Bcr-Abl regulates osteopontin transcription via Ras, PI-3K, aPKC, Raf-1, and MEK

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Abstract: Chronic myeloid leukemia (CML) is caused by the constitutively active Bcr-Abl tyrosine kinase. This fusion protein is generated by the Philadelphia translocation t(9;22). CML is a progressive condition that invariably advances from a drug-sensitive to a drug-resistant, aggressive, acute leukemia. The mechanisms responsible for this progression are largely unknown; however, in many cases, progression is accompanied by an increase in Bcr-Abl expression. Osteopontin (OPN) expression has been shown to be involved in the progression and increased aggression and invasiveness of many solid tumors. Here, we demonstrate that OPN expression is induced in a model of leukemia, and we describe the identification of specific signaling pathways required for the induction of OPN expression by p210 Bcr-Abl. We have determined that high levels of Bcr-Abl activate a signaling cascade involving the sequential activation of Ras, phosphatidylinositol-3 kinase, atypical protein kinase C, Raf-1, and mitogen-activated protein kinase kinase, leading to the ultimate expression of OPN. Our results suggest that these molecules represent a single pathway and also that there is no redundancy in this pathway, as inhibition of any individual component results in a block in the induction of OPN. The data presented here define for the first time the ability of Bcr-Abl to stimulate the expression of OPN and also identify the signaling pathway involved. This may not only prove important in understanding the mechanisms of progression of CML but also highlights a pathway that may prove significant in many other cases of oncogenesis, where OPN expression is implicated. ***J. Leukoc. Biol.*** **78: 289–300; 2005.**

Key Words: *chronic myeloid leukemia · cellular signaling · gene expression*

INTRODUCTION

Chronic myeloid leukemia (CML) accounts for approximately one-fifth of all leukemias. It is a myeloproliferative disorder caused by the t(9;22) translocation, also known as the Philadelphia chromosome [1]. This translocation juxtaposes sequences from the 5′ end of breakpoint cluster region (BCR) with sequences upstream of exon 2 of c-abelson (ABL), resulting in the production of a fusion gene and the resultant oncoprotein Bcr-Abl, which displays constitutive tyrosine kinase activity [2]. This constitutive tyrosine kinase activity has been shown to be crucial for the transforming ability of Bcr-Abl [3]. Bcr-Abl expression has been shown to be the sole requirement for leukemogenesis in animal models of CML [4, 5]. Depending on the breakpoint in BCR, the fusion protein may be 210 kD (p210) or 185 kD [6, 7]. p210 Bcr-Abl is seen in 95% of CML patients. CML is clinically characterized by the clonal expansion of a transformed hematopoietic stem cell and progresses from a chronic phase, which lasts 4–5 years, to an acute phase (blast crisis), which is invariably fatal. Cellular characteristics of CML include adhesion independence [8], growth factor independence [9, 10], and resistance to apoptosis [11, 12].

Bcr-Abl reportedly activates many signaling pathways, including the phosphatidylinositol-3 kinase (PI-3K)/AKT pathway [13]. This PI-3K activity is required for the proliferation of CML cells. Other downstream effectors of Bcr-Abl include Ras [14] and Myc [15]. The Janus kinase/signal transducer and activator of transcription pathway (STAT) has also been shown to play a key role in human CML [16], and STATs are the only transcription factors known to be regulated by tyrosine phosphorylation. It is hoped that the identification of genes associated with Bcr-Abl expression will potentially lead to new molecular targets for therapy.

Osteopontin (OPN) is a secreted, acidic phosphoprotein, which binds to various cell-surface receptors including integrins, e.g., $\alpha_v\beta_3$ [17] and CD44 [18]. One well-documented function of OPN is as a cell adhesion molecule [19, 20]. Its expression has been shown to be increased by phorbol esters, growth factors, and hormones [21]. It has also been demonstrated that OPN is involved in many disease states including atherosclerosis [22], kidney disease [23], and the growth and survival of metastatic cells [24]. Other studies indicate increased levels of OPN in the blood of patients with metastatic disease [25]. However, the function of OPN in tumorigenesis is unclear. In addition, the upstream signaling pathways regulating OPN expression in response to various stimuli are poorly characterized.

Here, we demonstrate that OPN expression is up-regulated transcriptionally in response to high levels of p210 Bcr-Abl. We also describe a systematic evaluation of the major signaling

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molecules required for this up-regulation of OPN. Analysis of the mitogen-activated protein kinase (MAPK) cascade reveals that this pathway is required for OPN expression in our model of CML. Additionally, we demonstrate involvement of atypical protein kinase C (aPKC) and PI-3K. We determine that activity of PI-3K and aPKC is required for optimal activation of Raf-1, even in the presence of active Ras. The requirement of aPKC activity represents a novel pathway in the up-regulation of OPN. As such, this may represent a potentially globally important pathway in the induction of OPN expression in other disease states. These data not only provide insights into the regulation of OPN but also identify key signaling cascades initiated by the oncogene Bcr-Abl.

MATERIALS AND METHODS

Cell culture/reagents

The 32D cell line was maintained in RPMI 1640 (Gibco Invitrogen Corp., Paisley, UK), 10% fetal calf serum (FCS), 100 U/ml penicillin, 1 mg/ml streptomycin (Sigma-Aldrich, Dublin, Ireland), and 10% WEHI-conditioned media (CM) as a source of interleukin (IL)-3. The Bcr-Abl expressing C2, C4, and C5 clones were maintained in RPMI 1640, 10% FCS, 100 U/ml penicillin, 1 mg/ml streptomycin, and 0.2 mg/ml puromycin (Sigma-Aldrich). Radiochemicals were obtained from Amersham Biosciences (Little Chalfont, UK). Inhibitors included STI571, kindly provided by Novartis (Basel, Switzerland); Etoposide (VP-16), purchased from Sigma-Aldrich; S-trans, trans-farnesylthiosalicyclic acid (FTS), obtained from Affiniti Research Products (Exeter, UK); UO126, from Cell Signaling Technology (Hertfordshire, UK); and 8-bromocyclic adenosine monophosphate (cAMP), 5-Iodo-3-[(3,5-dibromo-4-hydroxyphenyl)methylene]-2-indolinone (5-Iodo), PKCζ pseudosubstrate inhibitor, and LY294002, purchased from Calbiochem (Merck Biosciences, Nottingham, UK).

Cell lysis and immunoblotting

Cells were lysed in radio immunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 1 μg/ml antipain, 1 μg/ml aprotinin, 1 μg/ml chymostatin, 0.1 μg/ml leupeptin, 1 μg/ml pepstatin, and 100 μM phenylmethylsulfonyl fluoride). The lysates were centrifuged at 20,000 *g* (4°C) for 15 min to remove insoluble debris. Equivalent amounts of protein, as determined by the Bio-Rad protein assay, were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). All secondary antibodies were peroxidase-conjugated, and proteins were detected using enhanced chemiluminescence (Amersham Biosciences). Antibodies included anti-c-Abl (AB-3; Calbiochem); antiphosphotyrosine (PY20; Transduction Laboratories, San Diego, CA); anti-OPN (R&D Systems, Abingdon, UK); anti-Ras (clone RAS10), anti-MAPK kinase (MEK), and anti-Raf-1 (Upstate Biotechnology Ltd., Milton Keynes, UK); anti-nPKCζ, also detects PKCλ/ι (Santa Cruz Biotechnology, Heidelberg, Germany); antiphospho-AKT (ser 473) and anti-AKT (Cell Signaling Technology); and anti β-actin (Sigma-Aldrich).

