CTLA-4: Acting at the Synapse

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Successful immune cell control requires a delicate balance of positive and negative regulatory signals. Costimulatory pathways involving molecules such as CD28, inducible costimulator (ICOS), 4-1BB, and CD40L are essential coactivators of proliferation, cytokine production, and cell migration. To balance these signals, cell surface molecules like Fas, tumor necrosis factor-α receptor (TNFR), and programmed death-1 (PD-1) decrease T cell responses. Most prominent among these T cell regulators is cytotoxic T lymphocyte antigen-4 (CTLA-4), a homolog of CD28 that can interact with the CD28 ligands CD80 and CD86 *(1)*. CTLA-4 inhibits T cell responses in a T cell receptor- (TCR)-dependent manner. For example, T cells treated with soluble CTLA-4 monoclonal antibody (mAb) have enhanced T cell proliferation and cytokine production, whereas surface-immobilized CTLA-4 mAb inhibits interleukin-2 (IL-2) production and cell cycle progression both in vitro and in vivo *(2, 3)*. In addition, CTLA-4–deficient mice exhibit a $CD4^+$ T cell lymphoproliferative disorder that leads to death of the animals a few weeks after birth *(4, 5)*.

Several models have been proposed to explain the molecular basis for CTLA-4 inhibition (Figure 1A). The first model proposes that CTLA-4 successfully competes against CD28 for CD80 or CD86 binding because CTLA-4 binds to them with much stronger avidity than does the CD28 costimulatory molecule (*Ligand Competition Model*) *(6)*. Support for this model comes from studies showing that mutant CTLA-4 lacking a functional intracytoplasmic domain can suppress T cell function both in vitro *(7)*, and in vivo *(8)*. A second model suggests that CTLA-4 inhibits important downstream signaling pathways involving the extracellular signal-regulated kinase (ERK) and nuclear factor-kappa B (NF-κB) pathways *(9, 10)*, subsequent to TCR-CD3 association and CD28 receptor engagement *(Distal Blockade Model)*. In this model, CTLA-4-mediated inhibition depends on, but is downstream of, the TCR signal. Finally, a third model suggests that CTLA-4 associates with the immunological synapse and attenuates proximal signal initiated by the TCR *(Proximal Blockade Model) (11, 12)*.

The immunological synapse is formed at the interface between the T cell and antigen-presenting cell (APC) membranes and is the hot spot for T cell activation. Upon T-cell–APC engagement, critical components of the TCR signal transduction machinery relocalize to this region initiating a biochemical cascade that leads to full T cell activation. In many instances, the interface forms highly organized scaffolds that create an immunological synapse *(13, 14)*. Several early studies suggested that upon T-cell–APC engagement, CTLA-4 is exported to the cell surface at the site of cell–cell interaction, where CTLA-4 can regulate TCR-proximal signals *(15, 16)*. These studies have been confirmed and extended by the work of Egen and Allison who demonstrate that CTLA-4 is at first localized in the uropod of activated T cells, where the microtubule organizing center (MTOC) is located *(11)*. When T cells are restimulated with weak-agonist peptide-bearing APCs, CTLA-4–containing vesicles in the T cells rapidly relocalize just beneath the APC–T cell contact site. By comparison, stimulation with a strong agonist peptide induces translocation of CTLA-4 to the cell surface and association with the immunological synapse. At least two mechanisms regulate the cell surface expression of CTLA-4: tyrosine phosphorylation-dependent clathrin-mediated internalization *(17–21)*, and the active release of the molecule from the intracellular compartment to the cell surface *(22, 23)*. CTLA-4 has a tyrosine-based FVYVKM motif within its cytoplasmic tail that strongly interacts—in the dephosphorylated state—with the clathrin adaptor molecules AP1 and AP2. This interaction results in rapid internalization and trafficking of CTLA-4 to intracellular compartments *(16–21)*. Many kinases can phosphorylate this tyrosine-based motif in vitro, and therefore prevent CTLA-4 from associating with clathrin and becoming internalized. The TCR-associated Src family kinases, Lck and Fyn, are the most likely tyrosine kinases involved, considering their localization within the immunological synapse *(24, 25)*. This hypothesis is also consistent with observations showing that active translocation of CTLA-4 depends on TCR signal and is thus ZAP-70– and Lck-dependent *(22)*, because Lck activation causes surface stabilization of CTLA-4 by direct phosphorylation of a regulatory tyrosine within the CTLA-4 tail *(24, 25)*. Thus, the TCR signal might influence CTLA-4 expression by both translocation and stabilization, although whether this active transport depends on lysosome secretion or other mechanisms remains unclear *(23)*.

CTLA-4 has been observed at the immunological synapse; however, there has been no direct biochemical basis to explain its inhibitory activity. CTLA-4 can directly associate with the TCRζ chain of the TCR–CD3 complex, and subsequent dephosphorylation of the ζ chain has been observed *(12)*. These observations provided the basis for a model suggesting that the negative regulatory activity of this cell surface protein depends on the association of a tyrosine phosphatase because CTLA-4 lacks known intrinsic enzymatic activity. In this regard, two protein tyrosine phosphatases—Src homology 2 (SH2) domain–containing tyrosine phosphatase-1 (SHP-1) and SHP-2—can associate with the cytoplasmic tail of CTLA-4 *(19, 26, 27)*; however, the role of these enzymes in the function of CTLA-4 is controversial because SHP-2 can positively regulate cell activities *(28)*, and CTLA-4, in some cellular contexts, negatively regulates signals in the absence of SHP-1 *(29)*.

PP2A, a protein serine-threonine phosphatase can associate with CTLA-4 *(30)*. This protein phosphatase is known to negatively regulate the Ras-ERK pathway in some systems *(31)*. This finding also fits with the localization of CTLA-4 to the immunological synapse, because Ras is activated within the

