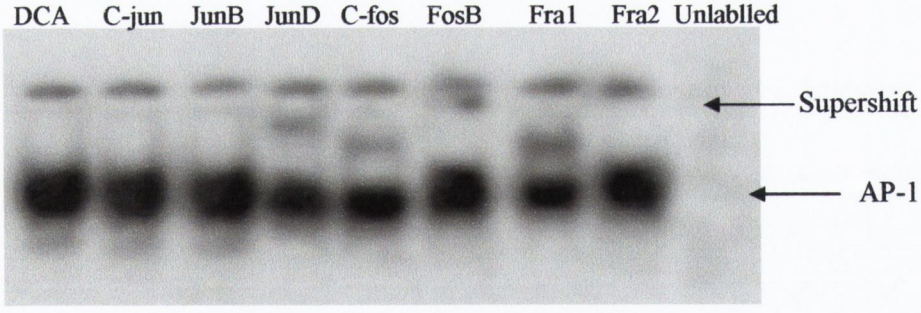


Fig. 4.5b Supershift assays on nuclear extracts from HCT116 cells stimulated with 300 μM DCA for 4-6 hours. A panel of antibodies directed against various AP-1 subunits (*c-jun, junB, junD, c-fos, fosB, Fra1, Fra2*), 45O ng were preincubated with nuclear extracts from DCA treated HCT116 cells for 30 minutes.

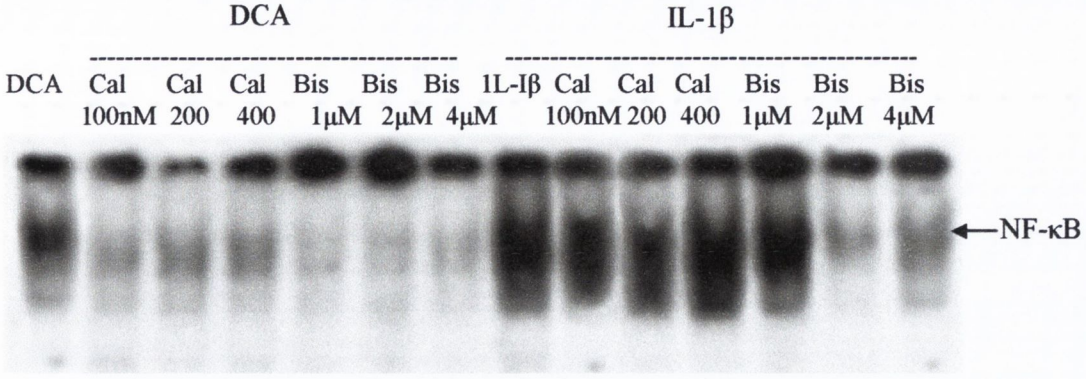


FIG 4.6 a

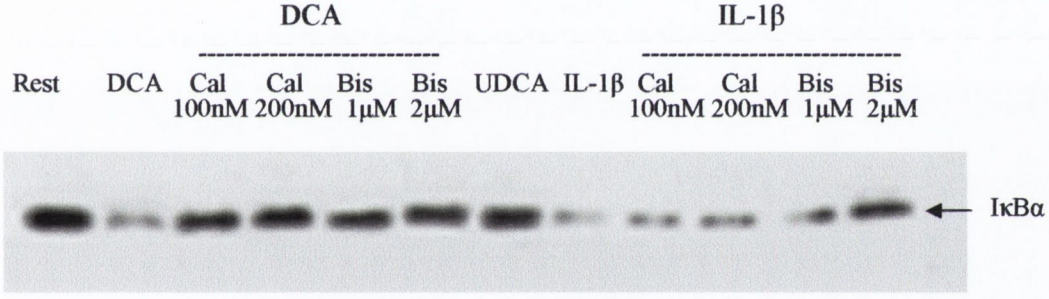


FIG 4.6 b

Fig. 4.6 a,b HCT116 cells were pretreated with PKC inhibitors Calphostin C (Cal) and Bisindolylmaleimide (Bis) at different doses prior to stimulation with either DCA or IL-1β. PKC inhibitors inhibited DCA-induced NF-κB DNA binding but fail prevent NF-κB DNA binding caused by IL-1β (Fig 4.6a). Western blot analysis on the total cell extracts shows that IκB-α degradation is inhibited by PKC inhibitors in DCA treated cells (Fig 4.6b).

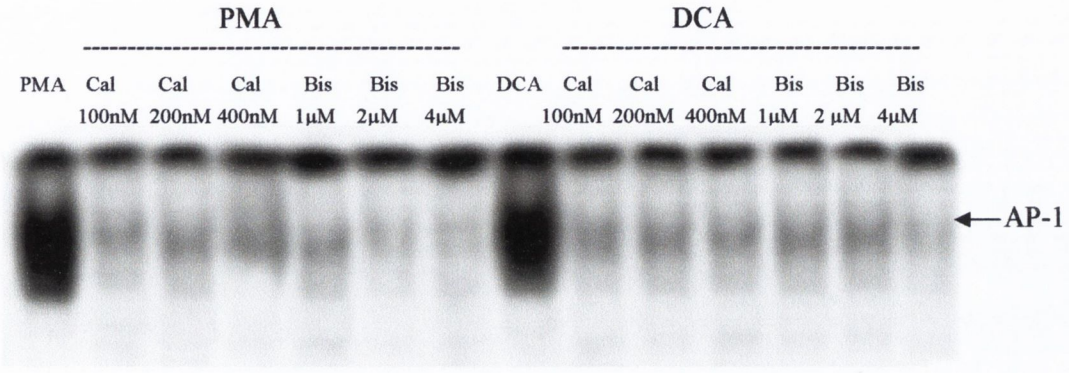


Fig. 4.6c HCT116 cells were pretreated with PKC inhibitors Calphostin C and Bisindolylmaleimide for 30 minutes at different doses prior to stimulation with either DCA or PMA. Gel shift assays were performed on the nuclear extracts of treated cell.

inflammatory response and its activators include tumour necrosis factor alpha, interleukin beta as well as many mitogenic stimuli. NF-κB has been reported to be upregulated in colorectal cancer. It is thought that high levels of NF-κB may confer survival benefit to colorectal tumour cells and rendering them more resistant to apoptosis (Gilmore *et al.*, 1996; Gilmore *et al.*, 2001; Yu *et al.*, 2003). Many anti-apoptotic genes are under the transcriptional regulation of NF-κB, which also regulates genes involved in oncogenesis, angiogenesis and proliferation (Wang *et al.*, 2003; Huang *et al.*, 2001). Tumours expressing high levels of NF-κB are resistance to apoptosis and inhibition of NF-κB has been reported to enhance sensitivity of the colonic tumour cells to chemotherapeutic agents (Cusack 2003; Uetsuka *et al.*, 2003). This hypothesis is supported by the finding that proteasome inhibitor CPT-11, used in colorectal cancer is reported to increase IκB-α. This inhibitory protein keeps NF-κB in check by binding it and inhibiting its release. Hence, NF-κB can not translocated in to the nucleus and its effects are blocked. By doing so, CPT-11 is reported to increase its increase chemosensitivity (Cusack *et al.*, 2001). Cyclooxygenase-2 (COX-2) implicated in colorectal cancers and high levels of COX-2 in colon cancer are associated with more aggressive tumours. NF-κB is involved in the regulation of COX-2 (Liu *et al.*, 2003).

AP-1 is another transcription factor implicated in colorectal carcinogenesis. Recent data has demonstrated that AP-1 is critical modulator of colorectal cancer proliferation and that dominant negative *c-jun* significantly blocked both cell proliferation and tumour growth (Suto *et al.*, 2004). Like NF-κB, AP-1 regulates many genes involved in cell transformation, oncogenesis including COX-2 in intestinal epithelial cells (Karin *et al.*, 1997; Guo *et al.*, 2001). These two transcription factors are reported to play a synergistic role in many biological processes such as cell proliferation and cross-coupling of these transcription factors is thought to enhance their effects on cell proliferation (Glinghammer *et al.*, 2002; Li 1997). Thus, agents inhibiting the activation of these two transcription factors may have a potential role in suppressing the tumour growth in colorectal cancer.

Our study indicates that bile acids DCA and UDCA have distinct and differential effects on the DNA binding of, NF-κB and AP-1 in colon cancer cell lines. DCA induced NF-κB and AP-1 DNA binding and hence caused their activation. This may be one of the mechanisms behind the tumour promoting effects of DCA reported by many researchers. UDCA on the other hand did not induce DNA binding of either of the

transcription factor. By contrast it inhibited DCA and IL-1β-induced NF-κB DNA binding and DCA, IL-1β and PMA-induced AP-1 DNA binding. The effects on IκB-α levels were also distinct. DCA treatment caused reduction in IκB-α levels, indicating degradation of IκB-α by DCA, while UDCA pre-treatment blocked IκB-α degradation induced by DCA. This may suggest a role for UDCA at cytoplasmic levels, where it may inhibit some of the kinases responsible for the phosphorylation and degradation of IκBα. One of these kinases, PKC and other members of kinase family are involved in the regulation of NF-κB. To investigate the role for PKC in modulating NF-κB and AP-, the cells were treated with various PKC inhibitors at different concentrations. The data shows that pre-treatment of the cells with PKC inhibitors prevented DCA-induced NF-κB DNA binding and inhibited IκB-α degradation, suggesting a possible role for PKC in DCA-induced NF-κB activation. An interesting observation was that IL-1β-induced NF-κB DNA binding was not blocked by the PKC inhibitors except partial inhibition at high concentration of Bisindolylmaleimide. These findings are consistent with previous reports that NF-κB binding may be induced by multiple and diverse signalling pathways. Phosphatidylinositol 3-kinase (PI 3-kinase) is involved in IL-1β-induce NF-κB DNA binding while phorbol esters may induce NF-κB DNA binding through activation of PKC (Sizemore *et al.*, 1999; Hirano *et al.*, 1995). The inhibition of IL-1β-induced NF-κB activation at high doe of Bisindolylmaleimide may be non-specific as it may inhibit other kinases at such concentration. Hence, these data suggest that PKC signalling pathway may play a significant role in the bile acid-induced NF-κB activation in colorectal cancer cells. It has been reported that, DCA, like phorbol esters induces AP-1 DNA binding in colon cancer cells and that the protein components of AP-1 induced by DCA are the products of oncogenes c-fos and c-jun (Qiao *et al.*, 2000; Matheson *et al.*, 1996). UDCA did not induce AP-1 and pre-treatment of the cells with UDCA inhibited AP-1 DNA binding caused by DCA and PMA.

UDCA has been used for more than a decade for cholestatic liver disease. More recently, UDCA is shown to have protective effect against colorectal cancer in animals as well as humans (Momen *et al.*, 2002; Pardi *et al.*, 2003). Despite abundant evidence for the cytoprotective and chemoprotective effects of UDCA in colorectal cancer, both the target molecules and pathways for UDCA effects remain elusive. Our data shows UDCA blocks DCA-induced activation of both transcription factors, AP-1 and NF-κB possibly through PKC signalling pathways in colorectal cancer cells. This may be one

of the mechanism through which UDCA is reported to provide chemoprotection against colorectal cancer.

CHAPTER V

EFFECTS OF CONJUGATED LINOLEIC ACID ON PROTEIN KINASE C AND NUCLEAR FACTOR KAPPA B TRANSLOCATION IN COLON CANCER CELLS

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5.1 INTRODUCTION

Protein kinase C are lipid dependent serine-threonine kinases, established as key regulators of signal transduction in cell proliferation, differentiation and apoptosis. Classical PKC are diacylglycerol (DAG) and calcium dependant, novel PKC calcium dependant and atypical PKC are both DAG and calcium independent (Nishizuka 1988). PKC have been reported as important factors in cell proliferation and survival pathways in colorectal cancer cells (Hochegger *et al.*, 1999). Intestinal neoplasms express reduced PKC activity relative to normal mucosa and attenuated activity of PKC has been reported in preneoplastic colonic mucosa suggesting that alterations in PKC activity predate the well-defined genetic mutations in colorectal cancer (Baum *et al.*, 1990; Guillem et *al.*, 1987; Kopp *et al.*, 1991). PKC isoenzymes are located in the cytoplasm in resting state of the cells and on activation translocate to different intracellular sites including membrane and nuclei (Nishizuka 1988).