Measurement of apoptosis [phosphatidyl serine (PS) exposure]

The exposure of PS on the extracellular surface of the plasma membrane was monitored by the binding of annexin V-fluorescein isothiocyanate (FITC), according to the manufacturer's instructions (IQ Products, Labron Ltd., Dublin, Ireland). Briefly, 5×10^5/ml cells were resuspended in calcium-binding buffer (10 mM Hepes, 2.5 mM $CaCl_2$, 140 mM NaCl) and incubated with annexin V-FITC for 5 min at room temperature in the dark. Cells were incubated with 50 μg/ml propidium iodide (PI) at room temperature before analysis. Fluorescence resulting from FITC and PI was measured at 530 nm (FL1) and 590 nm (FL2), respectively, and analyzed using CellQuest software on a FACScan flow cytometer (Becton Dickinson, Oxford, UK) using an excitation of 488 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was prepared using Tri Reagent (Molecular Research Centre Inc, Dublin, Ireland). Single-stranded cDNA was synthesized according to the Moloney murine leukemia virus (M-MLV) RT protocol (Promega, Southampton, UK). Oligo dT, $MgCl_2$, and RNAsin were also purchased from Promega. Deoxy-nucleoside 5′-triphosphates (dNTPs) were obtained from Sigma-Aldrich. cDNA was amplified using Taq DNA polymerase (Promega), according to the manufacturer's instructions. PCR reactions were carried out as follows: 95°C for 5 min, 22 cycles of –95°C for 1 min, T_{anneal} for 1 min, 72°C for 1 min, followed by 72°C for 5 min. Primers included OPN (forward 5′-TTCACAGCCACAAGGACAAG-3′, reverse 5′-TGCAGAAGCTTTTGGTTACAA-3′, T_{anneal}, 55°C) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; forward 5′-ACCACAGTCCATGCCATCAC-3′, reverse 5′-TCCACCACCCAGTTGCTGTA-3′, T_{anneal}, 58°C). To control for DNA contamination of RNA samples, PCR was also carried out in the absence of RT (see **Fig. 1a**). In this case, M-MLV was omitted from the RT reaction. Control reactions were performed initially to ensure linearity of amplification over the number of cycles used.

Barium citrate precipitation of proteins

CM was precipitated prior to electrophoresis, essentially as described by Senger et al. [25]. Briefly, phosphoproteins were precipitated by the addition of one-tenth volume sodium citrate solution (130 mM) and one-tenth volume barium chloride solution (615 mM), purchased from Sigma-Aldrich. Precipitated proteins were eluted in electrophoresis sample buffer containing 0.2 M sodium citrate.

Kinase assays

Ras activity was measured using the Ras activation assay kit from Upstate Biotechnology Ltd. and following the manufacturer's instructions. Guanosine 5′-triphosphate (GTP)-bound Ras from cell lysates was "pulled down" using the glutathione *S*-transferase fusion protein corresponding to the Ras-binding domain (RBD) of Raf-1, bound to agarose. The presence of active Ras was detected by Western blotting using anti-Ras antibody. Raf-1 kinase assays were performed using the Raf-1 kinase cascade assay kit from Upstate Biotechnology Ltd., according to the manufacturer's instructions. Raf-1 was immunoprecipitated from cell lysates, and kinase activity was measured using MEK, extracellular signal-regulated kinase (ERK), and myelin basic protein (MBP) as sequential substrates. MEK kinase assays were performed essentially as Raf-1 kinase assays with some modifications. MEK was immunoprecipitated from cell lysates, and kinase activity was measured using ERK and MBP as sequential substrates. aPKC activity was assessed according to Takeda et al. [26]. Briefly, aPKC was immunoprecipitated from whole cell lysates, and kinase activity was assessed using MBP as a substrate. Assay products for Raf-1, MEK, and aPKC assays were assessed using P81 phosphocellulose paper (Upstate Biotechnology Ltd.) and counting on a Beckman Coulter LS 6500 scintillation counter.

Measurement of cell death

Cell death was assessed by PI uptake on a FACScan (Becton Dickinson) flow cytometer at 590 nm (FL2). Cells were incubated with 5 μg/ml PI at room temperature prior to analysis. The criteria for cell death, as measured by flow cytometry, were based on changes in light-scattering properties of dead cells as a result of cell shrinkage and increased granularity and also, permeability to PI.

RESULTS

p210 Bcr-Abl expression leads to increased levels of OPN mRNA and protein

OPN has been shown to be involved in the growth and survival of metastatic cells and to be present at increased levels in the

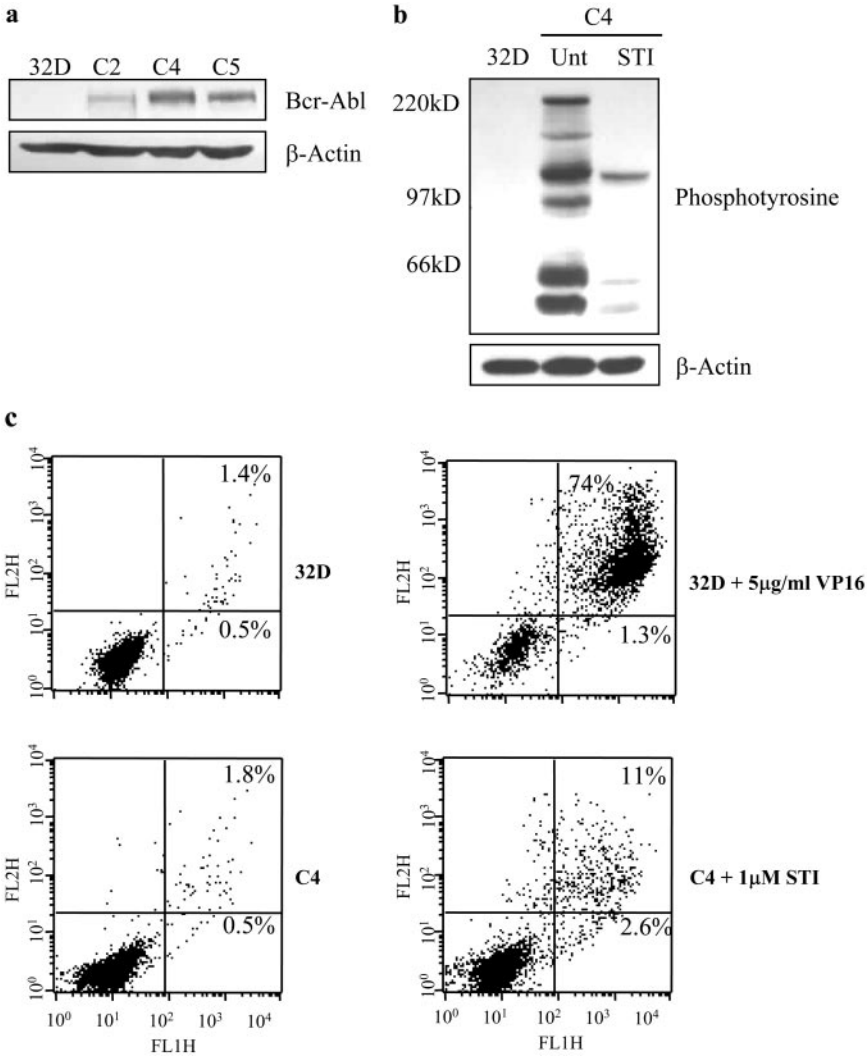

Fig. 1. Expression of p210 Bcr-Abl in 32D cells. (a) Western blot analysis of Bcr-Abl expression in whole cell lysates from 32D parental cells and Bcr-Abl-transfected C2, C4, and C5 cells. Actin antibody-binding demonstrates equal protein loading. (b) Western blot analysis of phosphotyrosine levels in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated with 1 μM STI571 (STI) for 48 h. (c) Untreated 32D and C4 cells and C4 cells treated for 48 h with 1 μM STI571 were assessed for apoptotic cell death by annexin V/PI staining. Percentages in the bottom-right quadrants indicate annexin V-positive, PI-negative cells, and percentages in the top-right quadrants indicate annexin V-positive, PI-positive cells. 32D cells treated for 48 h with 5 μg/ml VP16 are included as a positive control for high levels of apoptotic cell death. Data are representative of three independent experiments.