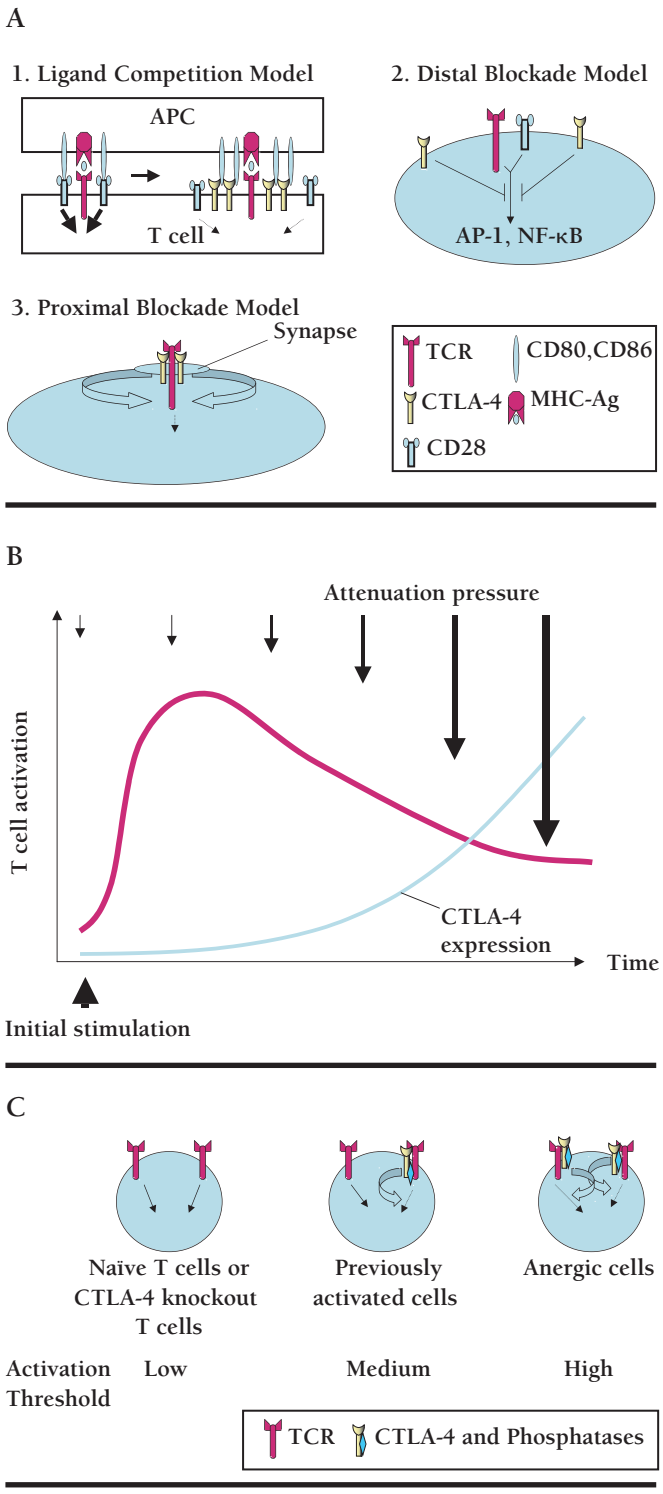

FIGURE 1. A. Three models for T cell inhibition by CTLA-4. 1. Ligand Competition Model wherein CTLA-4 can successfully compete with CD28 for CD80 and CD86 binding because of CTLA-4's stronger avidity. 2. Distal Blockade Model wherein CTLA-4 inhibits distal pathways of TCR–CD28 signaling. 3. Proximal Blockade Model wherein CTLA-4 is recruited to the immunological synapse and blocks early signal transduction via the TCR. **B.** Attenuation model: In single T cells, CTLA-4 is synthesized during the time course of T cell activation and is recruited to the T cell surface. As a consequence, the T cell signal is attenuated and T cell activation is inhibited. **C.** Threshold model: In the absence of CTLA-4 (naïve cells and CTLA-4 knockout cells), the minimal requirement for T cell activation is very low. However, once CTLA-4 is induced (e.g., in previously activated cells or anergic cells), CTLA-4 is recruited to the immunological synapse where it increases the activation threshold either at the level of antigen specific (TCR) or co-stimulatory (CD28) signals.

synapse. However, PP2A also associates with CD28 *(30)*, and is considered an activating molecule in T cell costimulation, possibly by dephosphorylating actin cytoskeleton–associated molecules *(32)*. Thus, although CTLA-4 appears to effect early events in TCR signaling within the immunological synapse, the role of protein phosphatases in CTLA-4–mediated T cell inhibition remains unclear.

CTLA-4 appears to function at various stages of immune activation. CTLA-4–dependent blockade of TCR signaling effects the induction of tolerance following exposure to soluble antigens *(33)*, reverses tolerance in experimental models of autoimmunity, and inhibits effector T cell function *(34)*. In this regard, it is interesting to note that CTLA-4 is constitutively expressed on certain T cell subsets. Although naïve T cells do not express CTLA-4, memory $CD4^+$ T cells do express low levels of the protein intracellularly *(35)*. Moreover, both regulatory and tolerant T cells appear to express CTLA-4, and treatment of these cells with CTLA-4 mAbs long after antigen exposure can reverse tolerance. Thus, CTLA-4 might either inhibit T cell activation by increasing the threshold for productive T cell signaling activation, or attenuate ongoing immune responses by "putting the brakes" on the activation process.

During a primary T cell response to antigen presentation or during sustained T cell activation, CTLA-4 may participate more as an attenuator than as a threshold regulator because little CTLA-4 is expressed on the surface of these cells (Figure 1B). This idea is supported by several recent findings. In one study, more CTLA-4 protein was expressed at the cell surface of proliferating T cells as compared to resting cells *(36)*. Additionally, Egen and Allison showed that stimulation of T cells by a strong agonistic peptide caused the enhanced accumulation of CTLA-4 at the immunological synapse, where CTLA-4 inhibited further T cell activation *(11)*.

By comparison, CTLA-4 is likely to increase the threshold for T cell activation under conditions where CTLA-4 is already present at the immunological synapse (Figure 1C), for example, in recently activated memory T cells, tolerant T cells, regulatory T cells, or upon continued T cell stimulation. Once T cells are activated through antigen presentation, CTLA-4 becomes constitutively expressed, thus setting a signal threshold for

secondary activation. In this case, the very small fraction of CTLA-4 molecules constitutively found on the surface of T cells blocks weak activation signals. Thus, the association of CTLA-4 at the immunological synapse in memory T cells or tolerant T cells may increase the signaling threshold for T cell activation by modulating either antigen-dependent (TCR) or -independent (CD28) signal transduction at the cell surface. The uncontrolled polyclonal expansion of autoreactive T cells in CTLA-4 knockout mice strongly suggests that CTLA-4 may increase the signaling threshold for activation to prevent the activation of T cells which have weak affinity to self ligands. In this context, the threshold will be set by the surface availability of CTLA-4, which is determined by the total amount and the spatial localization of CTLA-4 within one T cell.

When all the data are examined together, a picture unfolds suggesting that indeed the inhibitory function of CTLA-4 is not likely to be strictly a consequence of competition with CD28 for ligands, or inhibition of distal signaling cascades, but rather a very early event proximal to the signaling events following TCR engagement with antigen–major histocompatibility complexes (MHC). However, future studies of CTLA-4 regulation of T cell functions will be needed to gain better understanding of immune cell homeostasis as well as new insights for therapeutic manipulation.

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Transactivation of the EGF Receptor: Is the PDGF Receptor an Unexpected Accomplice?

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The transactivation of the cytoplasmic tyrosine kinase domain of the epidermal growth factor receptor (EGFR) by heterologous signals has become an emerging theme in the complex field of

receptor-mediated signal transduction *(1)*. Evidence for both direct and indirect EGFR transactivation mechanisms has been proposed, thereby expanding the traditional view of highly specific receptor–ligand interactions. In fact, current data now suggest a wealth of signals impinging on the EGFR, making this receptor a central player in cellular responses.