Nuclear factor-kappa B is eukaryotic transcription factor and regulates many biological functions such as inflammation, immunity, cell proliferation and apoptosis. NF-κB consists of p50, p52 and p65, Rel B and c-Rel subunits (Ghosh *et al.*, 1998; Miyamoto *et al.*, 1995; Siebenlist *et al.*, 1994). NF-κB resides in the cytoplasm in an inactive form bound to the inhibitory molecule IκB. Once a stimulus is received, phosphorylation and degradation of IκB occurs and released NF-kB translocates to the nucleus and induces genes involved in cell transformation, proliferation, invasion and angiogenesis (Baldwin 2001; Bharti *et al.*, 2002; Martin *et al.*, 2001; Mayo *et al.*, 1997). Upregulation of NF-κB has been reported in human cancer including colon cancer (Lind *et al.*, 2001, Rayet *et al.*, 1999). It also has been reported that NF-κB activation induces resistance to apoptosis and confers survival to transforming cells (Gilmore *et al.*, 1996, 2001).

Many epidemiological studies have shown that a diet in high fat predisposes to colorectal cancer (Armstrong *et al.*, 1975). It has been suggested that large amounts of secondary bile acids are secreted in response to high fat content in diet. Predominant component of these bile acids is the deoxycholic acid (DCA) which is regarded as tumour promoter in colorectal cancer (Bayerdorffer *et al.*, 1993; Eynard *et al.*, 1997; Imary *et al.*, 1992, Lapre *et al.*, 1992; Reddy *et al.*, 1977). High serum as well as faecal

levels of secondary bile acids is found in patients with adenomas and colorectal carcinomas (Bayerdorffer *et al.*, 1993; Reddy *et al.*, 1977). In Animal studies have shown that carcinogen-treated rats, fed a diet supplemented with DCA developed more colonic tumours than those treated with carcinogen alone (Hori *et al.*, 1998; Narisawa *et al.*, 1974). The hyperproliferation of the colonic mucosa is regarded as the first step in colorectal carcinogenesis and DCA has been reported to induce colonic mucosal proliferation (Ochsenkuhn *et al.*, 1999; Hori *et al.*, 1998). Furthermore, surgically altering the bile acid flow in rat colon, hence changing the faecal bile acid excretion also altered the incidence of tumors (Morvay *et al.*, 1989). DCA has been reported to activated PKC and NF-κB and cyclooxygenase 2 (COX-2), all implicated in colorectal carcinogenesis (Fitzer *et al.*, 1987; Huang *et al.*, 1992; Payne *et al.*, 1998; Zhang *et al.*, 1998).

CLA are the geometrical and position isomers of the essential fatty acid, linoleic acid (LA), formed as result of rumen gut microbial isomerisation. (Bauman *et al.*, 2000; Griinary *et al.*, 2000). It is found in dairy products and meat of ruminant animals. Most common isomers in diet are the *c9,t11-CLA and t10,c12-CLA* and are present in equal amounts in synthesised CLA (Kritchevsky 2000). CLA are reported to have growth inhibitory effects in many types of cancers including colorectal cancer (Yeung *et al.*, 2000; Igarashi *et al.*, 2001; Cesano *et al.*, 1998; Schonberg *et al.*, 1995; Liew *et al.*, 1995). CLA are reported to inhibit cell proliferation and induce apoptosis in colon cancer cells (Miller *et al.*, 2002; Kim *et al.*, 2002). CLA administration has also been reported to protect against carcinogen-induced colon tumours in rats (Liew *et al.*, 1995; Park *et al.*, 2001). A number of mechanisms have been suggested for anticancer effects of CLA, including reduction in arachidonic acid and prostaglandin E-2 (PEG-2) levels and inhibition of eicosanoid synthesis (Banni *et al.*, 1999; Belury *et al.*, 1997; Sugano *et al.*, 1998; Liu *et al.*, 1997). CLA feeding has also been demonstrated to reduce colon tumours with effects on diacyglycerol (DAG) levels (Kim *et al.*, 2003; Park *et al.*, 2001). DAG is a co-factor for PKC activation and hence CLA might modulate PKC signalling in colorectal cancer. There are many reports of alterations in cell signal transduction by CLA (IPC *et al.*, 1991; Liu *et al.*, 1997, Moya-Camarena *et al.*, 1999) but precise signalling pathways remain elusive. In our study transfection of the cells with GFP tagged PKC isoezymes made it possible to visualize some of these signalling dynamics in living colon cancer cells. Oleic acid is a cis- monounsaturated fatty acid and is thought to be involved in mitogenic signalling pathways. Oleic acid is reported to

activate PKC directly and is a more potent activator of the Ca^{2+}-independent PKC isoforms suggesting that that fatty acids are physiological regulators of Ca^{2+}-independent PKC activity (Murakami *et al.*, 1986; Khan *et al.*, 1993).

5.2 OBJECTIVES

1 To investigate the effects of the acute treatment with CLA Isomers *c9,t11-CLA and t10,c12-CLA* on the PKC isoenzymes activation
2 To investigate the effects of the chronic treatment with CLA Isomers *c9,t11-CLA and t10,c12-CLA* on the PKC isoenzymes activation
3 To investigate the effects of the acute treatment with CLA Isomers *c9,t11-CLA and t10,c12-CLA* on the NF-κB signalling in colon cancer cells.
4 To investigate the effects of the chronic treatment with CLA Isomers *c9,t11-CLA and t10,c12-CLA* on the NF-κB signalling in colon cancer cells.
5 To investigate the effects of OA on PKC and NF-κB expression

5.3 RESULTS

The effects of acute and chronic and acute treatments of *c9,t11*-CLA and *t10,c12*-CLA on EGFP-tagged PKC isoenzymes and transcription factors NF-κB are described. HCT116 cells were treated with CLA or OA or LA for 24 hours for acute treatment and 14 days for chronic treatment. The cells were then transfected with PKC isoenzymes and stimulated with DCA, PMA or with DCA, IL-1β in untransfected cells for NF-κB. The cells were examined with real time photomicroscopy and immunofluorescent microscopy to investigate the effects on PKC and gel shift assays on nuclear extracts of the cells for NF-κB DNA binding.

5.3.1 Effects of acute *c9,t11*-CLA and *t10,c12*-CLA treatment on PKC β1 translocation (Fig 5.1)

PKC β1 is found in the cytoplasm of the cells in resting cells. On stimulation, it translocates to the cell membrane as shown in PMA treated cells. EGFP only transfected cells were treated with PMA and DCA in a similar fashion for control. The two CLA isomers used *c9,t11-CLA and t10,c12-CLA* and LA or OA did not cause translocation of PKC β. Both PMA and DCA caused translocation of PKC β1 to the cell membrane but had no effect on EGFP only transfected cells. Pretreatment of the

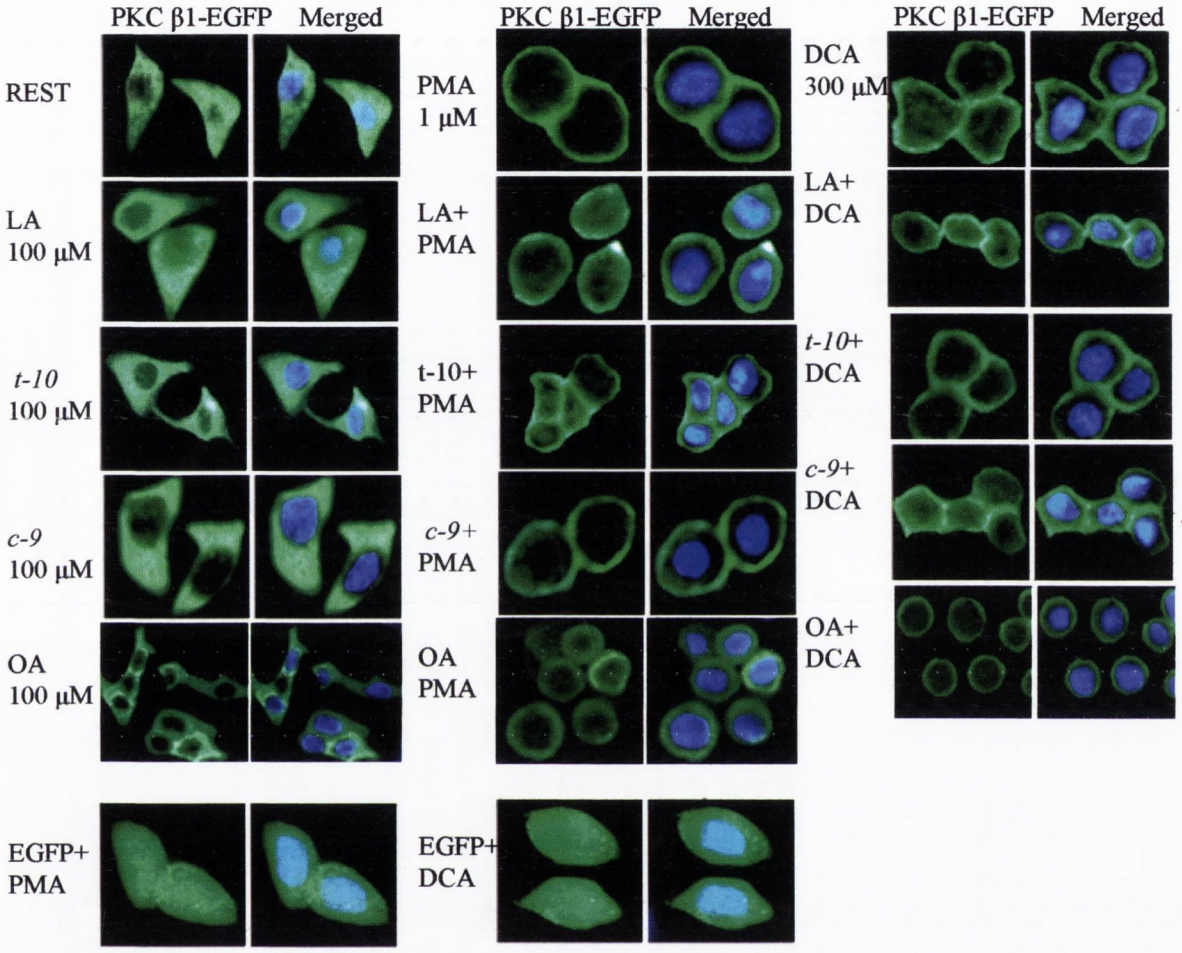

Fig 5.1 HCT116 cells were treated with *c-9, t-11, t-10, c-12* CLA, OA or LA for 24 hours. The cells were transfected with enhanced green fluorescent protein (EGFP) or PKC β1-EGFP and then DCA or PMA for 2 hours. Untreated cells are labelled as Rest. EGFP only transfected cells were treated with DCA or PMA. Nuclei are stained with DAPI.

cells with *c9,t11-CLA and t10,c12-CLA*, LA or OA for 24 hour did not block DCA and PMA-induced PKC β1 translocation.

5.3.2 Effects of chronic *cis-9, trans-11* and *trans-10, cis-12* CLA treatment on PKC β1 translocation (Fig 5.2)

PKC β1 transfected cells were treated with *c9,t11-CLA and t10,c12-CLA* for 14 days and then effects of DCA on PKC β1 translocation observed. The results show that chronic pre-treatment of the cells with these two CLA isomers inhibited DCA-induced PKC β1 translocation. Chronic pre-treatment with LA or OA did not inhibit DCA-induced PKC β1 translocation. EGFP transfected cells are shown for control.

5.3.3 Effects of acute *c9,t11*-CLA and *t10,c12*-CLA treatment on PKC δ translocation (Fig 5.3)

PKC δ is novel PKC isoenzymes and translocates to the membrane as well as nucleus on activation. Both PMA and DCA caused translocation of the PKC δ to the cell membrane and to the nucleus in untreated cells. Pre-treatment of the cells with *c9,t11-CLA and t10,c12-CLA,* LA or OA for 24 hours, did not inhibit DCA-induced PKC δ translocation. Acute treatment with CLA had no effect on PKC δ translocation in these cells.

5.3.4 Effects of chronic *c9,t11*-CLA and *t10,c12*-CLA treatment on PKC δ translocation (Fig 5.4)

In the cells, chronically treated with *c9,t11*-CLA and *t10,c12*-CLA show inhibition of PKC δ translocation caused by the DCA. LA or OA pre-treatment for 14 days failed to inhibit DCA-induced PKC δ translocation. EGFP transfected cells are also shown.