blood of patients with metastatic disease. In vitro studies have shown it to be associated with proliferation [27] and the prevention of apoptosis [28]. Increased OPN often correlates with increased metastatic potential of cancers. We hypothesized that the up-regulation of OPN may not be restricted to solid tumors but may also occur in leukemias. To investigate this possibility, we used 32D cells and a transfected clone of this cell line expressing high levels of Bcr-Abl (C4; Fig. 1a, lanes 1 and 3). 32D cells are IL-3-dependent myeloid progenitor cells; transfection with Bcr-Abl renders these cells IL-3-independent and drug-resistant [12]. RT-PCR was used to compare levels of OPN mRNA between 32D and C4 cells. We found that OPN was expressed at undetectable levels in 32D cells but at high levels in C4 cells (**Fig. 2a**). STI571 (Gleevec) is a specific inhibitor of Bcr-Abl tyrosine kinase activity [29]. When C4 cells are treated with 1 μM STI571 for 48 h, the phenotype is reverted to that of 32D cells. That is, levels of phosphotyrosine are reduced (Fig. 1b), and the cells become

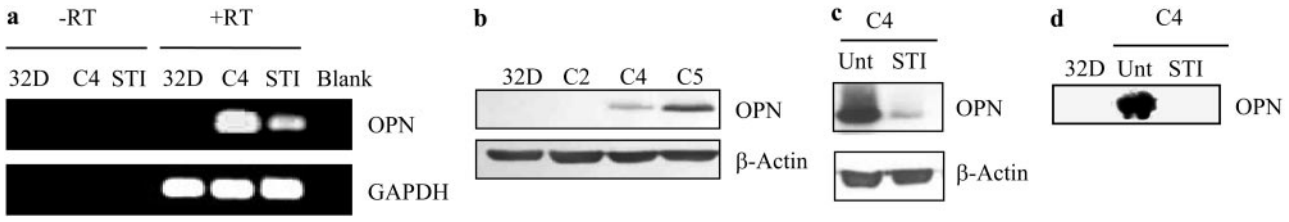

Fig. 2. Bcr-Abl expression leads to increased levels of OPN mRNA and protein. (a) RT-PCR experiments, using cDNA from 32D, untreated C4 cells, and C4 cells treated for 48 h with 1 μM STI571 (STI), show expression levels of OPN in the presence (+RT) and absence (–RT) of RT. Levels of GAPDH-PCR product demonstrate equal quantities of cDNA. (b) Western blot analysis of OPN expression in total cell lysates from 32D, C2, C4, and C5 cells. Actin antibody-binding demonstrates equal protein loading. (c) Western blot analysis of OPN expression in cell lysates from untreated C4 cells (Unt) and C4 cells treated for 48 h with 1 μM STI571. (d) Western blot analysis of OPN expression in barium citrate precipitates from culture media of 32D, untreated C4 cells, and C4 cells treated for 48 h with 1 μM STI571.

dependent on IL-3 for survival (data not shown). This treatment does not result in significant cytotoxicity, as demonstrated by annexin V/PI staining (Fig. 1c). This eliminates the possibility that effects seen in cells treated with STI571 are a result of cell death rather than Bcr-Abl inhibition. As such, STI571 was used to confirm that differences between 32D and C4 cells are in fact a result of the activity of Bcr-Abl, and indeed, treatment of C4 cells with STI571 resulted in a decrease in the levels of expression of OPN mRNA (Fig. 2a). The increased OPN mRNA seen in C4 cells is reflected by an increased level of OPN protein, as seen by Western blotting of whole cell lysates from 32D and C4 cells (Fig. 2b, lanes 1 and 3). To further confirm the induction of OPN expression in response to high levels of Bcr-Abl, we analyzed another transfected clone of 32D cells (C5) expressing levels of Bcr-Abl similar to that seen in C4 cells (Fig. 1a, lane 4). We found that expression of OPN also occurs in this clone (Fig. 2b, lane 4) and to an even greater extent than in C4 cells. It is also shown that treatment with STI571 results in a decrease in levels of OPN protein in C4 cells (Fig. 2c).

CML is a progressive disease, and progression, in most cases, is linked to increased expression of Bcr-Abl. As OPN has been linked to disease progression in tumorigenesis, we investigated the possibility that the level of Bcr-Abl expression is involved in the up-regulation of OPN expression and used a transfected clone of 32D cells expressing low levels of Bcr-Abl (C2; Fig. 1a, lane 2). OPN protein was undetectable in whole cell lysates from C2 cells (Fig. 2b, lane 2), suggesting that, as with solid tumors, in leukemia OPN expression is associated with disease progression and increased aggressiveness.

OPN is reportedly a secreted protein. Barium citrate precipitation was used to precipitate OPN from CM obtained from 32D and C4 cells and C4 cells treated with STI571. High levels of OPN could be precipitated from the CM of untreated C4 cells alone with undetectable levels in the media from 32D- and STI571-treated C4 cells (Fig. 2d).

The up-regulation of OPN is dependent on Ras activity

The ability of high levels of Bcr-Abl to up-regulate the expression of OPN represents a novel and potentially important downstream affect of this oncogene. As such, we aimed to determine the pathways and signaling molecules required for this induction. A potential role for Ras was first examined, as OPN expression in other models has previously been shown to be dependent on Ras [30], and also, as Ras is well-documented as a target of Bcr-Abl [14, 31, 32]. Western blot analysis revealed that C4 cells express higher levels of Ras than do 32D cells and that this increase in expression is Bcr-Abl tyrosine kinase-dependent, as it can be reversed by treatment with STI571. Treatment of C4 cells with 10 μM FTS for 48 h does not significantly reduce Ras expression (**Fig. 3a**). C4 cells also display a higher level of Ras activity than 32D cells, as determined by a Ras activity assay (Fig. 3b). It is shown that this increase in Ras activity seen in C4 cells can be reversed by STI571 treatment and also by treatment with 10 μM FTS (Fig. 3b). As shown in Figure 3a and b, levels of Ras protein and activity are increased in C4 cells in a Bcr-Abl-dependent manner. These results indicate that Bcr-Abl up-regulates this pathway at two levels—first, by increasing expression of Ras and then, by leading to its increased activation. Treatment with FTS blocked the induction of OPN expression in response to Bcr-Abl (Fig. 3c), indicating that Ras activity is essential for its up-regulation.

The up-regulation of OPN is dependent on MEK activity

Ras is known to activate the Raf/MEK/ERK pathway, and also, induction of OPN expression has been shown to be MEK-dependent [33]. We investigated the involvement of MEK in the up-regulation of OPN by Bcr-Abl. C4 cells express higher levels of MEK protein than 32D cells, and this increased expression can be reduced by treatment of STI571. Treatment of C4 cells with the ERK1/2-specific MEK1/2 inhibitor UO126 (5 μM for 24 h) results in a slight down-regulation in levels of MEK protein (**Fig. 4a**). Along with increased levels of MEK protein, C4 cells also display higher levels of MEK activity than 32D cells, as determined by MEK kinase activity assay (Fig. 4b). These results show that as seen for Ras above, Bcr-Abl up-regulates MEK at the level of expression and activation in a tyrosine kinase-dependent manner. Western blot analysis shows that treatment of C4 cells with UO126 inhibits OPN expression (Fig. 4c), demonstrating that along with Ras, MEK activity is required for Bcr-Abl-mediated OPN expression. We also confirmed that MEK activity is required for the induction of OPN in C5 cells (data not shown).

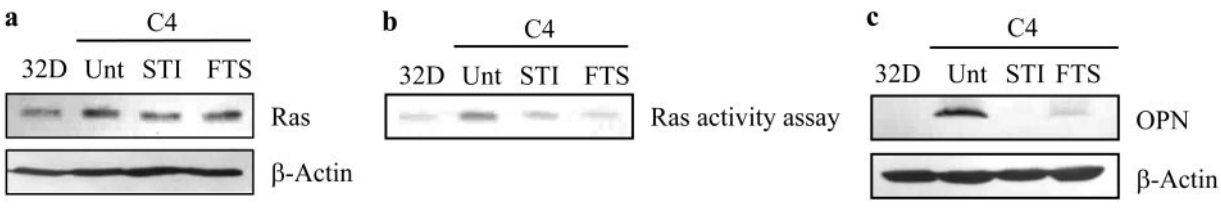

Fig. 3. The up-regulation of OPN is dependent on Ras activity. (a) Western blot analysis of Ras expression in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated for 48 h with 1 μM STI571 (STI) or 10 μM FTS. Actin antibody-binding demonstrates equal protein loading. (b) Cell lysates from (a) were incubated with Raf-1 RBD agarose-conjugated beads. Bound proteins were denatured by boiling in reducing sample buffer and then resolved by SDS-PAGE, followed by Western blotting to detect Ras-GTP using a monoclonal antibody to Ras. (c) Samples from (a) were harvested and separated by electrophoresis on a 10% polyacrylamide gel. The resulting membrane was immunoblotted using antibodies to OPN.

