Pulse-Chase in the Light Microscope

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To visualize specific proteins in living cells, cell biologists have fused the green fluorescent protein (GFP) or its analogs to the C termini of proteins and expressed these chimeras in culture and in embryos *(1–3)*. By using fluorescent proteins with different spectral properties *(4–6)*, researchers can follow exogenous proteins in real time and map their cellular locations and interactions.

These techniques have been complemented by the development of methods that permit the fluorescent labeling of recombinant proteins containing a tetracysteine sequence CCXXCC (where X is any amino acid). Proteins carrying this motif within an α-helix can be stained in vivo with the nonfluorescent, membrane-permeant biarsenical derivative of fluorescein, FlAsH-EDT_2 [4',5'-bis(1,3,2-dithioarsolan-2-yl)fluorescein-(1,2-ethanedithiol)2]. FlAsH-EDT_2 binds with high affinity (K_d ~ 10 pM) to the tetracysteine motif, resulting in strong green fluorescence *(7)*. Key advantages of this method are the smaller size of the tetracysteine tag, relative to the bulky ~27-kDa GFP moiety, and the possibility of pulse-labeling the tagged proteins.

Gaietta et al. *(8)* have developed a further variation on the tetracysteine theme. In addition to the FlAsH-EDT_2, they have synthesized ReAsH-EDT_2, a biarsenical derivative of the fluorophore resorufin, which, when bound to the tetracysteine tag, emits a red fluorescence. Gaietta et al. *(8)* use these two reagents to label tetracysteine motif–tagged intracellular proteins, at spaced temporal intervals, permitting a visual pulse-chase in living cells. The method is especially useful because the ReAsH-EDT_2 reagent is not only fluorescent, but also can be used, by photoconverting diaminobenzidine (DAB) to a highly insoluble reaction product, to visualize tagged proteins by electron microscopy *(9)*.

Gaietta et al. *(8)* demonstrate the power of their novel method in a study of the gap junction protein connexin43 (Cx43), stably expressed as a tetracysteine-containing, recombinant protein in HeLa cells. The choice of Cx43 in gap junctions as a test specimen is particularly appropriate: Cx43 has a rapid half-life [1.5 hours *(10)*] and the protein accumulates at very high concentration in maculae at cell–cell appositions, greatly facilitating its visualization at the cell surface. Using GFP and its cyan (CFP) and yellow (YFP) color variants, Falk *(11)* showed that the products of different connexin-encoding genes can either co-mingle or segregate within individual plaques. In the stably transfected HeLa cells, the high expression of Cx43 favors the assembly of unusually large gap junctions, resulting in dramatically clear images as observed through the confocal light microscope. Gaietta et al. *(8)* first pulse-label tetracysteine-containing Cx43 in HeLa cells with FlAsH-EDT_2. Following different chase times, newly synthesized, unlabeled Cx43 is then labeled with ReAsH-EDT_2, such that the old Cx43 pool fluoresces green and the newly synthesized pool, red. The authors show by confocal microscopy that newly synthesized Cx43 is added to gap junctional maculae from the edges; the older Cx43 gradually disappears from a central domain within each macula (Figure 1). These images are consistent with biochemical studies demonstrating that newly synthesized connexons (half-gap–junctional intercellular channels) are first inserted into non-junctional plasma membrane prior to their assembly into whole intercellular channels within the maculae *(12)*. The images presented by Gaietta et al. provide strong evidence for lateral diffusion of the connexons in the plasma membrane in order to accrete at the edges of pre-existing gap junctions.