5.3.5 Effects of acute *c9,t11*-CLA and *t10,c12*-CLA treatment on PKC ζ translocation (Fig 5.5)

PKC ζ is an atypical PKC isoform without DAG or calcium binding sites. It did not respond to stimulation with either PMA or DCA in our experiments (data not shown). C2-ceramide was used to observe the effects of PKC ζ translocation. C2-ceramide caused translocation of PKC ζ to the cell membrane and nucleus. Acute treatment with *c9,t11-CLA and t10,c12-CLA* did not inhibit C2-ceramide-induced PKC ζ translocation. An interesting observation was that a higher concentration of OA at 200 μM caused

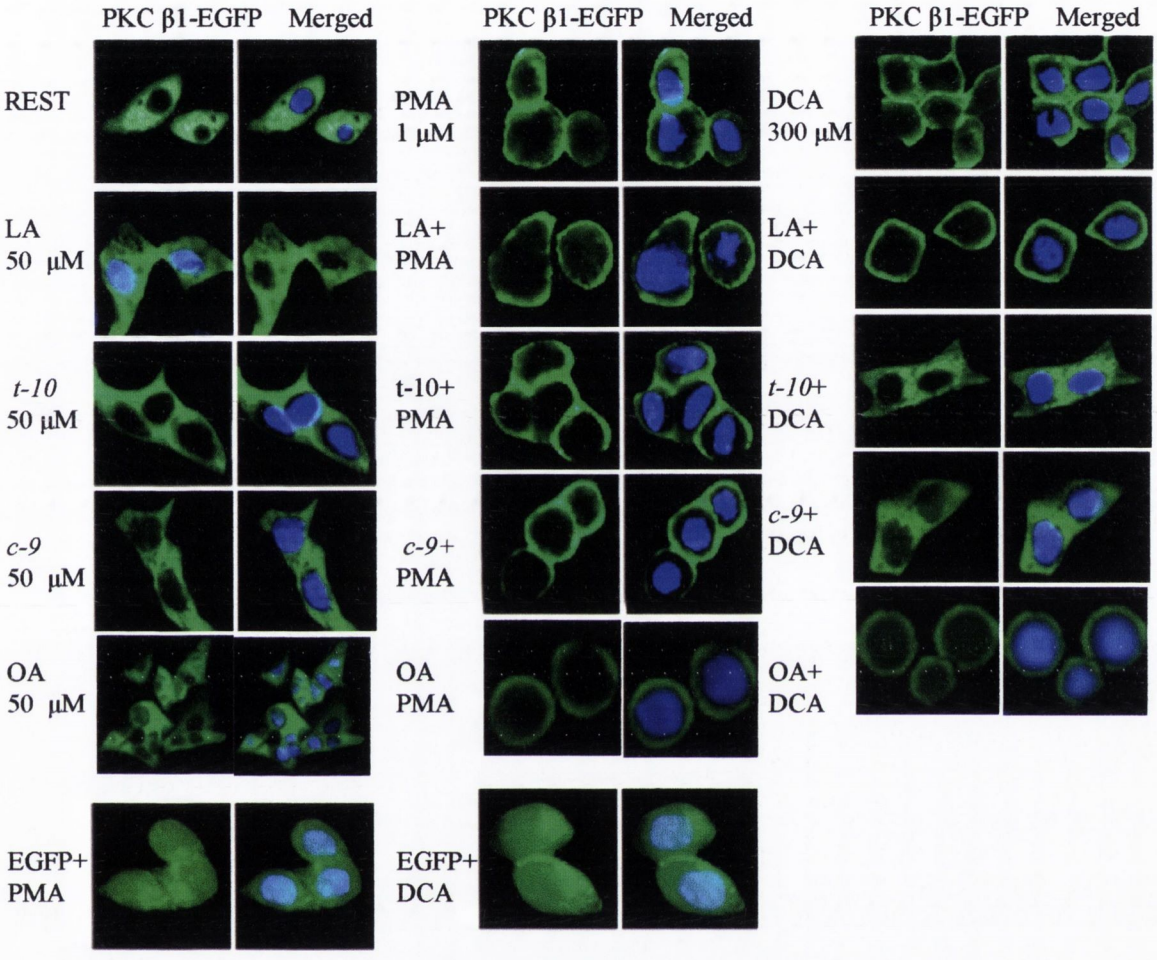

Fig. 5.2 HCT116 cells were treated with *c9,t11*-CLA , *t10,c12*-CLA, OA or LA for 14 days. These cells transfected with EGFP or PKC β1-EGFP and either treated with DCA or PMA for 2 hours. Untreated cells are labelled as Rest.

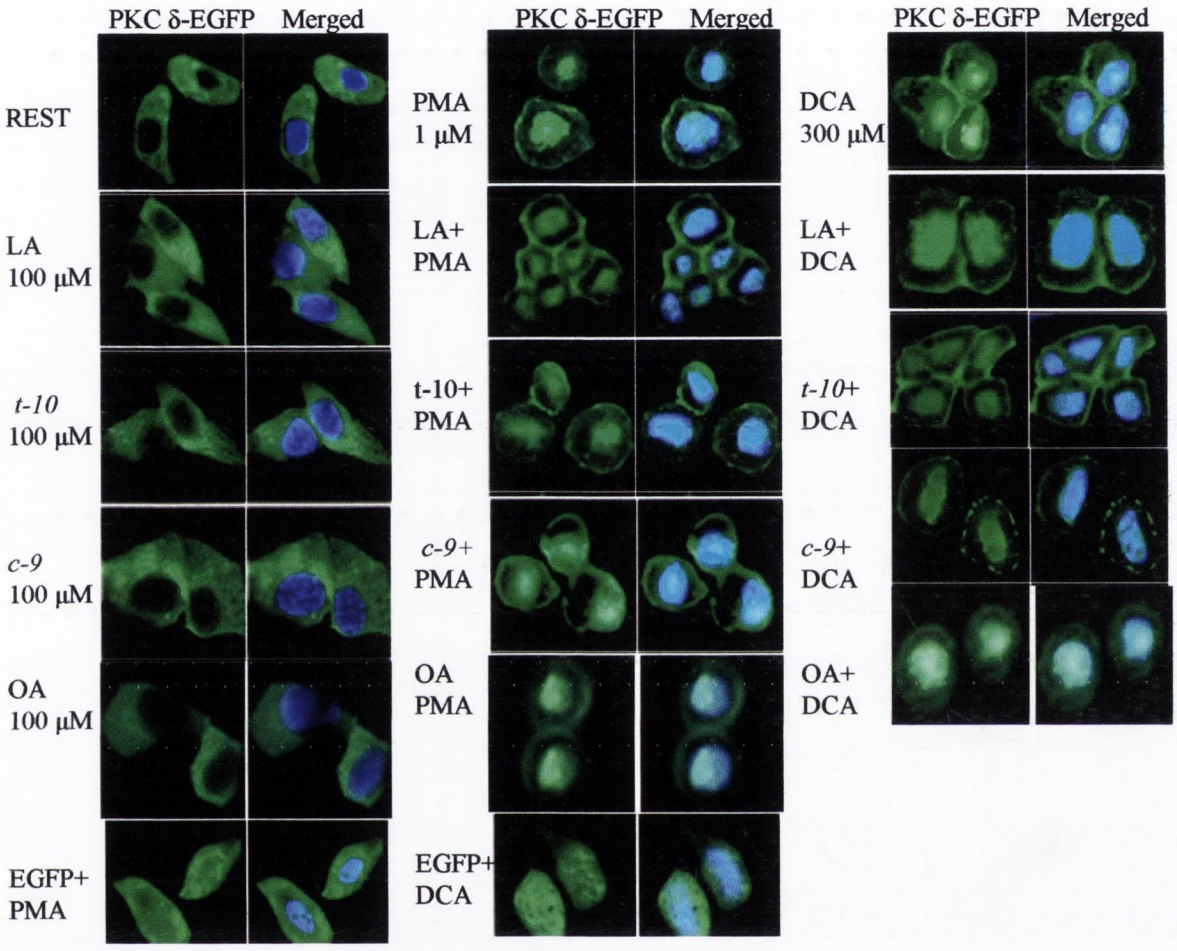

Fig. 5.3 HCT116 cells were treated with *c9,t11*-CLA , *t10,c12*-CLA, OA or LA for 24 hours. The cells were transfected with EGFP or PKC δ-EGFP, then either treated with DCA or PMA for 2 hours. Untreated cells are labelled as Rest. EGFP only transfected cells were treated with DCA or PMA.

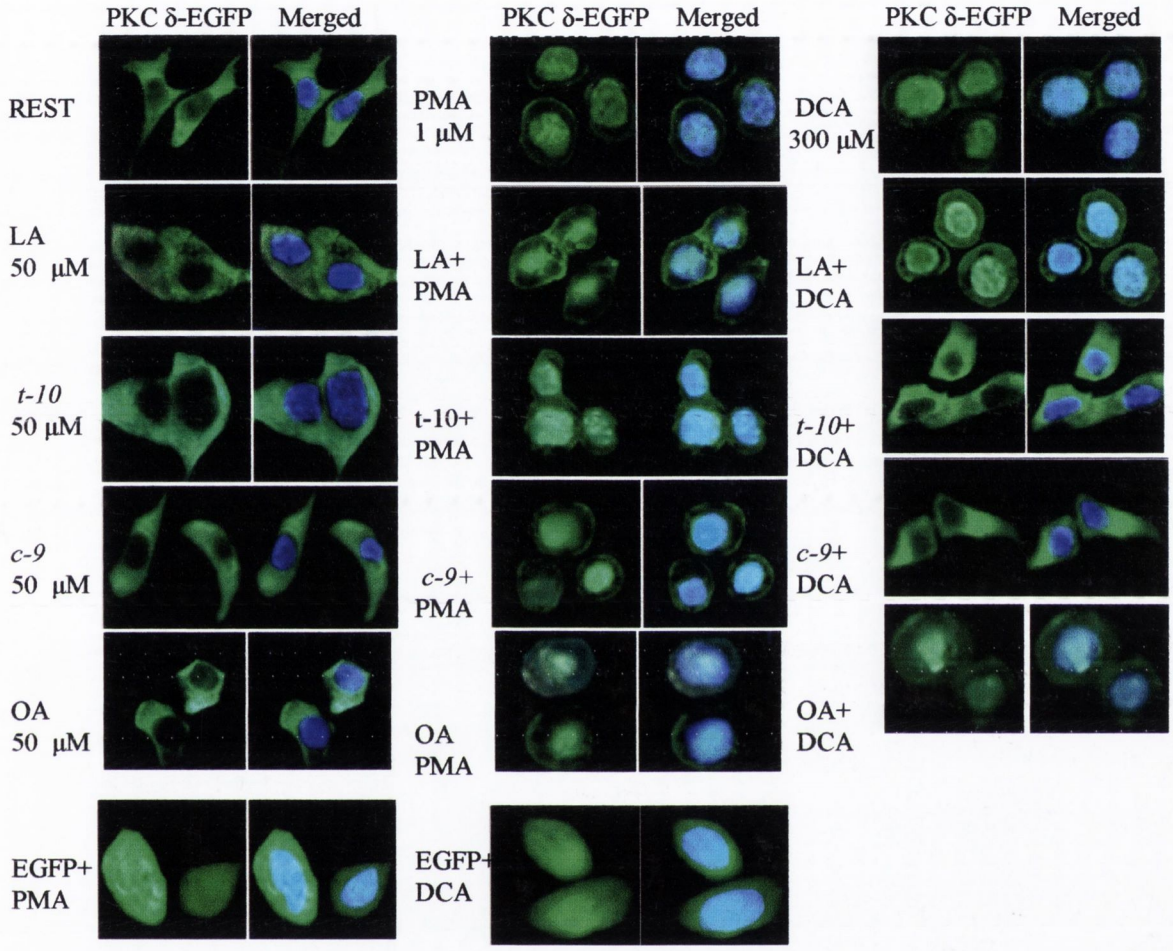

Fig. 5.4 HCT116 cells were treated with *c9,t11*-CLA , *t10,c12*-CLA, OA or LA for 14 days. The cells were transfected with EGFP or PKC δ-EGFP and treated with DCA or PMA for 2 hours. Untreated cells are labelled as Rest.