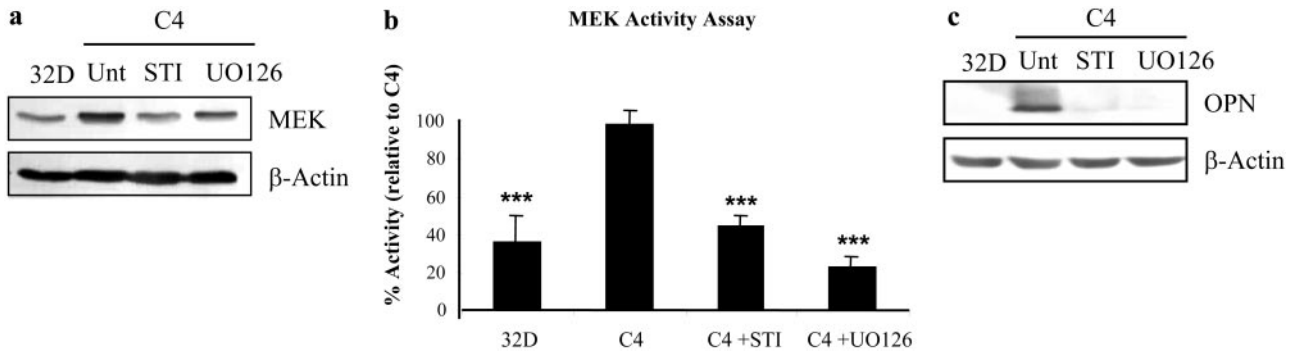

Fig. 4. The up-regulation of OPN is dependent on MEK activity. (a) Western blot analysis of MEK expression in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated for 48 h with 1 μM STI571 (STI) or for 24 h with 5 μM UO126 (ERK1/2-specific MEK1/2 inhibitor). Actin antibody-binding demonstrates equal protein loading. (b) Cell lysates from (a) were immunoprecipitated with MEK antibodies, and kinase activity was determined using an in vitro kinase assay in which recombinant ERK and MBP were used. MEK activity was determined quantitatively by scintillation counting of the P81 phosphocellulose squares spotted with the assay product. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (***, $P<0.005$, compared with levels of activity in C4 cells). (c) Western blot analysis of OPN expression in total cell lysates from samples used in (a).

The up-regulation of OPN is dependent on Raf-1 activity

Ras-dependent activation of MEK generally occurs via Raf-1 activation. Western blot analysis of whole cell lysates from 32D and C4 cells shows that as for Ras and MEK, C4 cells also express higher levels of Raf-1 than do 32D cells, and again, this is reversible by treatment with the Abl-specific inhibitor STI571. Treatment of C4 cells with 8-bromo-cAMP (cAMP activates PKA, resulting in the phosphorylation of Raf-1 on S43 and S621, inhibiting Raf-1 activity; ref. [34]) does not result in a significant reduction in Raf-1 protein expression (**Fig. 5a**). A Raf-1 activity assay confirmed that C4 cells have fivefold higher levels of Raf-1 activity than 32D cells and that this activity can be blocked by treatment with STI571 or 400 μM 8-bromo-cAMP (Fig. 5b). These findings demonstrate that Raf-1 up-regulation by Bcr-Abl is a result of increased expression and activation in a similar manner to Ras and MEK. As expected, Western analysis of untreated C4 cells and cells treated with 400 μM 8-bromo-cAMP for 24 h revealed that inhibition of Raf-1 activity results in a decrease in the expression levels of OPN (Fig. 5c). These results confirm that the Ras/Raf/MEK signaling cascade is up-regulated in C4 cells both at the level of expression and activation, and that up-regulation of OPN expression in response to the Bcr-Abl oncogene is dependent on this pathway.

Increasing Raf-1 activity in C4 cells leads to a further increase in OPN expression

We have previously noted that 5-Iodo, a compound that reportedly inhibits Raf-1 activity in vitro [35], actually results in increased Raf-1 activity when used to treat cells. This phenomenon has also been observed for other potential Raf-1 inhibitors and is thought to involve a feedback mechanism, whereby Raf suppresses its own activity, such that inhibition is counterbalanced by activation [36]. To further confirm the involvement of Raf-1 in the up-regulation of OPN, we treated

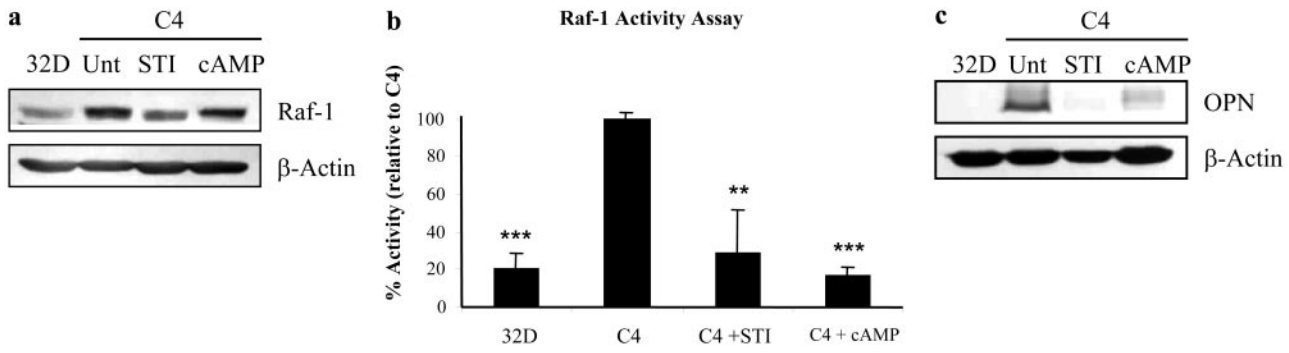

Fig. 5. The up-regulation of OPN is dependent on Raf-1 activity. (a) Western blot analysis of Raf-1 expression in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated for 48 h with 1 μM STI571 (STI) or for 24 h with 400 μM 8-bromo-cAMP (Raf-1 inhibitor). Actin antibody-binding demonstrates equal protein loading. (b) Cell lysates from (a) were immunoprecipitated with Raf-1 antibodies, and kinase activity was determined using an in vitro kinase assay. Raf activity was determined by scintillation counting. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of C4 cells. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (**, $P<0.01$; ***, $P<0.005$, compared with levels of activity in C4 cells). (c) Western blot analysis of OPN expression in total cell lysates from samples used in (a).