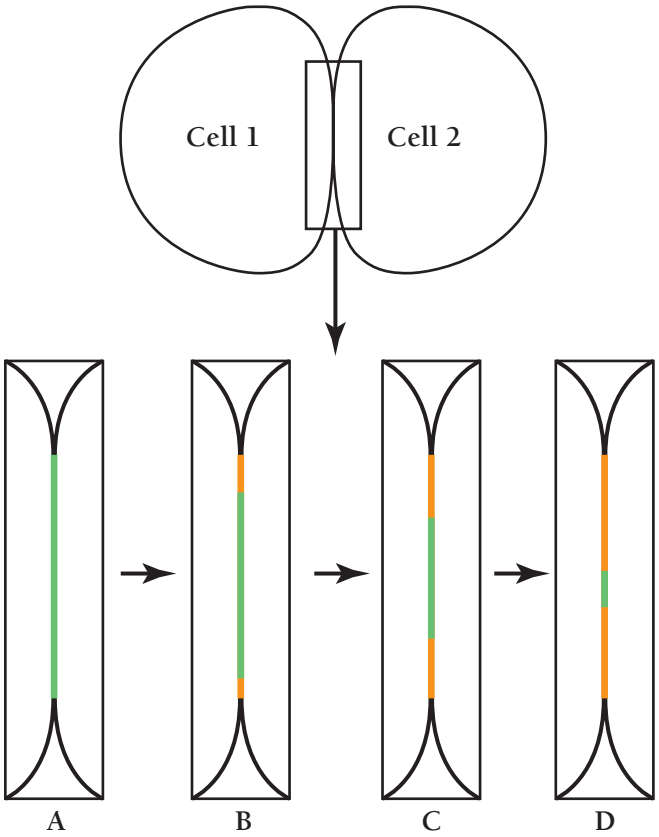

Figure 1. A diagram summarizing the visualization of pulse–chase of connexin 43 (Cx43) in gap junctions. Two cells are diagrammed; the boxed area of cell–cell interaction is enlarged in panels A–D. **(A)** Cells transfected with tetracysteine-tagged Cx43 are labeled with FlAsH-EDT_2, which stains the gap junction fluorescent green. Following a wash, to remove excess FlAsH-EDT_2, cells are treated with ReAsH-EDT_2 to label all newly synthesized Cx43 fluorescent red. **(B–D)** Over time, newly synthesized Cx43 (red) replaces pre-existing Cx43 (green) by accretion at the edges of the gap junction with concomitant removal of connexons from the center.

Gaietta et al. *(8)* further present data that exploit the ability to visualize ReAsH-EDT_2 in the electron microscope. In one study, they first observe a gap junction by confocal microscopy. Then, subsequent to the photoconversion of DAB, the same junction is imaged using electron microscopy. The two imaging strategies were thus exhibited as equivalent. By utilizing the increased resolution

inherent in electron microscopy on these overexpressing HeLa cells, they also detect newly synthesized Cx43 in small, putative post-Golgi transport vesicles [where Cx43 oligomerization is thought to occur *(13)*]. In addition, by reversing the staining order of the two reagents, senescent Cx43 in endocytic vesicles and secondary lysosomes can be clearly distinguished.

A powerful extension of the technique would be to use fluorescent resonance energy transfer (FRET) to monitor protein–protein interactions. In this approach, energy from one fluorescent molecule (the donor) is transferred by dipole–dipole interactions to a second molecule (the acceptor), provided that the two molecules are in a correct spatial orientation within 100 Å *(14, 15)*. This methodology has been used successfully by co-expressing GFP or its the cyan (CFP) or yellow (YFP) variants *(16–18)*. FRET could also be experimentally exploited by combining the CFP with FlAsH-EDT_2 fluorophores. As a proof of concept, the tetracysteine-containing α-helix was fused directly to the C terminus of CFP and the resulting protein was modified with FlAsH-EDT_2. The resultant complex displays FRET from CFP to FlAsH-EDT_2 *(7)*. The detailed time and spatial resolution achievable with the FlAsH-EDT_2 labeling, combined with FRET, would potentially permit detection of transient protein–protein interactions and pinpoint these events within a specific intercellular compartment.