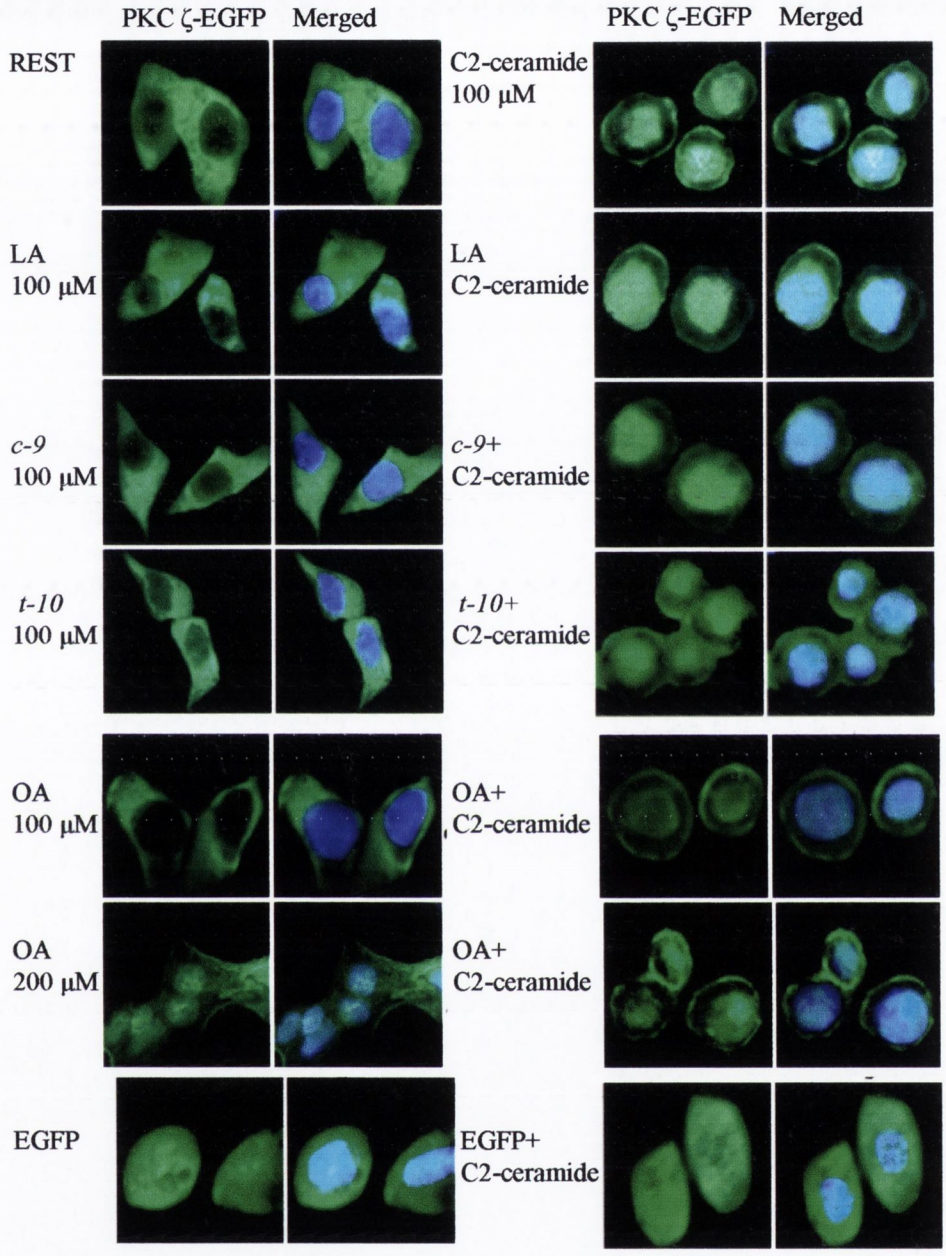

Fig. 5.5 HCT116 cells were treated with *c9,t11*-CLA , *t10,c12*-CLA, LA or 100-200 μM OA for 24 hours, transfected with EGFP or PKC ζ-EGFP and treated with C2-ceramide, DCA, PMA for 2 hours. Interestingly higher concentration of OA caused PKC ζ translocation. DCA and PMA had no effect (data not shown). Dihdroceramide used as control, did not cause PKC ζ translocation (data not shown).

PKC ζ translocation. The doses of all the agents CLA, LA and OA were titrated for the effects on PKC isoforms and this effect was only observed with OA.

5.3.6 Effects of chronic *c9,t11*-CLA and *t10,c12*-CLA treatment on PKC ζ translocation (Fig 5.6)

HCT 116 cells treated with *c9,t11-CLA and t10,c12-CLA* for 14 days inhibited C2-ceramide-induced PKC ζ membrane and nucleus translocation. LA pre-treatment did not inhibit PKC ζ translocation. C2-ceramide treatment had no effect on EGFP transfected cells. Chronic OA treatment at 50 μM did not cause PKC ζ translocation.

5.3.7 Effects of acute *c9,t11*-CLA and *t10,c12*-CLA treatment on NF-κB DNA binding (Fig 5.7)

NF-κB is located in the cytoplasm of the cell, bound to the inhibitory protein IκB α. On activation, it translocated to the nucleus. To observe the effects CLA on NF-κB DNA binding, gel shift assays were performed on the nuclear extracts of the cells, treated with either *c9,t11-CLA , t10,c12-CLA* or LA for 14 days and then activated with either DCA or IL-1β. The results show that CLA and LA did not induce NF-κB DNA binding. Both DCA and IL-1β cause NF-κB DNA binding in untreated cells. Acute treatment of the cells with either *c9,t11-CLA, t10,c12-CLA* or LA had no effect on DCA or IL-1β-induced NF-κB DNA binding.

5.3.8 Effects of chronic *c9,t11*-CLA and *t10,c12*-CLA treatment on NF-κB DNA binding (Fig 5.8)

These gel shift assay results show that chronic treatment of the cells with *c9,t11-CLA and t10,c12-CLA* inhibits DCA as well as IL-1β-induced NF-κB DNA binding. Pretreatment with LA failed to block NF-κB DNA binding in these cells.

5.3.9 Effects of acute *c9,t11-CLA* and *t10,c12-CLA* and OA on NF-κB DNA binding (Fig 5.9)

To investigate the effects CLA on NF-κB DNA binding and compare it with those of OA, gel shift assays were performed on the nuclear extracts of the cells, treated with either *c9,t11-CLA , t10,c12-CLA* or OA for 14 days and then activated with either DCA or IL-1β. The results show that both DCA and IL-1β cause NF-κB DNA binding in untreated cells that CLA did not induce NF-κB DNA binding. Acute treatment of the

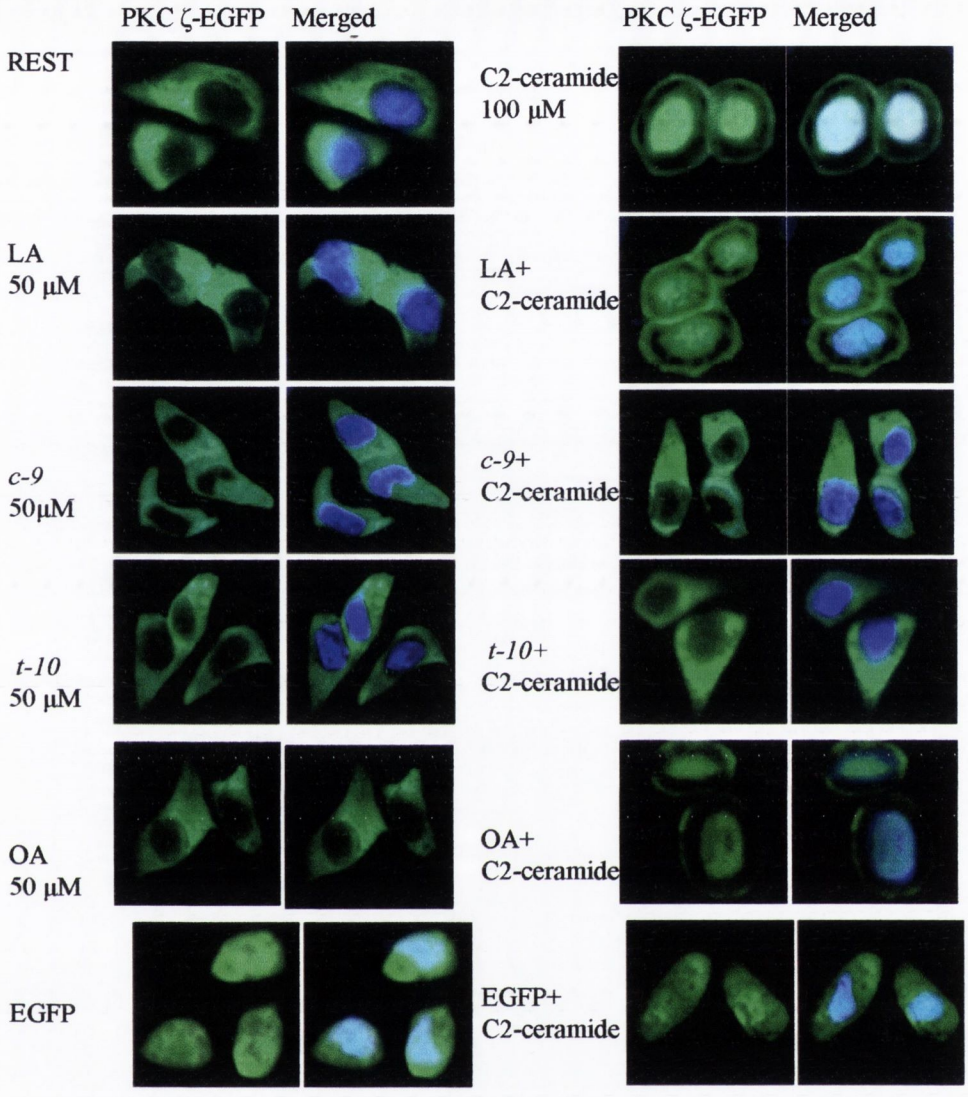

Fig. 5.6 HCT116 cells were pretreated with *c9,t11*-CLA , *t10,c12*-CLA, OA or LA for 14 days. The cells were transfected with PKC ζ-EGFP and treated with C2-ceramide for 2 hours. Untreated cells are labelled as Rest. DCA and PMA or Dihydroceramide had no effect on PKC ζ (data not shown).

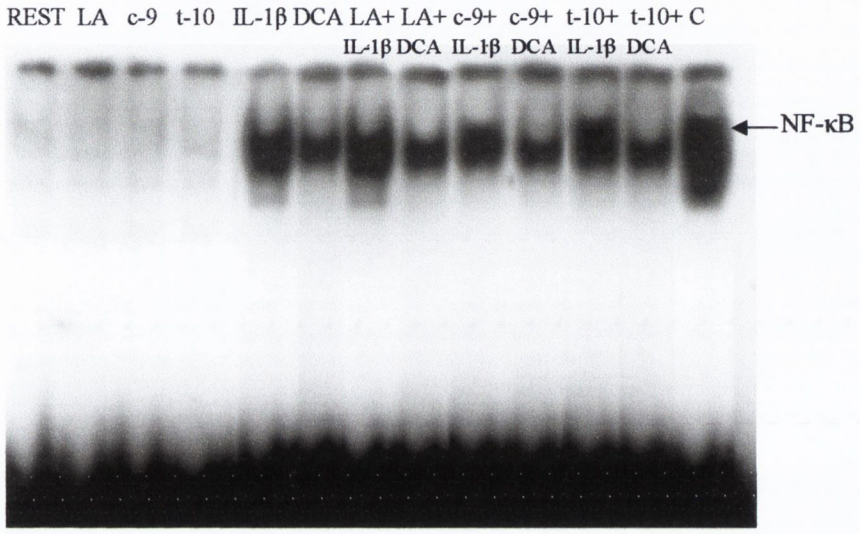

Fig. 5.7 HCT116 cells were treated with 100 μM c9,t11-CLA , t10,c12-CLA or LA for 24 hours. The cells were then treated with 300 μM DCA or 20 ng/ml IL-1β for 2 hours. Gel shift assays were performed on the nuclear extracts of these cells for NF-κB DNA binding. Untreated cells are labelled as Rest. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

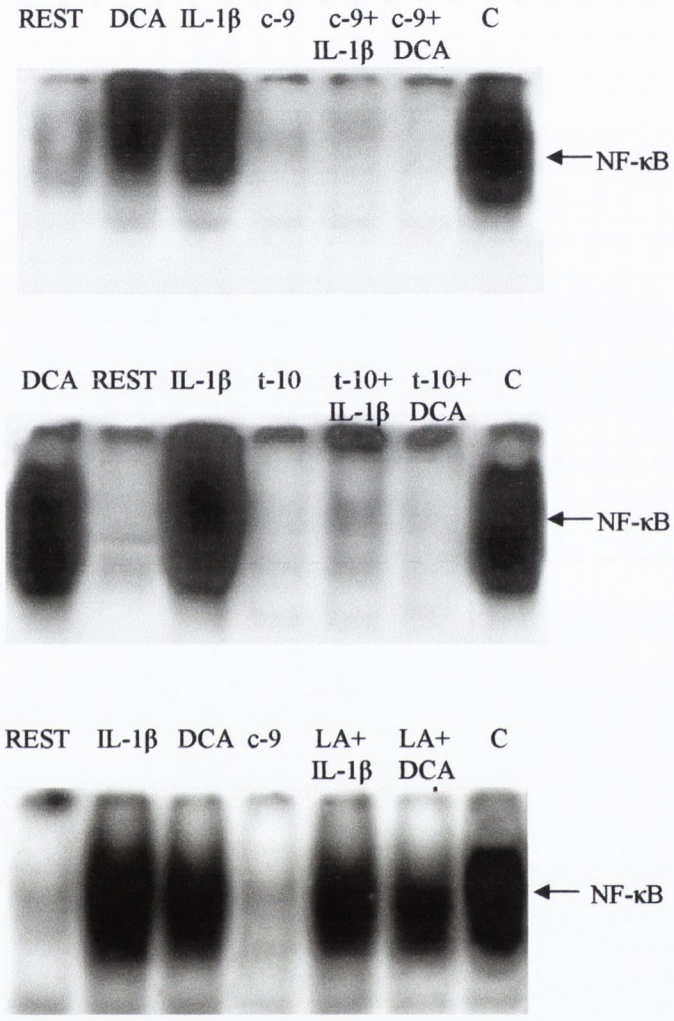

Fig. 5.8 HCT116 cells were treated with 50 μM *c9,t11*-CLA , *t10,c12*-CLA or LA for 14 days. The cells were then treated with 300 μM DCA or 20 ng/ml IL-1β for 2 hours. Gel shift assays were performed on the nuclear extracts of these cells for NF-κB DNA binding. Untreated cells are labelled as Rest. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

cells with OA caused NF-κB DNA binding though the effects were seen at a higher concentration as observed for PKC ζ.