Initially, the EGFR was found, using gradient centrifugation and chemical cross-linking techniques, to form specific, ligand-dependent, homodimers *(2, 3)*. Several years later the concept of heterodimers between EGFR family members was promulgated and proven *(4)*. This mechanism adds complexity to the family's signaling output. EGFR and HER2–4 can be detected in heterodimer complexes, each pair activated by one of multiple ligands in the EGF and neuregulin families. HER2, the one family member without a true direct ligand is the most promiscuous and the preferred heterodimerization partner of every other family member. By heterodimerizing with EGFR, HER3, or HER 4, HER2 can alter their signaling output and duration *(5, 6)*. Biological proof of the concept of heterodimerization was gained from mice deficient in HER2, HER4, or their ligand heregulin, which produced the same embryonic lethal phenotype: a failure in a specific step of cardiac development *(7, 8)*.

However, recent data have produced another surprise. The EGFR itself responds to a host of signals from outside the receptor-ligand family *(1)*. EGFR transactivation has been detected using a wide variety of G protein–coupled receptor agonists, phorbol esters, cytokines, chemokines, estrogen, and cell stress signals *(1, 9)*. When tyrosine becomes phosphorylated by these alternative pathways, EGFR behaves in a manner indistinguishable from that of activation by exogenous addition of EGF family ligands (Figure 1). EGFR transactivation has also been demonstrated through other receptor tyrosine kinases, including the insulin-like growth factor-1 receptor (IGF1R) and the platelet-derived growth factor receptor beta (PDGFβR) *(10, 11)*. Thus, crosstalk between receptor tyrosine kinase families provides yet another mechanism for diverse stimuli to tap into unique EGFR signaling and associated mitogenic pathways.

Saito et al. highlight the potential role the PDGFβR tyrosine kinase *(11)* plays in EGFR transactivation, and suggest a novel mechanism for these effects—there is historical precedence for these findings. Over a decade ago, PDGF was shown to stimulate the phosphorylation of serine residues on the EGFR. This effect, termed "transmodulation," serves to decrease the binding affinity of EGF to EGFR *(3, 12, 13)*. More recently, PDGFβR and EGFR were isolated from caveolae-containing membrane fractions, providing more evidence of "guilt by association" *(14, 15)*. Also, Habib et al. demonstrated a ligand-independent association of EGFR and PDGFβR when both were transfected into Cos-7 cells *(16)*. Interestingly, these researchers reported EGF-stimulated increases in PDGFβR tyrosine phosphorylation, in addition to the PDGFβR-dependent EGFR phosphorylation shown by Saito et al. and others *(11)*.

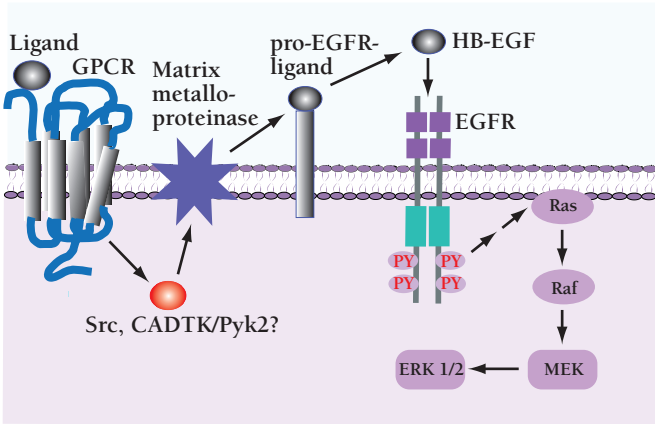

Figure 1. Signaling model of EGFR transactivation by crosstalk with a G protein–coupled receptor (GPCR). A mechanistic signaling model is described for heterologous GPCR coupling to an EGFR-dependent activation of the Ras/mitogen-activated protein kinase (MAPK) pathway (1). GPCR ligand-specific binding activates GPCR-Src– or calcium-dependent tyrosine kinase- (CADTK, or Pyk-2) –dependent intracellular signals that promote the release from the cell surface of pro-HB-EGF through the matrix metalloproteinase-mediated proteolytic cleavage of this and other EGF-like growth factor precursors. Free, active HB-EGF binds to EGFR, leading to transactivation of the EGFR.

However, the first demonstration of functional EGFR-PDGFβR receptor cooperation was observed using murine B82L fibroblasts *(17)*. The effects of PDGF on fibronectin-induced migration were enhanced by coexpression of a kinase-competent EGFR. Enhanced motility correlated with PDGF-stimulated EGFR tyrosine phosphorylation and was prevented by expression of a catalytically inactive or truncated EGFR. This PDGF-dependent EGFR activation was necessary to increase cellular motility. Similarly, He et al. showed that the PDGF-dependent stimulation of the p21-activated kinase (PAK) also required a functional EGFR *(18)*. Selective inhibition of the EGFR (with the chemical inhibitor AG1478) or the use of EGFR-deficient cells abolished the activation of PAK, indicating that PAK may be responsible for PDGF-dependent, EGFR-induced cell motility.

The current study of Saito et al. extends those observations showing parallel, PDGF-stimulated phosphorylation of both PDGFβR and EGFR in vascular smooth muscle cells *(11)*. The investigators used crosslinking reagents to stabilize and detect PDGFβR–EGFR "heterodimers;" this heterologous receptor interaction was ligand- and phosphorylation-independent. Surprisingly, whereas PDGF triggered EGFR tyrosine phosphorylation, the kinase activity of PDGFβR was dispensable for this effect. Incubation with antioxidants [e.g., *N*-acetylcysteine or Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid)] or a Src inhibitor (e.g., PP2) disrupted the constitutive PDGFβR–EGFR association, suggesting there may be unidentified factors involved in mediating heterologous receptor association. Lastly, inhibition of PDGF-dependent EGFR transactivation resulted in a partial diminution of extracellular signal-regulated kinase (ERK) activation, strengthening the biological relevance of EGFR