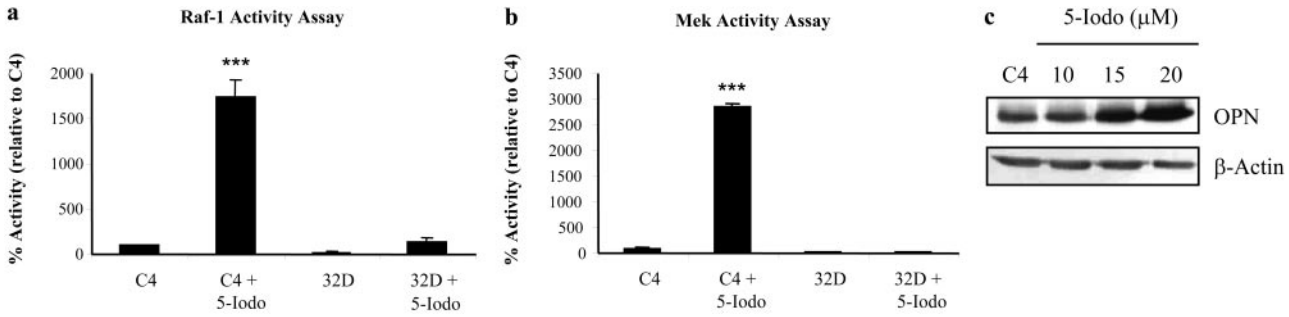

Fig. 6. Increasing Raf-1 activity in C4 cells leads to a further increase in OPN expression. (a) Lysates from untreated 32D and C4 cells or cells treated for 24 h with 5-Iodo were subjected to immunoprecipitation with Raf-1 antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (***, $P<0.005$, compared with levels of activity in untreated cells). (b) Lysates from (a) were immunoprecipitated with MEK antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (***, $P<0.005$, compared with levels of activity in untreated cells). (c) Western blot analysis of OPN levels in lysates from untreated C4 cells and C4 cells treated with the indicated concentration of 5-Iodo for 24 h. Actin antibody-binding demonstrates equal protein loading.

C4 cells with 20 μM 5-Iodo for 24 h. This resulted in an 18-fold increase in Raf-1 activity in these cells (**Fig. 6a**, first two columns). To confirm that this increased Raf-1 activity was in fact leading to increased activity of the Raf/MEK/ERK pathway, we also measured MEK activity in C4 cells in response to 5-Iodo treatment. The increased Raf-1 activity was found to result in a subsequent increase in MEK activity of 30-fold (Fig. 6b, first two columns). This increased Raf-1/MEK activity in response to 5-Iodo was shown to up-regulate OPN expression in a dose-dependent manner by Western analysis (Fig. 6c). This further confirms that Raf-1 plays a role in the up-regulation of OPN in response to Bcr-Abl expression.

32D cells were also treated with 20 μM 5-Iodo for 24 h. However, treatment with 20 μM led to only a modest increase in Raf-1 activity (less than twofold) in 32D cells (Fig. 6a, second two columns) and no downstream activation of MEK (Fig. 6b, second two columns). As a result, no increase was seen in levels of OPN protein in 32D cells (data not shown). Similar results were seen when C2 cells were treated with 5-Iodo. That is, no induction of OPN was observed (data not shown). The inability of 5-Iodo to activate Raf-1 activity in 32D cells was surprising and suggests differences in the activation of Raf-1 between 32D and C4 cells. This could possibly be a result of the fact that the Raf-1 protein is expressed at higher levels in C4 cells (Fig. 5a) or that basal levels of Raf activity are fivefold higher in C4 cells than 32D cells (Fig. 5b). It is also possible that other pathways are signaling to Raf-1 in C4 cells and that these pathways are necessary for the increased activity seen in response to 5-Iodo.

The up-regulation of OPN is dependent on aPKC, which lies upstream of Raf-1 and MEK

PKC has been shown previously to be involved in the up-regulation of OPN [37]. PKC represents a family of at least 11 members that can be divided into three distinct classes: classical PKC (cPKC), nPKC, and aPKC. cPKC and nPKC members are activated by diacylglycerol (DAG) and phorbol esters in vitro and in vivo [38, 39]. In contrast, aPKC members require neither DAG nor phorbol esters for activation [40, 41]. There are currently two members of the aPKC subfamily, ζ and ι. The murine ortholog of PKCι is termed PKCλ. In particular, the aPKCζ isoform has been shown to activate Raf-1 directly in the absence of Ras in Rat-1 fibroblasts [42]. We investigated the possibility that a signaling pathway involving aPKC is activated in C4 cells. Western blot analysis showed that C4 cells express higher levels of aPKC than 32D cells, and STI571 treatment confirmed that this up-regulation was dependent on Bcr-Abl tyrosine kinase activity. Treatment with the myristoylated PKCζ pseudosubstrate peptide (10 μM for 24 h) did not lead to any significant alteration in the expression levels of aPKC in C4 cells (**Fig. 7a**). We also found that the basal level of aPKC activity in C4 cells is approximately fourfold greater than that of 32D cells (Fig. 7b). Following treatment with STI571, a large decrease in aPKC activity was seen, indicating that the kinase activity of Bcr-Abl is necessary for its activation. Treatment with the myristoylated PKCζ pseudosubstrate peptide (10 μM for 24 h) also inhibited aPKC activity in C4 cells (Fig. 7b). This peptide has been shown to inhibit PKCζ activity by interacting with the substrate-binding pocket in the catalytic domain [43, 44]. However, as the sequence of this peptide from PKCζ is identical to the corresponding sequence in PKCι/λ [45], it may be assumed that this PKCζ pseudosubstrate peptide acts as a general aPKC inhibitor. Once again, it is shown that Bcr-Abl up-regulates aPKC at the level of expression and activation, suggesting that this may be a common feature of Bcr-Abl signaling. Western analysis of whole cell lysates revealed that inhibition of aPKC activity in C4 cells led to a down-regulation in OPN expression (Fig. 7c), confirming that its activity is required for the induction of OPN in these cells.

We then investigated the possibility that aPKC lies upstream of Raf-1 in our system. We found that inhibition of aPKC led to an almost complete loss of Raf-1 activity in C4 cells (Fig. 7d) and a subsequent downstream reduction in MEK activity (Fig. 7e). This confirms that aPKC activity is required for Raf-1 activation in signaling downstream of Bcr-Abl. Treatment of 32D cells with the PKCζ pseudosubstrate inhibitor also led to a reduction in Raf-1 activity to undetectable levels (Fig. 7d)