5.3.10 Effects of chronic *c9,t11*-CLA and *t10,c12*-CLA or OA on NF-κB DNA binding (Fig 5.10)

The effects of chronic treatment with CLA on NF-κB compared with OA were investigated. These gel shift assay results showed that chronic treatment of the cells with *c9,t11-CLA and t10,c12-CLA* inhibited DCA as well as IL-1β-induced NF-κB DNA binding. Pretreatment with OA caused NF-κB DNA binding in these cells.

5.4 DISCUSSION

Our study demonstrates that chronic treatment of the HCT 116 colon cancer cell line with *c9, t11*-CLA and *t10,c12*-CLA isomers inhibits DCA-induced translocation of PKC β1, PKC δ and C2-ceramide-induced translocation of the PKC ζ. To date this is the first report of CLA effects on interaction between bile acids and GFP-tagged PKC isoforms in living cancer cells. PKC isoenzymes have been implicated in the regulation of the cellular and neoplastic transformation processes in the colon (Black 2000; Musashi *et al.*, 2000). A number of studies have reported reduced expression of PKC in human colonic carcinomas and adenomas as well as preneoplastic mucosa compared with colonic mucosa. (Kahl-Rainer *et al.*, 1994; Kopp *et al.*, 1991) PKC α, βI, and ζ seem to be lost early during intestinal carcinogenesis (Klein *et al.*, 2000). Overexpression of PKC β1 and γ isoforms had resulted in increased growth rate, high saturation densities, anchorage-independent growth and enhanced tumorigenicity in rodent fibroblasts (Housey *et al*, 1988). PKC β1 overexpression has also been associated with resistance to apoptosis (Cesaro *et al.*, 2001). Although PKC βI expression is modestly decreased in aberrant crypt foci, it is markedly reduced in APC^{min} mouse adenomas and in azoxymethane-induced colon carcinomas in mice(Klein *et al.*, 2000; Gokmen-Polar *et al.*, 2001). PKC δ is involved in the regulation of cell growth and differentiation and apoptosis and tumour development. (Emoto *et al.*, *1995*; Ghayur *et al.*, 1996). Decreased expression of PKC ζ has been reported in both human and experimental colon cancers (Kahl-Rainer *et al.*, 1994).

It has been suggested that bile acids act as tumour promoters by disturbing the balance between proliferation, differentiation and apoptosis in colonic epithelial cells (Hoffman 1999). High serum and faecal levels of secondary bile acids are found in patients with

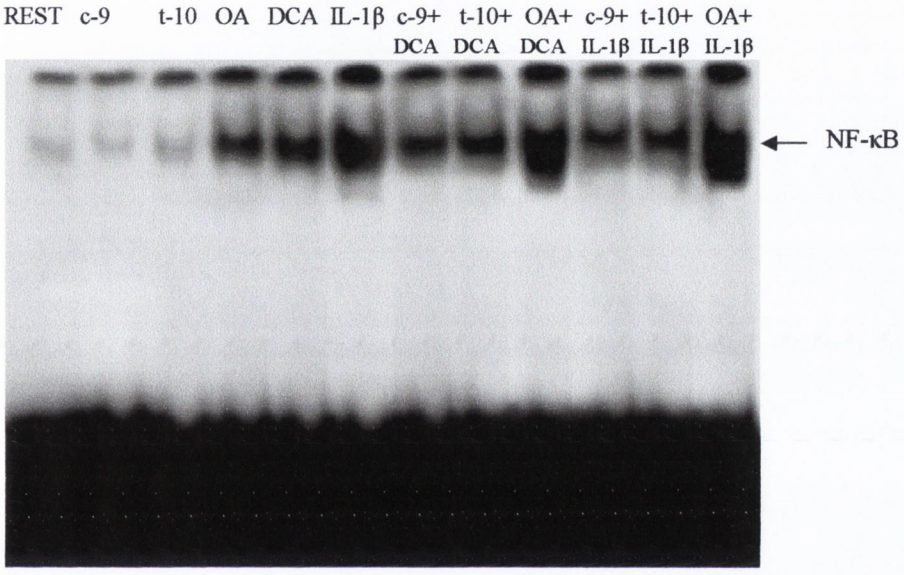

Fig. 5.9 HCT116 cells were treated with 100 μM *c9,t11*-CLA , *t10,c12*-CLA or OA for 24 hours. The cells were then treated with 300 μM DCA or 20 ng/ml IL-1β for 2 hours. Gel shift assays were performed on the nuclear extracts of these cells for NF-κB DNA binding. Untreated cells are labelled as Rest.

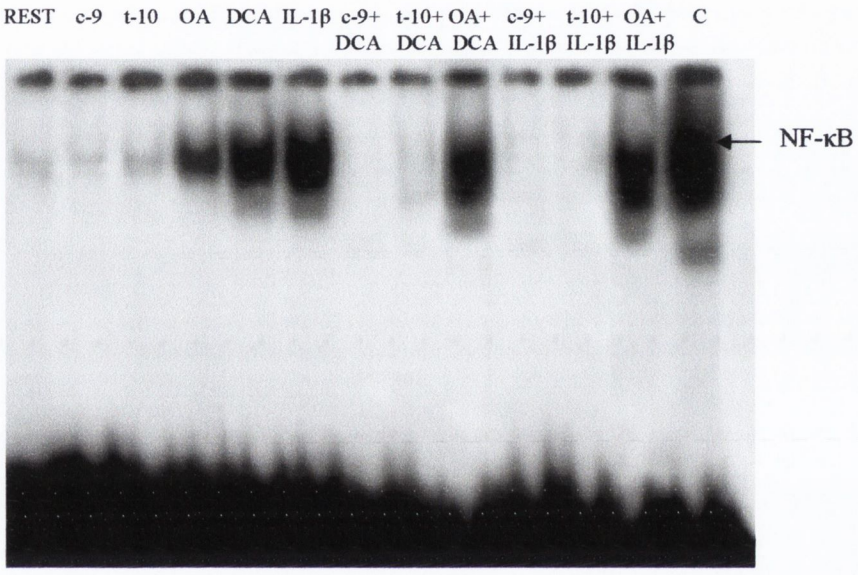

Fig. 5.10 HCT116 cells were treated with 50 μM *c9,t11*-CLA , *t10,c12*-CLA or OA for 14 days. The cells were then treated with 300 μM DCA or 20 ng/ml IL-1β for 2 hours. Gel shift assays were performed on the nuclear extracts of these cells for NF-κB DNA binding. Untreated cells are labelled as Rest. The Human T cell leukaemic HuT 78 cell line constitutively expressing NF-κB, was used as positive control (C).

colorectal adenomas and carcinomas.(Reddy *et al.*, 1977; Bayerdorffer *et al.*, 1993). Carcinogen-treated rats, fed a diet supplemented with deoxycholic acid (DCA) developed more colonic tumours than those treated with carcinogen alone (Hori *et al.*, 1998; Narisawa *et al.*, 1974). Bile acids are thought to induce hyperproliferation in colon mucosal cells (Ochsenkuhn *et al.*, 1999). Others suggest that bile acids may exert their cytotoxic effects through activation of intracelluar signalling pathways such as Protein Kinase C (PKC) and Map Kinases (MAPK) (Qiao *et al.*, 2000; Huang *et al.*, 1992). Bile acids are reported to cause DNA damage in colon cells leading to genetic mutations (Venturi *et al.*, 1997). In addition bile acids induce transcription factors, Nuclear Factor Kappa B (NF-κB) and Activator Protein 1 (AP-1), both implicated in the pathogenesis of colon cancer (Glinghammer *et al.*, 2002).

Our study also investigated the role of CLA treatment on transcription factor NF-κB DNA binding in colon cancer cells. Our data shows that chronic treatment of the cells with either *c9,t11*-CLA, *t10,c12*-CLA inhibits both DCA and IL-1β-induced NF-κB DNA binding. LA on the other hand did not prevent NF-κB DNA binding. None of the CLA or LA activated NF-κB DNA binding either with acute or chronic treatment. CLA has recently been reported to inhibit LPS-induced NF-κB activation in RAW macrophages (Cheng *et al.*, 2004). NF-κB has a significant role in the pathogenesis of colon cancer. The upregulation of NF-κB have been reported in colon cancer and high levels of this transcription are associated with resistance to apoptosis by the chemotherapeutic agents (Yu *et al.*, 2003; Cusack *et al.*, 2003). It has been demonstrated that colon tumour are rendered more chemosensitivity by increasing the levels of inhibitory protein IκB (Cusack *et al.*, 2001). IκB proteins bind to and prevent NF-κB activation. Once degradation of IκB occurs, NF-κB nuclear is released and becomes activated. NF-κB regulates many important genes involved in apoptosis, oncogenesis, angiogenesis and proliferation (Huang *et al.*, 2001; Bharti *et al.*, 2002). Cyclooxygenase-2 (COX-2) overexpression is associated with aggressive colorectal tumours and COX-2 expression is under the regulatory control of NF-κB (Liu *et al.* 2003). It has previously been reported that CLA modulates NF-κB and COX- 2 in macrophages, thus affecting the inflammatory cascade (Cheng *et al.*, 2004). LA is reported activate NF-κB in endothelial cells (Park *et al.*, 2001; Toborek *et al,*. 1996). LA has also been associated with increased risk of colon cancer (Miller *et al.*, 2001), hence inhibition of NF-κB activation by the CLA may be a significant mechanism in the anticarcingenic effects of CLA in colon cancer. An interesting observation was that OA

treatment caused PKC ζ translocation and activated NF-κB. This effect occurred at a higher concentration of the OA. The concentrations of all the CLA, LA and OA were tirated in our experiments. Only OA caused activation of PKC and NF-κB and others at similar concentrations did not activate either PKC or NF-κB. This might have implications for carcinogenesis as *cis*-PUFAs have been reported to enhance human breast cancer cell adhesion to collagen IV by selectively activating specific PKC isoenzymes (Palmantier *et al.*, 2001). Studies have demonstrated that oleic acid activates calcium-independent PKC causing the phosphorylation of the 40-kDa substrate (Parka *et al.*, 2003).

Previous studies have reported that several saturated and unsaturated fatty acids potentiate PKC activity (Shirai *et al.,* 1998; Nishizuka 1995). Unsaturated fatty acids such as arachidonic and oleic acid are reported to enhance the activity of several PKC isoenzymes (Khan *et al.* 1993; Diaz-Guerra *et al.,* 1991). However, much less information is available on the effects of CLA in PKC signalling. However, there is abundant of data on anticarcinogenic effects of CLA in colorectal cancer and several mechanisms have been described. It has been reported that CLA inhibit cell proliferation and stimulate apoptosis in colon cancer cells by downregulating ErbB3 signalling and the PI3-kinase/Akt pathway (Cho *et al.*, 2003). In a recent study CLA feeding has been shown to significantly increase natural killer (NK) cytotoxicity in azoxymethane-induced aberrant crypt foci formation in a rat model of colon carcinogenesis (Nichenametla *et al.*, 2004). It has also been reported that CLA may inhibit colon cancer growth through *p53* dependant mechanism. (Kemp *et al.* 2003). CLA is also thought to be a ligand for peroxisome proliferator-activated receptor (PPARγ), a nuclear hormone receptor with growth inhibitory effects in cancer cells (Bull *et al.* 2003; Yu *et al.* 2002).