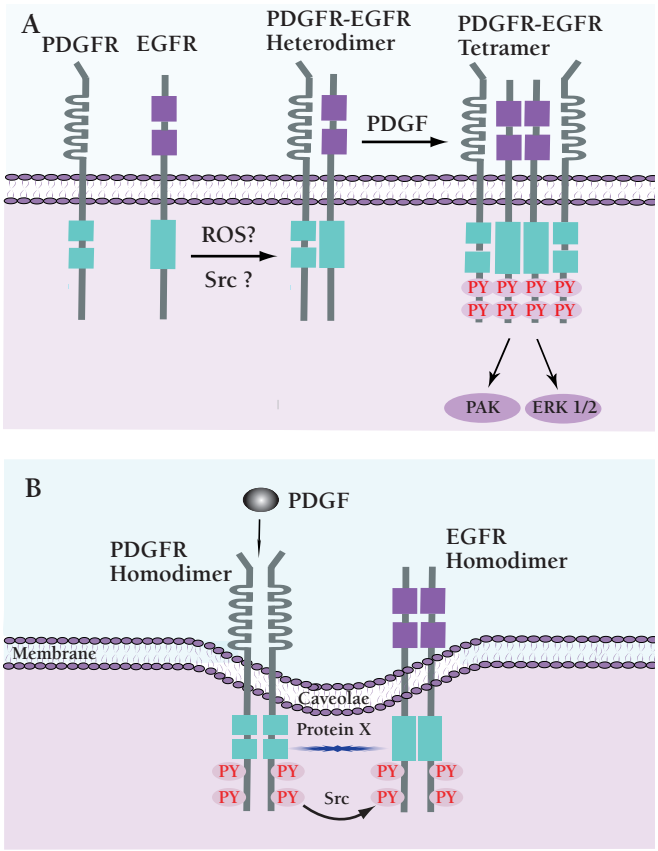

Figure 2. Mechanism of PDGF-induced EGFR transactivation. A. Model of basal PDGFβR-EGFR heterodimer formation and signal transduction. As proposed by Saito et al., PDGFβR-EGFR heterodimers exist in unstimulated cells *(11)*. The formation of the heterodimer provides a scaffold for other molecules that induces EGFR transactivation and downstream signaling. The constitutive interaction between PDGFβR and EGFR is regulated by ROS and Src family kinases. **B.** Alternative mechanism of EGFR transactivation in caveolae. Caveolae are the principal location of PDGFβR *(14)*. Upon PDGF binding to PDGFβR, EGFR is activated by Src-mediated phosphorylation prior to the formation of EGFR homodimers. The heterodimers between EGFR and PDGFβR may bind each other indirectly as a part of a large cross-linked complex involving unknown proteins.

transactivation.

The process by which EGFR is transactivated is potentially very important with regard to normal physiology and carcinogenesis; however, the mechanisms of transactivation are numerous, and there are many unresolved issues regarding hormone, cytokine, and PDGF-dependent EGFR activation. The model proposed by Saito and coworkers suggest that a Src- or reactive oxygen species-(ROS)-dependent constitutive heterodimerization of the PDGFβR–EGFR exists and facilitates PDGF-stimulated increases in EGFR tyrosine phosphorylation (Figure 2A). However, although the data on PDGF, Src, or ROS-dependent transactivation of the EGFR and the biological consequences appear firm *(11)*, formal acceptance of constitutive, specific EGFR–PDGFβR heterodimers requires additional experimental proof. The coexistence of PDGFβR and EGFR in caveolae-controlling microdomains, their association with cytoskeletal elements, and the chemical nature of the cross-linking experiments allow for the possibility that the complex is large, indirect, and may depend upon other non-receptor proteins (Figure 2B). These caveats leave open the specificity of PDGFβR–EGFR association but not the transactivation consequences.

The fact that transactivation required PDGF but not the kinase activity of PDGFβR is fascinating. How does the PDGFβR affect EGFR in the absence of its kinase activity? The authors suggest that pre-existing PDGFβR–EGFR heterodimers are present. Does PDGF simply facilitate EGFR homodimerization and autophosphorylation in large receptor oligomers? The involvement of Src or the antioxidant-sensitive step in this process may provide another explanation. The authors suggest that active Src is required for maintenance of the PDGFβR–EGFR heterodimer, possibly through site-specific EGFR phosphorylation. However, this would require a constant level of receptor phosphorylation in the unliganded state. Others have identified EGFR Tyr^{845} as the site of Src-activating stimuli in B82L fibroblasts *(19)*. Therefore, instead of Src maintaining heterodimers between two different receptor tyrosine kinase families, perhaps PDGFβR, with its known interaction of Src, can be induced to activate Src-mediated EGFR tyrosine phosphorylation without the requirement for PDGFβR tyrosine kinase activity. In other words, PDGFβR homodimers, even without tyrosine kinase activity, might direct Src to EGFR. Antioxidants may prevent ROS-dependent Src activation. Ultimately, experiments involving kinase-inactive versions of the PDGFβR, $Src^{-/-}$ cells, or other chemical techniques, will provide additional means by which to examine putative PDGFβR–EGFR heterodimers and insight into the mechanism of this complex and important regulatory schema. In addition, other kinases must still be considered as indirect participants in EGFR transactivation by PDGF.

Other indirect routes of EGFR activation have been identified. One prevailing model for EGFR transactivation by G protein–coupled receptors or other signals involves a phosphatidylinositol-3' kinase (PI3K), Src, or calcium-dependent tyrosine kinase- (CADTK, or Pyk2)-dependent activation of an ADAM family metalloproteinase *(20, 21)*. These membrane-based metalloproteinases can cleave membrane-anchored growth functions including the EGFR ligands heparin bound-(HB)-EGF and tumor growth factor alpha (TGF-α). The ligand is released from its membrane anchor and thereby provides autocrine or paracrine activation of the EGFR *(22–24)* (Figure 1).

So what is the motive for this seemingly indiscreet collaboration—EGFR transactivation by multiple signals? As pointed out by Li et al., the combination of these signals may facilitate unique aspects of mitogenesis and migration. Because the EGFR contains a specific actin-binding motif not found in the

PDGFβR *(25, 26)*, coactivation of these EGFR-containing heterodimeric receptors may enhance the ability of PDGF to mediate cell motility. In addition, the cooperative PDGFβR–EGFR–dependent activation of PAK may further modulate actin dynamics that are necessary for cell migration *(18)*. Lastly, many experiments in the "early days" of growth factor research indicated that cells need twelve to twenty-four hours of serum stimulation in order to traverse the cell cycle *(27–29)*. In defined cell culture media that contain more physiologically relevant doses of growth factors, a requirement for distinct signals from each of the PDGFβR, EGFR, and IGFR was demonstrated for cell cycle traversal. However, high doses of PDGF can overcome the need for EGF. Perhaps EGFR transactivation by one of the multiple mechanisms noted above short-circuits the need for exogenous EGF ligands (see Figures 1 and 2). Whatever the mechanism, the phenomenon of EGFR transactivation has been reported in some cell systems and is likely to have a basis in the physiology of growth control, tissue formation, or stromal epithelial interaction. Whatever the purpose (and mechanism), further investigations will no doubt result in other intriguing relations.