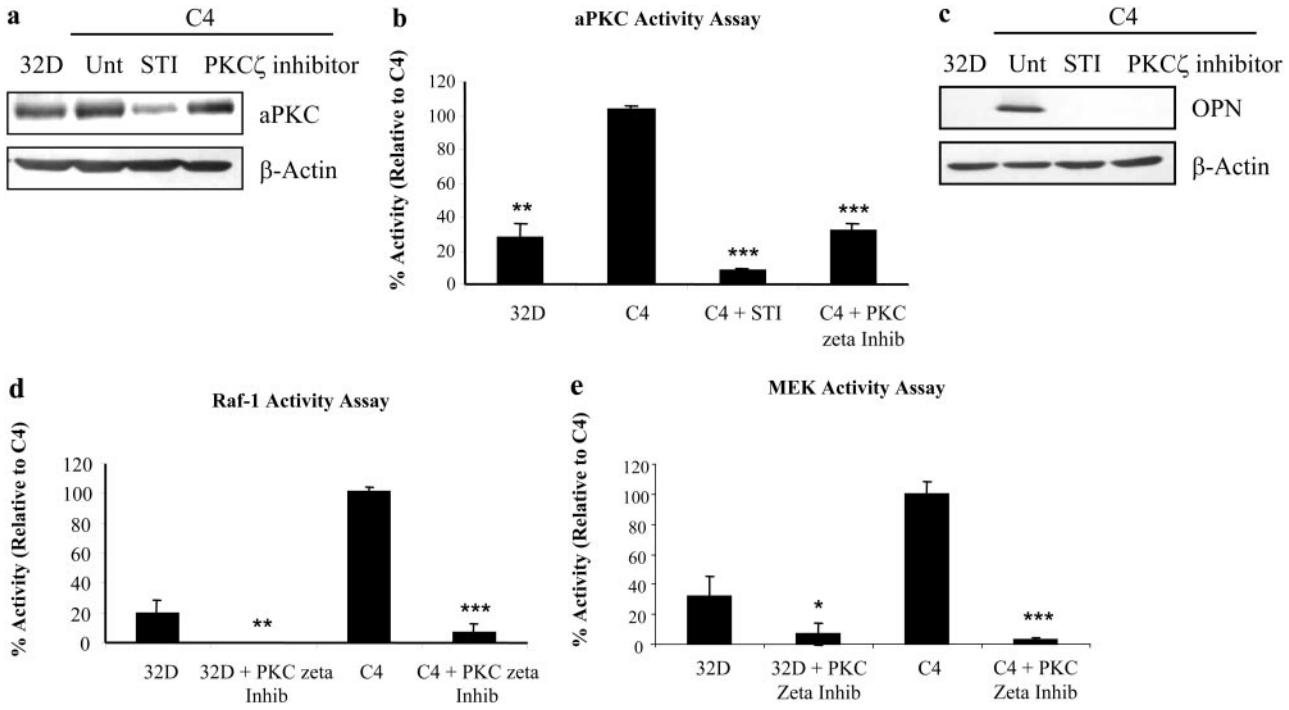

Fig. 7. The up-regulation of OPN is dependent on aPKC, which lies upstream of Raf-1 and MEK. (a) Western blot analysis of aPKC expression in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated for 48 h with 1 μM STI571 (STI) or for 24 h with 10 μM PKCζ pseudosubstrate inhibitor. Actin antibody-binding demonstrates equal protein loading. (b) Cell lysates from (a) were immunoprecipitated with aPKC antibodies, and kinase activity was determined using an in vitro kinase assay. aPKC activity was determined by scintillation counting. Background counts were subtracted from each sample, and the resultant net counts were expressed as a percentage of the values obtained for C4 cells. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (**, $P<0.01$; ***, $P<0.005$, compared with levels of activity in C4 cells). (c) Western blot analysis of OPN expression in total cell lysates from samples used in (a). (d) Untreated 32D and C4 cells and cells treated with 10 μM PKCζ pseudosubstrate inhibitor for 24 h were subjected to immunoprecipitation with Raf-1 antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (**, $P<0.01$; ***, $P<0.005$, compared with levels of activity in untreated cells). (e) Lysates from (d) were immunoprecipitated with MEK antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (*, $P<0.05$; ***, $P<0.005$, compared with levels of activity in untreated cells).

and to a downstream reduction in MEK activity levels (Fig. 7e). This indicates that the pathway from aPKC to Raf-1 and MEK is active in the parental cell line. We have also shown that 32D cells have significantly lower levels of aPKC activity than do C4 cells, and this may explain our inability to activate Raf-1 in 32D cells using a compound 5-Iodo, which led to a strong activation in C4 cells. This potentially highlights the importance of increased expression as well as activation of signaling molecules downstream of Bcr-Abl.

Ras lies upstream of aPKC activation

Our results thus far demonstrate that in response to Bcr-Abl tyrosine kinase activity, expression of the OPN gene is activated. We have shown that the signaling pathways involved require Ras, PKC (specifically aPKC), MEK, and Raf-1 activities and that aPKC lies upstream of Raf-1 activation, which leads to downstream activation of MEK. This led us to question where Ras lies in this signaling pathway. We considered two possibilities: First, Ras and aPKC are required for activation of Raf-1, and second, Ras is not, in fact, required for the activation of Raf directly but lies upstream and is responsible for the activation of aPKC. To investigate the latter possibility, untreated C4 cells and C4 cells treated with 10 μM FTS for 48 h were analyzed for aPKC activity. We found that inhibition of Ras activity led to a significant reduction in aPKC activity (**Fig. 8**). This indicates that in response to Bcr-Abl expression,

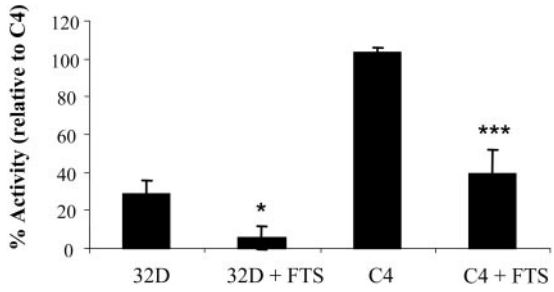

Fig. 8. Ras lies upstream of aPKC activation. Cell lysates from untreated 32D and C4 cells and cells treated for 48 h with 10 μM FTS were immunoprecipitated with aPKC antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (*, $P<0.05$; ***, $P<0.005$, compared with levels of activity in untreated cells).

Ras is activated, leading to a downstream activation of the aPKC, Raf-1, and MEK. This does not rule out the possibility that signals from Ras directly and aPKC are required for Raf-1 activation. We also found that treatment of 32D cells with 10 μM FTS led to a significant reduction in aPKC activity (Fig. 8). This indicates that the same pathway exists in the parental cell line.

The up-regulation of OPN is dependent on PI-3K, which lies upstream of aPKC

Activation of aPKC by Bcr-Abl has not been reported to date, and as such, we questioned the possible pathways involved in this activation. It has been reported that the major activation pathway of PKCζ depends on phosphatidylinositol phosphate 3 (PIP3), produced mainly by PI-3K [46]. It has also been extensively reported that PI-3K activity is increased in response to Bcr-Abl tyrosine kinase activity [13, 31, 47]. To confirm that this is the case, in our system, we performed Western blot analysis to study levels of AKT phosphorylation (ser 473) in 32D and C4 cells (**Fig. 9a**). We show that this increase in p-Akt is Bcr-Abl-dependent by treatment with STI571 and that it is PI-3K-dependent by treatment for 48 h with the PI-3K inhibitor LY294002 (Fig. 9a).

To determine the affect of PI-3K on aPKC activity, C4 cells were treated for 48 h with 20 μM LY294002 and analyzed for aPKC activity. LY294002 led to a marked decrease in aPKC activity in C4 cells (Fig. 9b). A similar result was seen when 32D cells were treated with 20 μM LY294002 (Fig. 9b). This, once again, confirms that the signaling pathway required for the induction of OPN expression in C4 cells is also present in the parental 32D cell line but is activated to a lesser extent. If, as we hypothesize, PI-3K leads to increased Raf-1 and MEK activity via aPKC, then it would be expected that inhibition of PI-3K would lead to decreased levels of OPN protein. Western blot analysis of untreated C4 cells and C4 cells treated with 20 μM LY294002 for 48 h showed that this pathway is indeed involved in OPN expression, as treatment with the PI-3K inhibitor completely abolished OPN expression in C4 cells (Fig. 9c). This led us to question whether Ras lies upstream of PI-3K or vice versa in this signaling cascade. Treatment of C4 cells with LY294002 did not lead to any decrease in Ras activity (Fig. 9d), indicating that PI-3K does not lie upstream of Ras and suggesting that Ras is in fact responsible for the activation of PI-3K in our system.

These results indicate that the pathway involved in OPN expression response to Bcr-Abl involves activation of Ras by Bcr-Abl and subsequent downstream activation of PI-3K, aPKC, Raf-1, and MEK (**Fig. 10**).

OPN expression correlates with drug resistance

We have found that high levels of Bcr-Abl (found in C4 and C5 cells) lead to the expression of OPN, and lower levels of Bcr-Abl (seen in C2 cells) do not. The main difference seen between these cell lines is in their response to treatment with chemotherapeutic drugs. We find that resistance to treatment with VP16 correlates with the expression of OPN in cells, that is, high levels of death are seen in 32D and C2 cells that lack OPN, and significantly reduced death is seen in C4 and C5 cells (**Fig. 11**). It is also shown that treatment of C4 cells with the STI571, FTS, UO126, or PKCζ pseudosubstrate inhibitor results in increased levels of death in response to VP16 treatment. All of these treatments also lead to the reduction of OPN expression in C4 cells.