PKC is an upstream modulator for NF-κB activation and our data suggests that chronic CLA treatment may affect NF-κB through modulation of PKC. It has been reported in previous studies that CLA are incorporated in the cell membrane phospholipids, with the potential to modulate cell signalling. (Kim *et al.*, 2003). The membrane phospholipids play a significant role in signal transduction and inhibition of phospholipase A2 by other chemoprotective agents has been reported (Ikegami *et al.,* 1998). The diacylglycerol (DAG) is an activator of PKC and CLA have been reported to reduce CLA levels. We suggest that chronic treatment with CLA may affect PKC signalling through modification of membrane phospholipids or reduction of DAG

levels. The PKC isoenzymes and NF-κB play a pivotal role in many events leading to carcinogenesis including cell growth, proliferation, cell migration and apoptosis. The alteration of these signalling pathways by the CLA isomers may be significant mechanism in the growth inhibitory effects of CLA reported in many epidemiological and animal studies.

High fat diets particularly saturated fatty acids and cholesterol has been reported to play an important role in increased colon cancer risk (Navarro *et al.,* 1995)., Conjugated linoleic acid (CLA), on the other hand is the only fatty acid reported, unequivocally to inhibit carcinogenesis in experimental animals (National Research Council 1996 USA). Diets high in animal fat seem to modulate opposite effects in human colon cancer development and relative concentrations of the saturated fats and CLA may play a crucial role in preventing this common malignancy. Inhibition of PKC and NF-κB by the two isomers *c9,t11-CLA and t10,c12-CLA* may be a significant mechanism in the protective effect of these ruminant fats in colon cancer reported in many studied.

CHAPTER VI

GENERAL DISCUSSION

CHAPTER VI

GENERAL DISCUSSION

8.1 Bile acids, PKC and colon cancer

Bile acids, particularly deoxycholic acid (DCA), are implicated in promoting colon cancer growth and progression. The mechanisms by which secondary bile acids such as DCA exert their tumour promoting are not clear. However, a number of mechanisms and pathways have been suggested. The interactions between bile acids and PKC signalling pathways have long been the target of research and PKC pathway is probably the best studied signalling mechanisms in CRC (Huang *et al.*, 1992). This is because of the importance of the PKC in regulating cell proliferation, apoptosis and differentiation (Clemens *et al.*, 1992). Our study concentrated on these interactions but with a difference that they were studied in living cancer cells. As was described earlier PKC isoenzymes are probably secreted in pulses and are rapidly downgraded after their release. Their importance in tumour promotion became known by the finding that conventional PKC possess a phorbol ester-binding site, a known tumour promoter. PKCs are the key elements in cell growth proliferation and apoptosis (Black 2000; Clemens *et al.*, 1992; Musashi *et al.*, 2000). The levels of many PKC isoforms are reduced in human cancers and their overexpression in particular PKC α and PKC β1, are associated with enhanced cell growth and cell proliferation CRC (Kahl-Rainer *et al.*, 1994; Guillem *et al.*, 1987; Housey *et al.*, 1988; Krauss *et al.*, 1989). The other two PKC isoenzymes studied, PKC ε and PKC δ are involved in the cell growth, apoptosis and tumorigenesis and overexpression of PKC ε was oncogenic in a rat colonic epithelial cell line (Perletti *et al.*, 1998; Marras *et al.*, 2001). PKC ε overexpression is associated with increased cell growth and transformation *in vitro* as well as tumorigenic *in vivo* (Weller *et al.*, 1999; Mischak *et al.*, 1993). PKC ζ is also thought to be important in growth regulation in CRC and in an azoxymethane (AOM) model of colon carcinogenesis. A study has shown that three different chemoprotective agents markedly reduced the frequency of AOM-induced carcinomas by decreasing PKC ζ in levels (Wali *et al.*, 1996). In our study, PMA translocated all but the atypical PKC isoform. The activation caused by PMA happened in matter of seconds. Similarly, DCA induced PKC translocation of all the PKC isoenzymes to subcellular sites. This translocation caused by DCA, was as efficient as PMA. This observation suggests that DCA may be as potent a tumour promoter as PMA itself. The effects on endogenous PKC isoenzymes were studied first. PMA and DCA activated all the

endogenous PKC isoenzymes studied except PKC ζ. PKC β is a conventional isoform of PKC with important roles in cell proliferation and cell growth. It is also the isoform, which responded most efficiently to the effects of bile acids. This PKC isoform disappears early in the development of colon cancer and was not detected in the cell lines used in our studies. Hence, transfected PKC β1-EGFP was employed to observe these responses in real time. ALL the transfected EGFP-tagged PKC isoenzymes were translocated by both PMA and DCA, though the dose of PMA required for these PKCs was higher than that required for the endogenous PKC. The exact mechanisms for that effect are not clear, however, the suggestion is that EGFP tagging may confer some unknown conformational alterations to the PKC, rendering them resistant to the effects of PMA. This phenomenon needs further investigations. Real time microscopy was performed at the commencement of the treatment for up to 10 minutes and then periodically every 10-15 minutes for up to 2 hours. PKC δ responded slowly to DCA and overexpression of the PKC δ. UDCA treatment on its own, on the other hand, had no effect on either endogenous or EGFP-tagged PKC isoenzymes. This observation distinguished UDCA form DCA in its effects on PKC isoenzymes and encouraged further investigations. Dose titration was performed for DCA and UDCA with a maximum of 300 μM, as the concentration beyond this was toxic to the cells. The most interesting observation was that UDCA pre-treatment of the cells prevented DCA-induced translocation of the PKC isoenzymes. This was further explored with different concentrations of DCA and UDCA. In some experiments, UDCA was left in before DCA stimulation and others UDCA was taken out and then DCA added. UDCA pre-treatment inhibited DCA-induced translocation of PKC whether left in or taken out. This observation indicates that it is not only the dilution of toxic DCA by the UDCA responsible for its protective effects as described by other researchers. The concentrations of DCA used fall well within physiological range and efficiently caused translocation of the PKC isoenzymes. DCA and other secondary bile acids are important in the pathogenesis of CRC as shown by multitude of epidemiological and animal studies. Bile acids seem to play important role in all the stages of histopathological alterations in colon cancer from conversion of normal epithelium to metastatic cancer (Debruyne *et al.*, 1999; Janne *et al.*, 2000; Hanahan *et al.*, 2000). It has been reported that higher levels of these bile acids are found serum and stools of patients with adenomatous polyps and colon cancer (Imray *et al.*, 1992; Reddy *et al.*, 1976). Additionally bile acid-specific binding sites have been identified in mouse and human colorectal cancers (Summerton at *al.*, 1983, 1985; Bayerdorffer *et al.*, 1993). Unconjugated DCA and CDCA have been shown to be tumour promoters in animals (Reddy *et al.*, 1977; Mahmoud *et*

al., 1999). In rats, intrarectal instillation of bile acids promoted the development of colorectal adenomas and adenocarcinomas (Narisawa *et al.*, 1974; Reddy *et al.*, 1977). It has also been reported that bile acids may act as direct mitogens on colonic epithelial cells as shown by colonocyte proliferation in rat on DCA diet supplements (Barone *et al.*, 2002). The bile acids mediated a number of important molecules implicated in CRC. Cyclooxygenase 2 (COX-*2*) is probably the most important target gene of bile acid signalling in CRC. It may contribute to colon carcinogenesis in several ways. COX-2 inhibits apoptosis and stimulates expression of matrix-metalloproteinase (MMP-2) involved in invasion in human colorectal cancer cells (Tsujii *et al.*, 1995, 97, 98). DCA is reported to induce COX-2 promoter activity, which leads to the formation of the COX-2 enzyme and consequent increases in prostaglandin production (Zhang *et al.*, 1998). Bile acids are reported to increase expression of MMP-2 in Caco-2 cells (Halvorsen *et al.*, 2000). It was recently reported that low concentrations of DCA significantly increase tyrosine phosphorylation of beta-catenin, induce urokinase-type plasminogen activator, uPAR, and cyclin D1 expression and enhance CRC cell proliferation and invasiveness (Pai *et al.*, 2004). Beta-catenin and its target genes urokinase-type plasminogen activator receptor (uPAR) and cyclin D1 are overexpressed in colon cancers, and are linked to cancer growth, invasion, and metastasis. It was reported that DCA strongly inhibits BRCA-1 expression, which helps in repair of DNA and in induction of apoptosis. BRCA-1 was found to be substantially lower in colon adenocarcinomas. Compared with associated non-neoplastic colon tissue (Romagnolo *et al.*, 2003). In this context, UDCA's ability to block PKC signalling pathway activated by DCA may provide an important base to carry out further investigations into its effects on other signalling pathways involved in CRC.

Second important observation in our study was the effects of these treatments of F-actin localization of PKC and the shape change. DCA treatment induced shape change with appearance of rounded phenotypes with association of PKC isoenzymes to the membrane related cytoskeletal structures such as F-actin. The activation of the PKC isoenzymes seems to correspond to the association of these PKC to the F-actin. UDCA treatment in contrast to DCA did not induce these conformational changes and cell shape in the cells. UDCA pre-treatment also inhibited DCA induced cell shape changes. To confirm whether PKC activation actually is associated with these changes or not, experiments were performed in which broad spectrum PKC inhibitors were used prior to stimulation with DCA. These PKC inhibitors blocked the alterations in phenotype as well-associated conformational changes in F-actin. The experiments were performed in cells transfected

with PKC β1 because of the excellent transfection efficiency achieved with this PKC isoform and because of the fact that PKC β1 was not found in the cell line used and hence, interference with endogenous PKC was eliminated. We do not suggest that PKC β1 is the only PKC isoenzyme involved in the phenotype changes or alterations in F-actin and other PKC are also involved. The association of the PKC to the F-actin and inhibition of this effect by UDCA may have important implications invasion and metastasis of the tumour. Cytoskeletal structures including F-actin play an important role in cell shape and migration as well as their adherence to matrix through connections with surface molecules. Thus, conformational changes and remodelling of F-actin induced by the activation of PKC might have important implications in cell polarity, cell spreading, tight junction function and ultimately cancer cell growth and migration (Haller *et al.*, 1998; Saxon *et al.*, 1994). It is important to note that bile acids have been associated with cell migration and invasion of colorectal cancer cells. Studies have demonstrated that treatment of human colorectal cancer Caco-2 cells by LCA, resulted in an enhanced secretion of matrix metalloproteinase 2 (MMP-2) and an enhanced chemotaxis towards serum (Halvorsen *et al.*, 2000). Bile salts at physiologic conditions may increase cell migration, regulated by NF-κB and TGF-β (Strauch *et al.*, 2003; Debruyne *et al.*, 2002). It has been reported that bile acids stimulated invasion through stimulation of haptotaxis and it was dependent on the RhoA/Rho-kinase pathway and signalling cascades using PKC, MAPK, and COX-2. Further, in addition to its well-established role in the enhancement of proliferation, DCA also stimulated colon cancer-cell migration along the basement membrane matrix and this stimulation is probably dependent on protein kinase C (Milovic *et al.*, 2001). Hence, UDCA by blocking signalling pathways induced by DCA may have far-reaching effects on not only the cell proliferation but also invasion and metastasis of the tumour.

Finally, there is abundance of data for the protective effects UDCA in CRC in animal models of colon carcinogenesis as well as clinical studies in humans. UDCA improves the biochemical parameters of cholestasis in various cholestatic disorders including primary biliary cirrhosis and primary sclerosing cholangits (Trauner *et al.*, 1999). There is some evidence that UDCA may be protective in human colorectal cancer associated with inflammatory bowel disease (IBD), though most of the mechanistic evidence of chemopreventive role of UDCA in colorectal carcinoma has come from animal models. Rats treated with AOM and intrarectal N-methylnitrosourea and fed a diet supplemented with UDCA had a significant reduction in aberrant crypt formation (ACF) formation and a lower incidence of colon tumours (Momen *et*

al., 2002, Narisawa *et al.*, 2002). A study in rats has shown that UDCA significantly inhibited AOM-induced colonic carcinogenesis during either tumour initiation or in the promotion/progression phase (Wali *et al.*, 2002). UDCA has bee reported to inhibit COX-2 induction by *Ras*-dependent and -independent mechanisms (Khare *et al.*, 2003). Studies in humans have shown that UDCA pre-treatment suppresses DNA binding activity of AP-1, accompanied by downregulation of both extracellular signal-regulated kinase (ERK) and Raf-1 kinase activities stimulated by exposure to DCA. UDCA in contrast to DCA inhibited was found to inhibit EGFR activity (Im *et al.*, 2004). It has been reported that the prolonged administration of UDCA to patients with PBC significantly decreased the probability of colorectal adenoma recurrence following their removal (Serfaty *et al.*, 2003). A recent study has reported that UDCA significantly reduced the risk for developing colorectal dysplasia or cancer in patients with UC and PSC (Pardi *et al.*, 2003). These data has suggested a role for UDCA quite opposite to DCA in the pathogenesis of CRC. Despite plethora of evidence for UDCA's role in colorectal carcinogenesis, the mechanisms by which it might provide such protection largely remain unclear. This study gives insight into some of the molecular mechanisms behind UDCA's protective role in CRC. The precise site of action of UDCA in this signalling pathway is still unclear; some proposed sites of its action are illustrated in figure 6.1.