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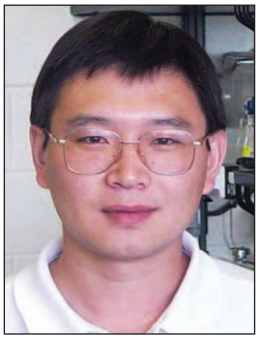

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IRAK-4: A New Drug Target in Inflammation, Sepsis, and Autoimmunity

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The cytokines interleukin-1 (IL-1) and IL-18 are important inflammatory mediators of such diseases as rheumatoid arthritis and inflammatory bowel disease. Their receptors, IL-1R–IL-1RAcP (IL-1R accessory protein) and IL-18R–IL-18RAcP, belong to a broader family of receptors that includes the Toll-like receptors (TLRs). This family is defined by a conserved cytosolic region termed the Toll-IL-1 receptor (TIR) domain. TLRs, crucially involved in the innate immune response to microbes, sense pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), peptidoglycan, dsRNA, and CpG DNA, which are recognized by TLR4, TLR2, TLR3, and TLR9, respectively. Additionally, TLR4 recognizes products of inflamed tissues such as the 60-kDa heat shock protein (HSP60).

Tremendous progress has recently been made in our understanding of how this family of receptors transmits signals. The TIR signaling system is attractive from a drug targeting point of view, given the role of IL-1 and IL-18 in inflammatory diseases, and the implication of TLRs in sepsis, autoimmunity (specifically TLR4 and TLR9), and possibly, atherosclerosis.

Recently, a key breakthrough has been made in the area of IL-1 and TLR signaling with the discovery of IRAK-4, which is

recruited to receptor TIR domains and appears to be the initiating kinase in the signaling pathways activated following stimulation *(1, 2)*. These receptors mediate cellular stimulation that culminates in the activation of well-known inflammatory signals such as the transcription factor NF-κB and stress-activated kinases, both of which are important drug targets with several inhibitors in Phase I clinical trials.

The TLR signaling pathways are strikingly complex. Ligand binding to the extracellular domain of TIR domain–containing receptors results in the recruitment of the soluble adapter molecule MyD88. The C-terminal TIR domain of MyD88 interacts with the receptor complexes, whereas the conserved death domain (DD) in its N terminus associates with the corresponding domains of IL-1R-associated kinases (IRAKs), homologs of the Drosophila protein kinase Pelle. Until recently, three mammalian members of this Ser-Thr kinase family were known: IRAK-1 *(3)*, IRAK-2 *(4)*, and IRAK-M *(5)*. IRAKs characteristically contain a DD in their N terminus, and a central kinase domain followed by a unique 90 to 170-residue C-terminal domain. Under resting conditions, unphosphorylated IRAKs are bound to the Toll-interacting protein (Tollip) in the cytosol through a DD–DD interaction. In this cytosolic complex, IRAK is prevented from being phosphorylated prior to stimulation of the cell *(6, 7)*. The binding of IL-1 to IL-1R leads to the recruitment of IL-1RAcP, which in turn transiently recruits the Tollip–IRAK complex resulting in activation. Furthermore, Tollip can directly associate with TLR2 or TLR4 *(6)*. Kinetic studies suggest that the recruitment of MyD88 and Tollip–IRAK to the receptor occur independently *(7)*. Moreover, no association between Tollip and MyD88 has been demonstrated, indicating that these proteins associate with distinct domains on the receptor *(6)*. The initial, activating phosphorylation of IRAK at the receptor complex weakens its affinity for Tollip, consequently making it more accessible for interaction with MyD88 *(7)*. Neither Tollip nor MyD88 are able to maintain interaction with fully activated, hyperphosphorylated IRAK-1, but can only associate with the unphosphorylated form *(7, 8)*. Thus, the association of MyD88 with IRAK-1 is disrupted upon further phosphorylation of IRAK-1, which results in the release of IRAK-1 from the receptor complex. The successive aggregation of activated IRAK-1 with the soluble tumor necrosis factor-α (TNF-α) receptor–associated factor 6 (TRAF6) is the initial step for assembly of the signalosome and downstream activation of either NF-κB or stress-activated kinases.

The reported high turnover rate of IRAK-1 and Tollip can be ascribed to phosphorylation and subsequent ubiquitination *(6)*. Rapid degradation of activated IRAK-1 ensures tight regulation of cytokine expression in response to IL-1R–TLR-mediated signaling. As opposed to IRAK-1, the IRAK-2 protein level remains constant after LPS treatment, which is an interesting indication of divergent roles of these homologs in the host defense against microbes *(9)*.

IRAK-1 itself is an active kinase and undergoes autophosphorylation. However, overexpression of a catalytically inactive IRAK-1 mutant, and characterization of a kinase-inactive splice variant of IRAK-1 revealed that kinase activity is dispensable for IL-1 induced NF-κB activation *(10, 11)*. Although IRAK-2 and IRAK-M both lack key catalytic residues, thus rendering them kinase-inactive, they nonetheless are both capable of signaling *(4, 5)*. The only known heterologous substrate of IRAK-1 is Tollip; phosphorylation of Tollip in the activated receptor complex is thought to facilitate the dissociation of IRAK-1 from Tollip *(6)*.

The nature of the IRAK-1–activating kinase at the receptor complex was, until recently, unknown. The fact that a heterologously overexpressed IRAK-1 kinase-negative mutant can still be phosphorylated in an IRAK-1–deficient cell line argues against self-activation (i.e., through autophosphorylation) *(10)*. The recently discovered new member of the IRAK family, IRAK-4, appears to bridge the gap between the stimulated receptor complex and IRAK-1 activation. The kinase was discovered in a database search for IRAK homologs. It is the closest human homolog to the Drosophila Pelle, a key kinase in Toll signaling in Drosophila, with which it shares twenty-two percent amino acid sequence identity. IRAK-4 consists of the DD, a linker domain, and the kinase domain, but unlike the other IRAKs, IRAK-4 does not have a C-terminal extension *(1)*. IRAK-4 knockout mice are resistant to LPS-induced septic shock, suggesting that the TLR4 signal pathway—known to be activated by LPS—was disrupted. Furthermore, the mice were resistant to shock induced by CpG DNA, peptidoglycan, and the dsRNA mimetic polyI:C, indicating that IRAK-4 is essential in TLR9, TLR2, and TLR3 signaling, respectively. In addition, the IRAK-4 knockout mice did not produce detectable amounts of TNF-α or IL-6 after IL-1 injection. This unresponsiveness to IL-1 stimulation was also observed in *IRAK4*$^{-/-}$ embryonic fibroblasts; however, full responsiveness to administered IL-1 was regained after stably transfecting IRAK-4 into these cells. Virus-infected IRAK-4–deficient mice were unable to produce interferon-γ (IFN-γ); because IFN-γ is produced in an IL-18–dependent manner after virus infection, signal transduction through the IL-18R also appears to be impaired. Activation of the mitogen-activated protein kinases (MAPKs) p38 and c-Jun N-terminal kinase by IL-1 or LPS is prevented in *IRAK4*$^{-/-}$ cells. Suzuki et al. furthermore demonstrated that IL-1 but not TNFα-induced NF-κB activity is dependent on IRAK-4 *(1)*. Compared to the effects observed in IRAK-1 deficiency, the loss of IRAK-4 more severely affects TIR-mediated signaling *(2)*. Partial redundancy of IRAK-1 with IRAK-2 and IRAK-M can explain this phenomenon; none of these IRAKs appear to functionally replace IRAK-4 in the knockout mice. For example, in contrast to the overexpression of IRAK-2 or IRAK-M, the overexpression of IRAK-4 in *IRAK-1*$^{-/-}$ cells does not activate NF-κB, suggesting that IRAK-4 might function upstream of IRAK-1. In addition, IRAK-4 appears to lie downstream of MyD88 and its homolog, the MyD88 adapter-like (Mal) protein, which has been implicated in TLR4 signals *(12)*. IRAK-4 is the only IRAK that relies on its kinase activity for NF-κB activation—a kinase-negative variant of this protein acts as a dominant negative in IL-1 signal transduction, and prevents the