DISCUSSION

OPN expression has been shown previously to correlate with solid tumor progression and metastasis in breast cancer [48], prostate cancer [49], and lung cancer [50] among others. Increased levels of OPN have also been demonstrated by Saeki et al. [51] in myeloma cells and in plasma from patients with multiple myeloma. This study also looked at OPN levels in patients with other haematological malignancies, including acute myeloid leukemia. This work by Saeki et al. [51] is the only reported study of OPN levels in haematological malignan-

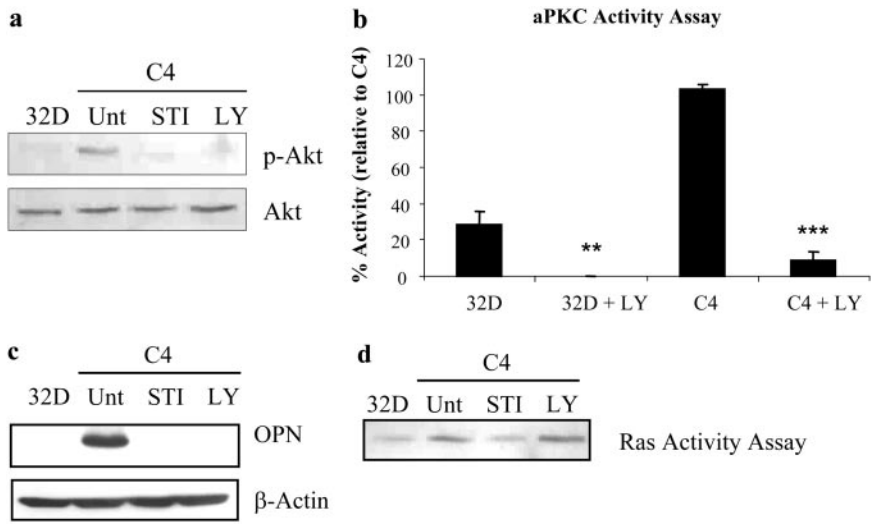

Fig. 9. The up-regulation of OPN is dependent on PI-3K, which lies upstream of aPKC. Western blot analysis of phosphor-AKT (p-Akt; ser 473) levels in total cell lysates from 32D, untreated C4 cells (Unt), and C4 cells treated for 48 h with 1 μM STI571 (STI) or 20 μM LY294002 (LY). AKT antibody-binding demonstrates equal protein loading. (b) Untreated 32D and C4 cells and cells treated with 20 μM LY294002 for 48 h were immunoprecipitated with aPKC antibodies, and kinase activity was determined using an in vitro kinase assay. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (**, $P<0.01$; ***, $P<0.005$, compared with levels of activity in untreated cells). (c) Western blot analysis of OPN levels in whole cell lysates from samples used in (a) above. Actin antibody-binding demonstrates equal protein loading. (d) Cell lysates from (a) were incubated with Raf-1 RBD agarose-conjugated beads. Bound proteins were denatured by boiling in reducing sample buffer and then resolved by SDS-PAGE, followed by Western blotting to detect Ras-GTP using a monoclonal antibody to Ras.

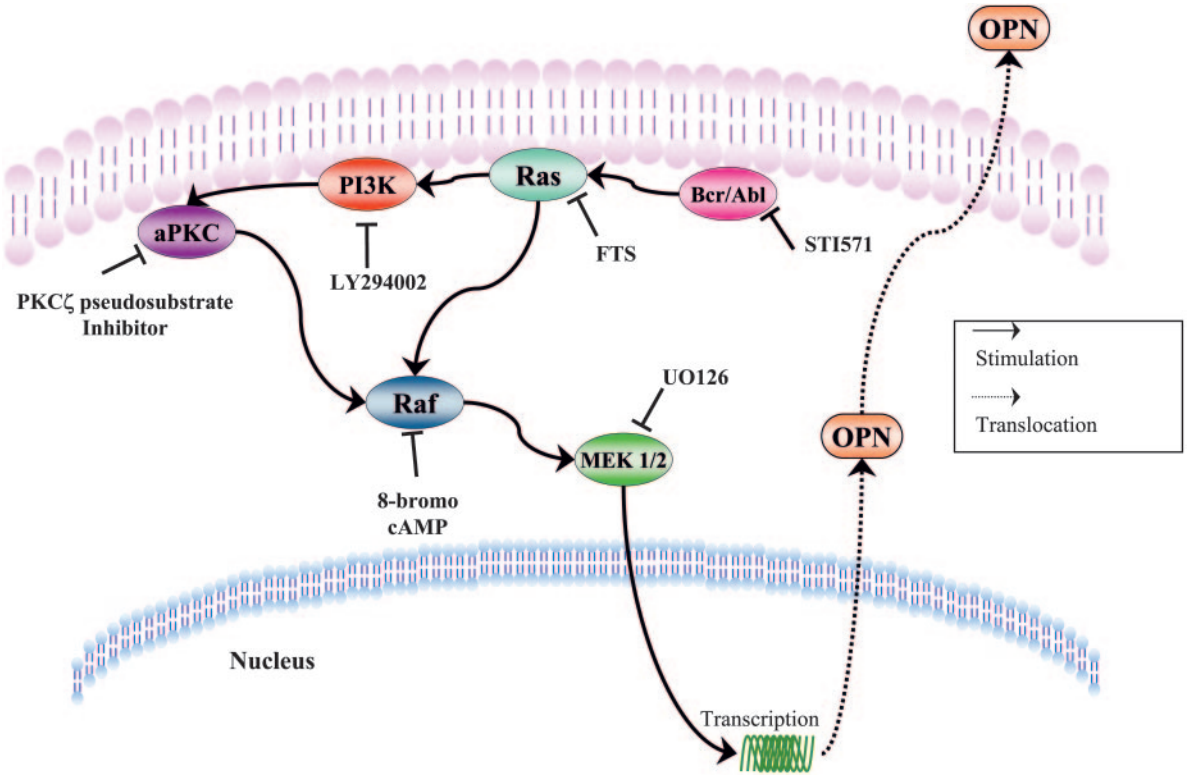

Fig. 10. Proposed mechanism of up-regulation of OPN by Bcr-Abl. This figure illustrates the pathway by which OPN expression is induced in a model of Bcr-Abl expression. Bcr-Abl activates Ras, which leads to the activation of a signaling cascade involving PI-3K, aPKC, Raf-1, and MEK. This ultimately leads to the transcriptional activation of the OPN gene and subsequent expression and secretion of the OPN protein.

cies to date and did not include analysis of CML samples. Therefore, our study represents the first investigation of the up-regulation of OPN by Bcr-Abl.

The work reported here used a model system of CML, whereby normal 32D cells were transfected with p210 Bcr-Abl. This model allows us to compare directly the effects of different levels of Bcr-Abl expression between the cell lines. Although this model is subject to the limitations of any cell line model, in all experiments, we have used the Bcr-Abl-specific inhibitor STI571 to confirm that all differences seen are in fact a result of Bcr-Abl tyrosine kinase activity. The data presented here demonstrate for the first time that OPN expression is induced (at the level of mRNA and secreted protein) in a leukemic cell line expressing high levels of p210 Bcr-Abl. Our inability to detect OPN in a cell line expressing lower levels of Bcr-Abl suggests that its expression is correlated with disease progression, as has been shown to be the case for many solid tumors. Results from this lab have previously demonstrated that although low levels of Bcr-Abl are sufficient to confer growth-factor independence on 32D cells, higher levels of Bcr-Abl expression are required for drug resistance [12]. As such, the C2 cells have a phenotype intermediate between that of 32D and C4 cells, being growth factor-independent but still susceptible to drug-induced apoptosis. The results presented here suggest that levels of Bcr-Abl, sufficient to induce drug resistance, are required for the induction of OPN expression. The potential importance of OPN up-regulation in CML prompted us to begin to define the signaling pathways by which the oncogene Bcr-Abl regulates OPN expression. Our results establish that high levels of Bcr-Abl, typically seen in CML patients in the blast-crisis stage of disease, result in the up-regulation of signaling pathways already existing in the cell

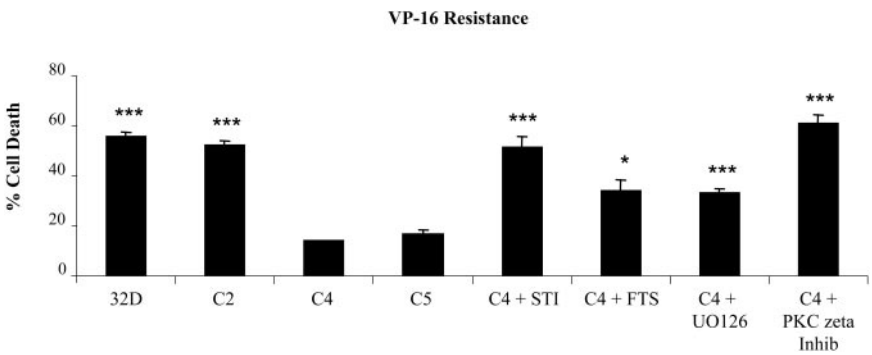

Fig. 11. OPN expression correlates with drug resistance. 32D, C2, C4, and C5 cells and C4 cells pretreated for 48 h with 1 μM STI571 (STI) or 10 μM FTS or for 24 h with 5 μM UO126 or 10 μM PKCζ pseudosubstrate inhibitor prior to treatment for 24 h with 5 μg/ml VP16. Levels of cell death were assessed by PI staining. Data are presented as the mean and standard deviation of the mean for independent experiments (n=4). Statistical analysis was performed using an unpaired Student's *t*-test (*, $P<0.05$; ***, $P<0.005$, compared with levels of cell death in C4 cells).

with the ultimate induction of OPN expression. We show that the pathway involved requires the increased activity of Ras, PI-3K, aPKC, Raf-1, and MEK.