8.2 Bile acids, AP-1, NF-κB and Colon Cancer

The investigations into the effects bile acids on the transcription factors NF-κB and AP-1 revealed interesting findings. The secondary bile acid DCA and UDCA had distinct and differential effects on the DNA binding of these transcription factors. DCA induced NF-κB and AP-1 DNA binding in colon cancer cells while UDCA treatment had no effect on these transcription factors. Furthermore, pre-treatment of the cells with UDCA blocked DCA and IL-1β-induced NF-κB and AP-1 DNA binding and PMA-induced AP-1 DNA binding. DCA also induced degradation of the inhibitory molecule IκB-α. Both transcription factors NF-κB and AP-1 play a significant role in colorectal carcinogenesis. Upregulation of NF-κB is has been reported in CRC and it is involved in the regulation of important genes associated with oncogenesis, angiogenesis and proliferation (Gilmore *et al.*, 1996; Yu *et al.*, 2003). AP-1 and NF-κB also regulate COX-2 with aggressive colorectal tumours. NF-κB and AP-1 regulate many genes involved in cell transformation, oncogenesis including COX-2 in intestinal epithelial cells (Karin *et al.*, 1997; Guo *et al.*, 2001). These two transcription factors may to play a synergistic role in carcinogenesis and cross coupling of these transcription factors is reported augment their effects (Li *et al.*, 1997). Bile acids activate AP-1 and NF-κB (Hirano *et al.*, 1996;

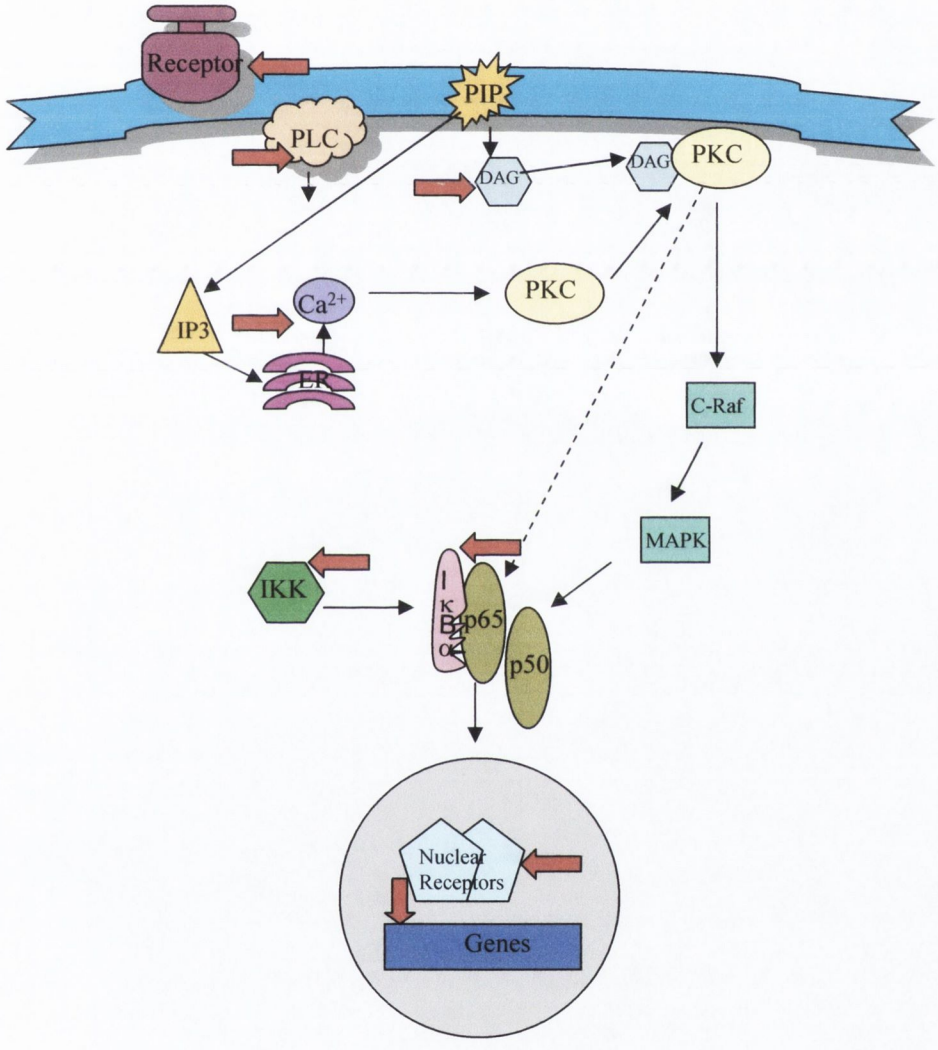

Fig. 6.1 Proposed sites of action of UDCA indicated by red arrows. UDCA may act at cell surface receptors, the membrane phospholipids such as phosphlipase A or C, may influence DAG levels, intracellular calcium levels. UDCA may also effect IκB phosporylation through PKC or IKK and may affect nuclear receptors or genes.

Matheson *et al.*, 1996; Glinghammar *et al.*, 1999). The putative mechanisms for UDCA on NF-κB expression and its effects on IκB-α were investigated. UDCA pre-treatment in contrast to DCA did not reduce IκB-α levels and UDCA treatment restored DCA-induced reduction in IκB-α levels. This may give some insight in to the effects of UDCA at cytoplasmic levels as inhibitors of IκB degradation in the cytoplasm have been reported to prevent NF-κB nuclear translocation and hence reduce tumour size (Cusack 2003; Uetsuka *et al.*, 2003). Many kinases take part in the regulation of NF-κB DNA binding and the use of PKC inhibitors have shed more light on this mechanism. PKC inhibitors prevented DCA-induced NF-κB activation and IκB-α degradation indicating that PKC signalling pathway may play a role in how bile acids influence NF-κB DNA binding in colorectal cancer. Immunofluorescence studies involving transfected PKC β1 and NF-κB further strengthened this argument. Similarly, the data on experiments with PKC inhibitor AP-1 indicated that bile acids might influence AP-1 DNA binding through PKC signalling pathways. Chronic inflammatory conditions such as ulcerative colitis (UC) are implicated in the pathogenesis of CRC and UC predisposes to colon cancer. NF-κB activation is one the most significant step in maintaining this inflammatory cascade in UC (O'Byrne *et al.*, 2001). By inhibiting NF-κB activation, UDCA might block inflammatory pathways orchestrated by NF-κB and NF-κB regulated genes such as oncogene *ras* and *cyclin D1* (Mayo *et al.*, 1997). Despite the cumulative evidence of the cytoprotective and chemoprotective effects of UDCA in animal as well as human studies, both the target molecule and pathway of UDCA action remain unknown. Our data has attempted to explore some of the most complex signalling pathways implicated in colorectal carcinogenesis and tried to establish a link between bile acids, PKC, NF-κB and AP-1. Further investigations are required, for example, to explore the effects of UDCA on cell surface receptors, its effects cytopalsmic events such as IκB-α phosphorylation and degradation and effects on nuclear receptors.

8.3 CLA and colon cancer

The investigation of effects of CLA isomers on PKC and NF-κB expression in colon cancer cells also resulted in interesting data. The effects of the CLA isomers and their precursor LA were compared with the effects of unsaturated fatty acid, OA on PKC isoenzymes and transcription factors NF-κB. Chronic by contrast with acute treatment with *c9, t11*-CLA and *t10,c12*-CLA isomers inhibited DCA-induced translocation of PKC β1, PKC δ and C2-ceramide-induced translocation of the PKC ζ. Chronic treatment with these CLA isomers inhibited DCA and IL-1β-induced NF-κB activation. On the other hand, LA did not prevent

PKC activation or NF-κB DNA binding. Conversely, LA is reported activate NF-κB in endothelial cells and has been associated with increased risk of colon cancer (Miller *et al.*, 2001; Park *et al.*, 2001; Toborek *et al.*, 1996). CLA has recently been reported to inhibit LPS-induced NF-κB activation in RAW macrophages and that CLA modulates NF-κB and COX-2 in macrophages, thus affecting the inflammatory cascade (Cheng *et al.* 2004). An interesting finding was the activation of PKC ζ and NF-κB by OA. The *cis*-PUFAs have been reported to enhance human breast cancer cell adhesion to collagen IV by selectively activating specific PKC isoenzymes (Palmantier *et al.*, 2001). Previous studies have demonstrated that oleic acid activates calcium-independent PKC and may also influence the transcription factors through PKC (Parka *et al.*, 2003).To date this is the first report of CLA effects on interaction between bile acids and GFP-tagged PKC isoforms in living cancer cells. Hence, inhibition of PKC and NF-κB activation by the CLA may be a significant mechanism in the anticarcinogenic effects of CLA in colon cancer.

Previous studies have reported that several saturated and unsaturated fatty acids potentiate PKC activity (Shirai *et al.*, 1998; Nishizuka 1995). Unsaturated fatty acids such as arachidonic and oleic acid are reported to enhance the activity of several PKC isoenzymes (Khan *et al.* 1993; Huang *et al.*, 1997; Diaz-Guerra *et al.*, 1991). However, much less information is available on the effects of CLA in PKC signalling. There is abundance of data on anticarcinogenic effects of CLA in colorectal cancer and several mechanisms have been described. It has been reported that CLA downregulates ErbB3 signalling and PI3-kinase/Akt pathway and may inhibit cell proliferation and stimulate apoptosis in colon cancer cells (Cho *et al.*, 2003). It has also been reported that CLA may inhibit colon cancer growth through *p53* dependant mechanisms (Kemp *et al.*, 2003). CLA is also thought to be a ligand for peroxisome proliferator-activated receptor (PPARγ), a nuclear hormone receptor with growth inhibitory effects in cancer cells (Bull *et al.*, 2003; Yu *et al.*, 2002). Conjugated linoleic acid (CLA) is the only fatty acid reported, unequivocally to inhibit carcinogenesis in experimental animals (National Research Council, USA, 1996). Diets high in animal fat seem to modulate opposite effects in human colon cancer development and relative concentrations of the saturated fats and CLA may play a crucial role in preventing this common malignancy. Our data has provided an alternate pathway for CLA action. As PKC is an upstream modulator for NF-κB activation and chronic CLA treatment may affect NF-κB through modulation of PKC. However, acute CLA treatment had no effect suggesting that CLA may not alter these pathways directly. It is known that CLA get incorporated in the cell membrane phospholipids and this alteration of membrane phospholipids

in particular phospholipase A2 may play a significant role in inhibition of the mitogenic stimuli such as DCA (Kim *et al.*, 2003). The other mechanism may be the reduction of DAG levels by CLA, an important cofactor involved in the activation of PKC. The PKC isoenzymes and NF-κB play a pivotal role in many events leading to carcinogenesis including cell growth, proliferation, cell migration and apoptosis. The alteration of these signalling pathways by the CLA isomers may be significant step in the growth inhibitory effects of CLA reported in many epidemiological and animal studies.

8.4 Future Prospects

Colorectal cancer is a common malignancy. There have been great advancements in the diagnosis and treatment of CRC. The clear definition of genetic basis of the disease has helped to devise new therapies for the disease. However, little is known of the cell signalling aspects leading to these well-known genetic mutations. The fact that majority of CRC are sporadic in nature with no definite association with the genetic alterations and that a long period of time is required to accumulate the genetic mutations, long-term preventative strategies in CRC have clear implications. The investigations of the signalling pathways and their interactions with known mitogens may provide a basis for these strategies. Our data attempts to provide insight in to some of the molecular mechanisms regarded as pivotal in the genesis of CRC. The present management strategies for CRC are inadequate, to say the least. The surveillance programmes and available therapies such as surgery and chemotherapy have made rapid progress in recent years but have not altered the prevalence of CRC. Probably the solution lies in the age-old dictum, that prevention is better than cure. A number of agents are being investigated for their potential effects in protection against CRC. Some have entered clinical trials but the search goes on for a perfect chemoprotective agent, which is effective, safe, cheap and easily available in the long-term. Our data concentrated on two such substances. UDCA and CLA are natural, endogenous substances with little or no toxicity. Data from many epidemiological and animal models studies supports their role in the prevention of CRC. UDCA is commonly used for cholestatic liver disease and CLA is available in the diet from animal sources. The effects of UDCA and CLA on PKC and NF-κB expression in CRC cells revealed encouraging results. PKC and NF-κB are important factors influencing downstream genetic events involved in CRC. However, PKC are heterogeneous group of enzymes. They are expressed in a variety of tissues with variety of functions. Earlier efforts at targeting PKC enzymes for the cancer therapy have not been very successful, partly because of lack of understanding of the dynamics of these enzymes in the malignant process and their expression and varied effects in different tissues.