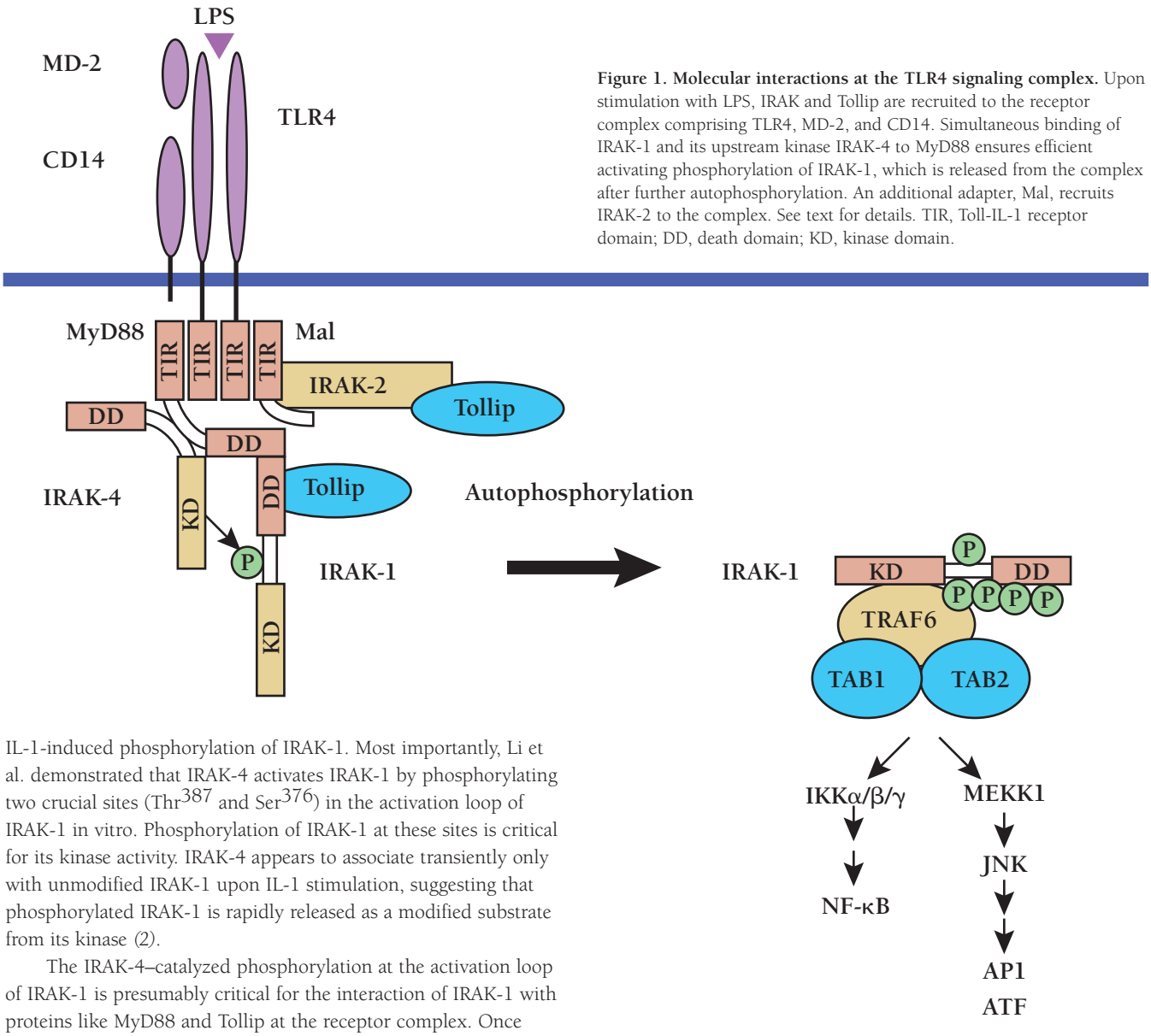

Figure 1. Molecular interactions at the TLR4 signaling complex. Upon stimulation with LPS, IRAK and Tollip are recruited to the receptor complex comprising TLR4, MD-2, and CD14. Simultaneous binding of IRAK-1 and its upstream kinase IRAK-4 to MyD88 ensures efficient activating phosphorylation of IRAK-1, which is released from the complex after further autophosphorylation. An additional adapter, Mal, recruits IRAK-2 to the complex. See text for details. TIR, Toll-IL-1 receptor domain; DD, death domain; KD, kinase domain.

IL-1-induced phosphorylation of IRAK-1. Most importantly, Li et al. demonstrated that IRAK-4 activates IRAK-1 by phosphorylating two crucial sites (Thr^{387} and Ser^{376}) in the activation loop of IRAK-1 in vitro. Phosphorylation of IRAK-1 at these sites is critical for its kinase activity. IRAK-4 appears to associate transiently only with unmodified IRAK-1 upon IL-1 stimulation, suggesting that phosphorylated IRAK-1 is rapidly released as a modified substrate from its kinase *(2)*.

The IRAK-4–catalyzed phosphorylation at the activation loop of IRAK-1 is presumably critical for the interaction of IRAK-1 with proteins like MyD88 and Tollip at the receptor complex. Once activated, IRAK-1 exhibits autophosphorylation and leaves the receptor complex in its hyperphosphorylated form to further associate with TRAF6.

Additional insights into the events at the activated IL-1R and TLRs have been gained in a study on MyD88s, an alternatively spliced form of MyD88 *(13)*. This truncated variant lacks the intermediate domain connecting the N-terminal DD with the C-terminal TIR domain. Its expression is carefully regulated, and it appears to be a natural repressor for IL-1 and LPS signaling by preventing IRAK-1 phosphorylation. However, IRAK-1 can still interact with MyD88s, but it does not become phosphorylated *(13)*. These observations imply that the MyD88 interdomain region, missing in MyD88s, is required for IRAK-1 phosphorylation possibly through the recruitment of IRAK-4. Simultaneous binding to distinct domains of MyD88 would bring IRAK-4 and its substrate IRAK-1 in close proximity and facilitate IRAK-1 phosphorylation. Thus, MyD88 could play an important role as a scaffolding protein in IL-1R–TLR signaling. A scheme describing TIR-dependent signaling is shown for TLR4 (Figure 1).