It has been shown that increased levels of OPN mRNA can be a result of increased mRNA stability [52]. This is unlikely to be the case in C4 cells, as the parental cell line 32D does not express detectable levels of OPN mRNA. It has also been demonstrated that the up-regulation of OPN in some cases is dependent on Ras [53]. It has been shown that this induction may be direct, via a Ras-activated enhancer, in the OPN promoter [54]. Here, we report that in response to Bcr-Abl expression, Ras activity is required for the induction of OPN expression. It is unlikely that this induction is direct in our model, as we have also shown that multiple signaling molecules are involved downstream of Ras.

The MAPK signaling cascade has also been implicated in OPN regulation in various systems including induction of OPN by inorganic phosphate in osteoblasts [33] and induction by 12-*O*-tetradecanoylphorbol 13-acetate (TPA) in HL-60 cells [55] and also in JB6 cells [56], in response to injury of rat arterial muscle cells [57] and to angiotensin II in cardiac cells [58]. The fact that the MAPK cascade has been implicated in the induction of OPN in response to so many varying stimuli may suggest that activation of the MAPK cascade is a crucial component in the regulation of OPN expression, regardless of the stimulus. These data support our findings that the expression of OPN in response to Bcr-Abl is dependent on MEK activity. As we have shown that Ras activity is required for OPN expression, we investigated the possibility that Raf-1 activity is also required, as Raf-1 is widely accepted to lead to MEK phosphorylation and activation in response to its own activation by Ras. The requirement for Raf-1 activity in our system represents a novel finding, as Raf-1 has not been implicated previously in the up-regulation of OPN.

The results presented here also identify the requirement for an atypical member of the PKC family of serine/threonine kinases in the up-regulation of OPN. To date, 11 PKC isozymes have been identified. They are classified into three groups based on their structure and mode of regulation. The conventional cPKCs are activated by calcium and DAG; the nPKCs can be activated by DAG alone (i.e., they do not require calcium); and the activators for the aPKCs remain unknown. There are currently two members of the aPKC subfamily, ζ and ι. The murine ortholog of PKCι is termed PKCλ. Members of the PKC family have been reported to be involved in OPN regulation, including induction by TPA in HL-60 [55] and JB6 cells [56]. These studies suggest that nPKC isoforms ε and δ are required for OPN expression. Beck and Knecht [33] demonstrated that a PKC isoform other than cPKCs is required for induction of OPN by inorganic phosphate in osteoblasts. We used a PKCζ pseudosubstrate inhibitor to show that in response to Bcr-Abl, aPKC is required for OPN expression. This represents the first demonstrated link between aPKC and OPN. Further investigation is required to determine which of the aPKC isoforms is specifically required. A study by Murray and Fields [59] has shown that K562 cells (a cell line derived from a patient in blast-crisis CML) express PKCι but undetectable levels of PKCζ. This group has also shown that PKCι activation in K562 cells requires Bcr-Abl activity [60] and furthermore, that Bcr-Abl regulates PKCι transcription [61]. All of these data support our finding that expression and activity of aPKC are increased in C4 cells in a Bcr-Abl-dependent manner. This may suggest that the aPKCλ/ι isoform is most likely to be involved in Bcr-Abl oncogenesis and indeed, in the up-regulation of OPN, although further investigation will be required to confirm this.

We have shown that Raf-1 activation in 32D and C4 cells occurs via aPKC. We have not ruled out the possibility that other signaling pathways also result in the activation of Raf-1 in these cells; however, we have shown that aPKC activation of Raf-1 is essential for the induction of OPN. Mas et al. [62] have demonstrated previously the activation of Raf-1 by PKCζ. They demonstrated that in a human lymphocytic cell line (U937), the anthracycline danorubicin leads to increased Raf-1 and MEK activities via a PKCζ-mediated pathway. To date, however, there have been no reports of Raf-1 activation by PKCλ/ι. Mas et al. [62] showed that the activation of PKCζ was sensitive to the PI-3K inhibitor Wortmannin. The activation of PKCλ/ι by PI-3K has also been demonstrated [63]. We have shown that in downstream signaling of Bcr-Abl, PI-3K is in fact responsible for the activation of aPKC. Our results also place Ras upstream of aPKC activation. Both aPKC isoforms have been reported previously to act as Ras effectors [64–66]. Inhibition of PI-3K had no affect on Ras activation, suggesting that Ras lies upstream of PI-3K in this pathway.

PKC and the MAPK pathway have been implicated in the up-regulation of OPN in a number of different cell lines and in response to various stimuli. This may suggest that these pathways are a common requirement for induction of OPN expression, although the particular PKC isozyme involved appears to be cell type-/stimulus-specific. There is also some discrepancy as to whether these signaling molecules form a single pathway or two individual pathways, and both are required for OPN expression. Our studies suggest that in a Bcr-Abl model, a single pathway is involved, with aPKC lying upstream of Raf-1. Similarly, Chang et al. [56] demonstrated a single pathway with PKC lying upstream of MEK, although in this case, a nPKC isozyme (δ and/or ε) was involved. Atkins et al. [55] demonstrated a similar single pathway. Beck and Knecht [33], however, have shown that PKC and MAPK form two distinct pathways in their model of OPN expression. This difference may again be dependent on the cell type or the stimulus used by the different groups.

The function of OPN, in tumor and in normal cells, remains poorly understood. The fact that OPN expression could only be detected in response to high levels of Bcr-Abl suggests that its role may be in disease progression or drug resistance. Our results demonstrate that levels of OPN correlate with the drug resistance of cells, suggesting that this may be its function in Bcr-Abl-mediated oncogenesis. We find that treatment of C4 cells with STI571 completely abolishes the drug resistance of these cells, indicating the crucial nature of Bcr-Abl tyrosine kinase activity. Treatment with Ras, MEK, and aPKC inhibitors reduces OPN expression in C4 cells and also sensitizes them to drug-induced apoptosis. However, levels of death seen in response to pretreatment with FTS and UO126 are not as high as those seen in 32D and C2 cells, suggesting that other pathways may also be involved in drug resistance. Our results

indicate that these pathways lie downstream of aPKC, as pretreatment with an inhibitor of aPKC blocks all resistance to VP16 treatment in C4 cells.

In summary, the data presented here establish for the first time that OPN expression is induced in a model of leukemia, specifically by high levels of p210 Bcr-Abl. We have used this model to determine the specific signaling pathways involved in this up-regulation. The signaling pathway appears to be initiated by Ras downstream of Bcr-Abl, leading to the subsequent activation of PI-3K, aPKC, Raf-1, and MEK. These findings should prove valuable in the understanding of signaling downstream of Bcr-Abl and also in the up-regulation of OPN. To date, it has been shown that OPN is up-regulated as tumors become more aggressive and metastasize. The finding that OPN is also induced in a model of late-stage, aggressive leukemia may suggest a role for OPN in oncogenesis in general.

ACKNOWLEDGMENTS

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