For example, the significance of pulsed secretion and translocation of PKC to different subcellular sites with subsequent rapid downregulation. The loss of some of the PKC isoenzymes in established malignancy may mean the failing of protective mechanisms against mitogenic stimuli resulting in loss of inhibitory controls on growth and the transformation of the epithelial cells with autonomous growth potential. On the other hand, retention of some of the enzymes may mean that, they have a permissive role and aid malignant cells to develop in to invasive cancer cells. The repercussions of PKC inhibition on the normal or non-malignant cells are not yet fully understood. Further, PKC has a role as apoptotic and anti-apoptotic molecules, the trigger and control being elusive. In nutshell, the mechanisms by which PKC are regulated are complex. Our data suggests that UDCA and CLA may have some role in the prevention of CRC with effects on PKC and other transcription factor expression. These transcription factors, NF-κB and AP-1 have recently emerged as promising targets in CRC. They have clear role in inflammation, are precursors for cancer and actively participate in the process of carcinogenesis themselves. Our data unveils the some of the mechanisms by which UDCA and CLA may influence these transcription factors but there are unanswered questions. For example how UDCA restores IκB-α levels. We investigated one of the signalling mechanisms, the PKC signalling pathways in the UDCA's effects on NF-κB activation. Other important questions remain. For example, the role of IKK activity in UDCA's effects on NF-κB needs to be elucidated. The kinase studies and phosphorylation studies of the IκB-α may be useful in this context. A major multi-centre trial is now underway to determine whether ursodeoxycholic acid can prevent sporadic adenoma formation in humans. Conjugated linoleic acids are the fatty acids obtained from animal sources. They are derived from linoleic acid, a fatty acid from plant origin. Ironically, LA from plant origin has been associated with increased incidence of colonic cancer whereas its derivatives from animal origin, the CLA seem to possess inhibitory properties against colon cancer growth. This is the paradox as fatty acids from animal origin have been blamed for high incidence of CRC in affluent countries.

Further studies are needed to clarify some of the ambiguities in the regulation of PKC and the transcription factors in CRC. The effects of bile acids and CLA on PKC and NF-κB need to be characterised further. For example to explore the exact site of action of UDCA or CLA in regulating these molecules. Whether they affect the cell surface receptors, their interaction with membrane phospholipids and effects on cytoplasmic events such as interactions with IκB, the DAG, the calcium signalling and the role of APC/β-Catenin in bile acid-induced PKC and transcription factors. One important unanswered question is the site of bile acid action. There

are a number of hypotheses regarding DCA action. It may modify cell signalling through its action on cell membrane phospholipds for example phospholipase C, D and through its actions on DAG levels. Others suggest its effects on calcium and hence PKC activation. However, the significance of nuclear orphan receptors in mediating bile acid effects cannot be ignored as DCA targets these receptors but UDCA does not signal through these nuclear receptors and may target the glucocorticoid receptors. Functional interactions between nuclear bile acid signaling pathways, PKC, and nuclear receptors for retinoic acid and vitamin D_3 has also been reported. The significance of these nuclear receptor interactions with bile acids need to be further characterized. The effects of UDCA and CLA on genes involved in CRC may be significant step and DNA microarrays may prove useful in this regard. Studies are required to explore the roles of UDCA and CLA in initiation and promotion of the malignancy and define the stage of the disease where these agents may be most effective. Fortunately, both these agents are non-toxic and pure forms of the CLA isomers are commercially available. Their role in three cohorts, at risk population, population at adenoma stage and established cancer need to be characterized. Another avenue for further investigations is their role as adjuvant therapy in CRC. The important role of proteasome inhibitors with chemotherapeutic agents in CRC has been elucidated and UDCA and CLA may provide relatively non-expensive and non-toxic option for adjuvant therapy.

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APPENDIX

Appendix A

Buffers and Reagents

Cell Culture Reagent

Culture cocktail	
Penicillin/Streptomycin	100 ml
L-glutamine	100 ml
2-mercaptoethanol	37 µl
This mixture was stored at 20°C in aliquots of 10 ml	

Complete RPMI1640 medium

RPMI 1640	500 ml
FCS	50 ml
Culture cocktail	10 ml

Complete McCoy's medium

McCoy's calcium free medium	500 ml
FCS	50 ml
Culture cocktail	10 ml

Complete L-15 medium

L-15 medium	500 ml
FCS	50 ml
Culture cocktail	10 ml

Hanks balanced salts washing solution

HBSS	500 ml
HEPES buffer (1M)	10 ml

Cryopreservative solution

Foetal calf serum	9 ml
Dimethylsulphoxide	1 ml

Ethidium bromide (EB) stock

EB	100 mg
PBS	20 ml

Acridine orange (AO) stock

AO	20 mg
PBS	20 ml

EBI/AO working solution

EB solution	4 ml
AO solution	4 ml
PBS	100 ml

Cell Protein Preparation

10 x Phosphate Buffered Saline (PBS)

$Na_2HP0_4.2H_20$ (8 mM)	14.24 g
KH_2P0_4 (1.5 mM)	2.04 g
NaCI (137 mM)	80 g
KCI (2.7 mM)	2 g

Adjust to pH 7.4 and made up to 1 litre

1 % Nonidet P40

PBS	100 ml
EGTA	0.038 g
Nonidet P40	946 µl

Protease inhibitors

Leupeptin	2mg/ml
PMSF	1 M

BSA buffer solution

Bovine serum albumin	50 mg

This solution was made up to 50 ml
with a 1:1 mixture of PBS and 1 % NP40

Bradford reagent

Coomassie Blue G	100 mg
95% ethanol	50 ml
0.85% orthophosphoric acid	100 ml

This solution was made up to 1 litre
with distilled water

Lowery Protein Assay Reagents

Reagent A

Na_2CO3	2 % w/v
NaOH	0.4 % w/v
K/Na Tartarate	0.16 % w/v
SDS	1 % w/v

Keeps indefinitely

Reagent B

$CuSO_4\ 5H_2O$	4 % w/v

Reagent C 1 volume B + 100 Volume A
Prepare fresh

Reagent D

Folins	dilute 1/3 with H2O

Total Protein Extraction Buffer C

20 mM Tris-Hcl PH 7.5
1 % SDS
150 mM NaCl

1 mM EGTA
1 mM EDTA

Polyacrylamide Gel Electrophoresis

5x Sample buffer

Glycerol	5 ml
2-mercaptoethanol	6.25 ml
20% SDS	5 ml
Stacking gel buffer	1.25 ml
0.2% Bromophenol blue	0.3 ml

Make up to 25 ml with distilled water

Resolving gel buffer

Tris base	36.6 g
Distilled water	100 ml

Adjust to pH 8.8 with concentrated HCI

Stacking gel buffer

Tris base	6 g
Distilled water	100 ml

Adjust to pH 6.8 with conc. HCI

1 % Ammonium persulphate (APS)

APS	0.1 g
Distilled water	10 ml

Make up fresh before use.

10% Sodium dodecyl sulphate (SDS)

SDS	1 g
Distilled water	10 ml

Acrylamide/Bisacrylamide Mix

Acrylamide	30 g
Bisacrylamide	0.8 g
Distilled water	100 ml

Water-saturated butanol

Butanol	10 ml
Distilled water	10 ml

This was mixed well, allowed to
settle and pipetted from top layer

10 x Electrode running buffer

Tris base	30 g
Glycine	114 g
SDS	5 g
Distilled water	1000 ml

Dilute 1/10 in distilled water before use

4-Semi-Dry Transfer buffer

Tris base	2.9 g
Glycine	1.45 g
SDS	0.185 g
Methanol	100 ml
Make up to 500 ml with distilled water	

Coomassie blue gel stain

Coomassie Brilliant Blue R	0.5 g
Methanol	200 ml
Glacial acetic acid	35 ml
Distilled water	265 ml

Gel destain solution

Methanol	400 ml
Glacial acetic acid	70 ml
Distilled water	530 ml

Immunoblotting

Blotto-Tween blocking solution

Skimmed dried milk (Marvel)	5 g
PBS	100 ml
Tween 20	46 µl
Make up fresh before use	

0.05% PBS-Tween washing solution

PBS	1000 ml
Tween 2	0.5 ml

Primary antibody solution

Primary antibody	10 µl
Blotto- Tween	10 ml

Secondary antibody solution

Swine anti-rabbit peroxidase-conjugated Ig	10 µl
Blotto-Tween	20 ml

Enhanced Chemiluminescence

Developing solution

Luminol	14 mg
Iodophenol	4 mg
DMSO	500 µl
H202	18 µl
0.1 M Tris-HCl (pH 8.8)	50 ml

5 % Para-formaldehyde solution

Para-formaldehyde	5 g
PBS	100 ml
The solution was heated up to 65°C to be dissolved, cooled, filtered and stored at 4°C	

Nuclear Extract Preparation

Extraction buffers

All stock solutions should be autoclaved

Buffer A	**Volume**	**Stock**
Hepes, pH 7.9 (10 mM)	100 µl	1M
Magensium chloride (1.5 mM)	15 µl	1M
Potassium chloride (10 mM)	100 µl	1M
PMSF	5 µl	1M
DTT	5 µl	1M
Make up to 10 ml with sterile water		

Buffer C		
Hepes, pH 7.9 (20 mM)	200 µl	1M
Magensium chloride (1.5 mM)	15 µl	1M
Sodium chloride (420 mM)	840 µl	5M
EDTA (0.2 mM)	4 µl	0.5M
Glycerol 25%	2.5 ml	----
PMSF (0.5 mM)	50 µl	100 mM
Make up to 10 ml with Sterile water		

Buffer D		
Hepes, pH 7.9 (10 mM)	100 µl	1M
Potassium chloride (50 mM)	400 µl	1M
EDTA (0.2 mM)	1.6 µl	0.5M
Gylcerol 20%	800 µl	----
PMSF (0.5 mM)	20 µl	100 mM
DTT (0.2 mM) 1M	2 µl	
Make up to 10 ml with sterile water		

Electrophoretic Mobility shift assay

I0 x Tris borate buffer (TBE)

Tris base	108 g
Boric acid	54 g
0.5M EDTA	40 ml
Make up to 1 litre with distilled water	

TE buffer (IX)

Tris-HCI (pH 8.0)	10 mM
EDTA	1 mM

Binding reaction buffer (10X)	**Volume**	**Stock**
Tris pH 7.5 (100 mM)	50 µl	1M
NaCI (1M)	100 µl	5M
Glycerol (40%)	200 µl	-----
EDTA (10 mM)	10 µl	0.5M
DTT (50 mM)	25 µl	1M
Nuclease free BSA (1 mg/ml)	50 µl	10 mg/ml
Make up to 500 1 µl with sterile water		

Plasmid Preparation

All stock solutions should be autoclaved

L-broth medium (LB)

Tryptamine	10 g
Yeast extract	5 g
Sodium chloride	5 g
Agar	15 g

Make up to 1 litre with distilled water

Agar Plates

LB Agar	4 gm
Distilled Water	200 ml

Autoclave

Storage of Plasmid DNA

BHI	9.4 ml
DMSO	200 µl

Loopful of colonies in 1 ml of the solution

Stored at -70°C

Antibiotic Selection

Ampicillin	100 mg/ml
Kanamycin	30mg/ml

STE solution

NaCI (0.1 M)	0.292 g
TE	50 ml

Acetic acid (57%)

Acetic acid	5.7 ml
Distilled water	4.3 ml

Potassium acetate solution (5 M)

Potassium acetate	4.907 g
Distilled water	10 ml

This solution should be filter sterilised

Agarose Gel Electrophoresis

TAE buffer (l0 x)

Tris base	24.2 g
Glacial acetic acid	5.71 ml
EDTA, pH 8.0 (0.5 M)	20 ml

Agarose gel (0.8%)

Agarose	0.32 g
TAE (1x)	40 ml

This solution was heated up until dissolved

Electrode running buffer

Distilled water	450 ml

Gel loading dye

Bromophenol blue (0.25%)	0.125 g
Xylene cyanol (0.25%)	0.125 g
Glycerol (30%)	15 g

Make up to 50 ml with distilled water