IRAK-4 is therefore the most receptor-proximal kinase so far described for IL-1, IL-18 and TLR signaling. Inhibiting its kinase activity would limit the pro-inflammatory effects of IL-1 and IL-18, and might also be beneficial in blocking chronic harmful TLR-dependent signaling in such conditions as sepsis or in autoimmunity.

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Act Locally: New Ways of Regulating Voltage-Gated Ion Channels

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In recent years, the modulation of ion channels by phosphorylation has been a major focus of research, and practically every category of channel possesses putative phosphorylation sites. Indeed, most channels are directly modified by kinases and phosphatases. Recent studies of the interactions of non-opioid sigma receptors with voltage-dependent ion channels suggest yet another mechanism of signal transduction that appears to be independent of phosphorylation. Sigma receptors were first characterized as opiate receptors based on their binding to the agonist SKF10047 *(1)*. However, this designation was abandoned when it was discovered that most other opiate receptor agonists and antagonists, including endogenous opiates, do not bind sigma receptors. In fact, characterizing sigma receptor binding became increasingly problematic, as pharmacological agents of very diverse chemical structure and receptor-selectivity were found to interact with sigma sites. To date, no endogenous sigma receptor ligands have been identified, although several categories of endogenous molecules can alter binding of canonical sigma receptor agonists. Candidate endogenous ligands include dopamine, serotonin, numerous steroids, and several peptides (most prominently neuropeptide Y)—their diversity further revealing the idiosyncratic binding properties of sigma receptors.

Indeed, the profligate binding of sigma receptors has made it difficult to ascribe functions to the receptor. Attributions range from higher-level cognitive processing to regulation of digestive, reproductive, and liver function. Nonetheless, sigma receptors are expressed in many tissues and probably play roles in several vital functions. Recently, several papers by Jackson and colleagues have elucidated a specific cellular function for sigma receptors in neuroendocrine cells of the pituitary gland. These findings not only offer to tie together the disparate proposed functions but,

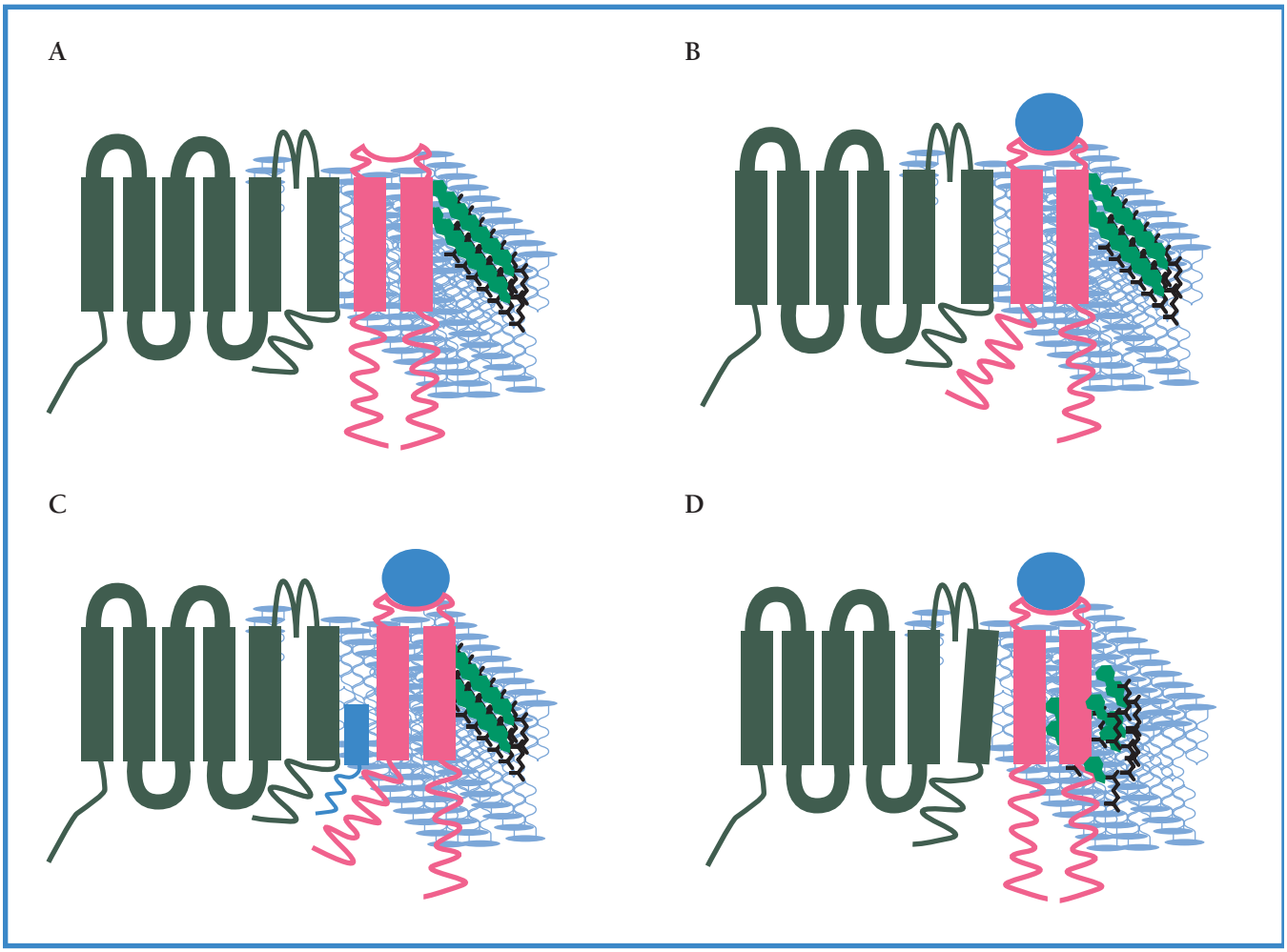

Figure 1. Putative models of sigma receptor modulation of ion channels. The plasma membrane is diagrammed to contain an idealized voltage-dependent ion channel (black) in close proximity to a sigma receptor (pink). The phopsholipid bilayer is depicted in blue and cholesterol molecules are green. When the unbound receptor and ion channel (as shown in **A**) binds to a ligand (blue circle), a conformational change occurs in the transmembrane or intracellular domains (or both) of the sigma receptor so that channel accessibility is either directly (as in **B**) or indirectly (as in **C**) regulated. Alternatively, ligand binding may result in local changes of cholesterol structure due to the activity of, or binding to, sterol isomerase-like sequences in the sigma receptors (**D**).

more interestingly, indicate a novel mechanism of cellular signal transduction. Originally, Wilke et al., using whole-terminal patch clamp in posterior pituitary slices, showed that the dopaminergic agonists (±)-2-(*N*-phenethyl-*N*-propyl)amino-5-hydroxytetralin (PPHT) and U101958 reduced two neurohypophysial potassium currents (*2*). Both drugs also bind to sigma receptors, and further studies with receptor-specific agonists and transgenic mice eliminated any role for dopamine in the effect on the pituitary currents. Additional work by the same group demonstrated that several other, non-dopaminergic, sigma receptor drugs (i.e., SKF10047, pentazocine, ditolylguanidine, haloperidol, and apomorphine) also reduced the neurohypophysial potassium currents (*3*). This specific and tractable cellular assay was then used to identify cellular signaling pathways that mediate sigma receptor effects. Evidence from earlier studies suggested that altering the function of G proteins could alter the binding of sigma ligands, although no unambiguous functional coupling between G proteins and sigma receptors was ever established (*4*). Using activators and inhibitors of G proteins as well as the non-hydrolyzable ATP analog AMPPcP, Lupardus et al. found the quite surprising and unexpected result that the sigma receptor–induced reduction of neurohypophysial potassium currents was independent of G protein activity and phosphorylation (*5*).

These studies suggest that the binding of sigma receptors to extracellular ligands could create a non-traditional cellular signal that alters the functions of ion channels. That this effect occurs locally in the membrane was demonstrated by comparing the efficacy of sigma agonists on channels in different patch conformations. For example, bath-applied SKF10047 reduced the current in channels from excised patches in the "outside-out" conformation, where bath-applied ligand is available directly to the extracellular binding domain of the receptor but cytoplasmic signaling intermediates are not available to the intracellular domains of the receptor. This occurred even when AMPPcP was included in the pipette to eliminate the possibility that small amounts of cytoplasm taken up during patch excision could

mediate the effect. Furthermore, when channels in a small patch of membrane were isolated from the bath but still in contact with cytoplasmic signaling molecules (the cell-attached patch conformation), bath-applied sigma agonists had no effect on the channels in the patch. Thus, the receptor and the channel are in close proximity and do not require any soluble factors for signal transmission *(5)*. In oocytes, heterologously expressed sigma receptors immunoprecipitate with the voltage-dependent potassium channels Kv1.4 and Kv1.5 *(6)*, further suggesting that sigma receptors and potassium channels interact directly or through closely associated intermediates. Like the two pituitary potassium currents, both channels also show reductions in peak current amplitude when coexpressed with sigma receptors and treated with sigma agonists. Furthermore, GFP-labeled sigma receptors are expressed at the cell surface where they can interact directly with coexpressed potassium channels. Thus, in response to extracellular signals, sigma receptors are capable of modulating channel function by interacting either with channel proteins or some very closely associated membrane constituent.

The most interesting unresolved questions from the new results on sigma receptors are concerned with the mechanism of signal transduction: How do sigma receptors act on ion channels to reduce current? The observations to date present several constraints. In addition to the lack of involvement of G proteins and kinases or phosphatases, the interaction occurs either within the membrane or among components that are closely associated with the inner surface of the membrane and does not include any diffusible second messengers. In spite of the close spatial requirement, it still is not clear whether a direct interaction exists between the sigma receptors and the ion channels. Given these constraints, receptor activation from the unliganded, resting state (Figure 1A) could result in a steric alteration of the receptor molecule that either allows or alleviates a specific interaction with a channel or a closely associated intermediate (Figure 1B and 1C). Intermediates could include ion channel β subunits or other components of the membrane such as lipids or cholesterol.

Another intriguing possibility arises from the amino acid sequence of sigma receptors *(7)*. The sigma-1 receptor, which is the isoform used in the studies of potassium channel regulation described above, shares some sequence similarity with sterol isomerases, particularly the yeast ergosterol biosynthetic enzyme ERG2 *(8, 9)*. Sterol isomerases catalyze the formation of cholesterol suggesting that cholesterol-rich membrane microdomains, such as lipid rafts, may be altered by activated sigma receptors. Although sigma receptors fail to rescue yeast ERG2 lethal mutants *(8)*, this might be explained by differences in yeast and mammalian sterol substrates, and cannot be used to definitively rule out sigma-mediated sterol isomerization in mammalian cells. It will be of considerable interest to determine if sigma receptors possess endogenous sterol isomerase activity with mammalian substrates. Interestingly, Kv1.5, one of the channels shown by Aydar et al. to be a target of sigma receptors *(6)*, is also associated with cholesterol-rich lipid rafts, and cholesterol depletion dramatically alters kinetic properties of Kv1.5 *(10, 11)*. Even if sigma receptors no longer retain isomerase activity, they may still retain the capacity to bind to cholesterol (Figure 1D) or its precursor, and thereby alter the organization of lipid domains within the plasma membrane as has been suggested in other raft-associated signaling systems *(12)*.

Sigma receptor modulation of potassium channels presents cellular biologists with a novel mechanism of signal transduction that is reminiscent of, but not identical to, the gating of the cardiac acetylcholine-regulated inward rectifier. Subsequent to ligand binding at muscarinic sites and the dissociation of G_i, potassium channels in heart cells are altered by a direct interaction with the βγ subunits (rather than with the GTPase-containing α subunit) *(13)*. Indeed, the mechanism proposed (Figure 1B) may reflect a similar type of protein–protein interaction between membrane-associate regions of sigma receptors and potassium channels as that observed to occur between βγ subunits and the delayed rectifier. The difference is that unlike G proteins, sigma receptors are not intermediates. Instead, the sigma receptor makes its own direct link from the extracellular ligand-binding event to a change in activity of a cellular target.

Are there other examples of the sigma type of signal transduction to be found? A number of proteins can interact with ion channels and possess extracellular domains of unknown function. These include the β subunit of the Slo Ca^{2+}-dependent K^+ channel *(14)*, and the small transmembrane protein minK which acts a subunit for some voltage-dependent potassium channels *(15)*. Although neither of these examples are known to possess extracellular binding partners, the new finding on sigma receptors has refined the theoretical framework for elucidating their mechanisms of action. More broadly, the sigma receptor form of signal transduction suggests that cells also have unique ways of responding within local microdomains. For example, although activation of traditional signaling pathways (i.e., utilizing kinases and phosphatases), can invoke global cellular activation such as the recruitment of new gene expression, it is undoubtedly beneficial for cells to respond to locally available cues only in the region of the cell exposed to the altered environment. This is especially relevant for large, complex cells that extend cellular domains over vast areas (e.g., neurons, glia, dendritic cells, fibroblasts) and for migratory developing cells that must respond to local guidance cues. Thus, it will be important to determine whether the functions of sigma receptors extend to other membrane proteins other than ion channels, and it will be of even more significance to find other local signal transducers.

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