There are a few limitations to the method in its current form. First, although the authors engineered the tetracysteine motif into α-helical secondary structures, it is clear that the dyes can bind to endogenous cysteine-containing proteins. Cell types other than HeLa may show considerable background signals, limiting the method to only those proteins expressed at very high levels *(19)*. It thus remains to be seen whether these probes can be effectively used in other cell types, or whether the method will be applicable to tetracysteine-tagged recombinant proteins expressed in transgenic animals. As seen in the study by Gaietta et al. *(8)*, non-junctional connexons *(12)* are not visualized; only those connexons collected at high density in gap junctional maculae or in intracellular vesicles are visible. Thus, this method may not be applicable to study membrane proteins found at lower densities at the cell surface. Other proteins that do accumulate at high concentration in specific sites, such as in desmosomes, microtubules, actin and intermediate filaments, focal adhesions, chemical synapses, and tight junctions, will be readily amenable for similar studies, and the new approaches pioneered by Gaietta et al. *(8)* should provide some spectacular images in the near future.

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The Near-Death Experience of Delta Opioid Receptors Leads to New Drug Targets

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The regulation of receptor expression at the cell surface is partially controlled by the endoplasmic reticulum (ER), which forms part of the secretory pathway. The ER is thought to process receptors constitutively and serve a passive role in signal transduction pathways, and as such, is not thought of as a target for direct therapeutic intervention. However, Petäjä-Repo and colleagues have challenged this assumption by demonstrating that cell-permeant pharmacological agents can interact directly with delta opioid receptors in the ER and rescue them from the degradative pathway.

The ER provides an environment that ensures the correct assembly and folding of proteins. Chaperones within the ER monitor the structure of newly synthesized proteins by recognizing properties, such as exposed hydrophobic sites, that distinguish misfolded proteins from their conformationally correct counterparts *(1–3)*. Proteins are sorted within the ER according to their folding and maturation status through a quality control process *(3)*. Folded proteins move relatively rapidly from the ER—aided by the exposed amino-acid sequence motifs on protein surfaces that direct their sorting—into nascent COPII-containing vesicles that evaginate and bud off from the ER *(1)*. Misfolded and unassembled proteins either aggregate or become degraded. The process of ER–associated protein degradation directs the movement of proteins from the ER to the cytosol where misfolded proteins are ubiquitinated and degraded by proteasomes *(4)*.

One of the most common examples of a disease resulting from protein misfolding is cystic fibrosis. Mutations in the cystic fibrosis transmembrane conductance rectifier (CFTR) gene can lead to misfolding that compromises the trafficking of CFTR through the ER, and destines the mutant protein for proteasome-directed proteolysis *(5)*. Chemical chaperones can rescue misfolded CFTRs retained in the ER *(6)*, and pharmacological chaperones, such as vasopressin 2 (V2) antagonists can rescue mutant V2 receptors normally retained in the ER *(2)*. Petäjä-Repo and colleagues now show that pharmacological chaperones can also rescue wild-type human delta opioid receptors (hδORs) *(7)*.

The hδOR is very inefficiently processed, with approximately forty percent of newly synthesized receptor reaching the cell surface. Petäjä-Repo et al. had previously proposed that this receptor is unable to efficiently adopt the correct conformation, leading to its targeted degradation *(8)*. Using pulse-chase labeling experiments, Petäjä-Repo et al. now demonstrate that cell-permeant opioid ligands interact with the intermediately folded precursors of the hδOR located on the surface of the ER *(7)*. This treatment rescues the receptors from the degradative pathway and results in more receptors available for signaling at the cell surface. Both lipophilic opioid antagonists and agonists were able to rescue hδOR from the ER, suggesting that either ligand class could induce a more stable receptor conformation. Naltrexone treatment led to a twofold increase in the amount of mature (M_r ~ 55-kDa) hδOR at the cell surface, while a concomitant decrease in the half-life of the core glycosylated precursor of hδOR (M_r ~ 45-kDa) was observed at the ER. Additionally, naltrexone was able to rescue precursor hδOR that had been retained in the ER for up to four hours. It is provocative to note that ligands such as naltrexone function intracellularly at receptors located within the ER: cell-impermeant ligands, such as [Leu]enkephalin, had no effect on the trafficking of the mature receptor to the cell surface. Thus, the transport of receptors out of the ER using cell-permeant ligands may provide a new target for therapeutic interventions aimed at

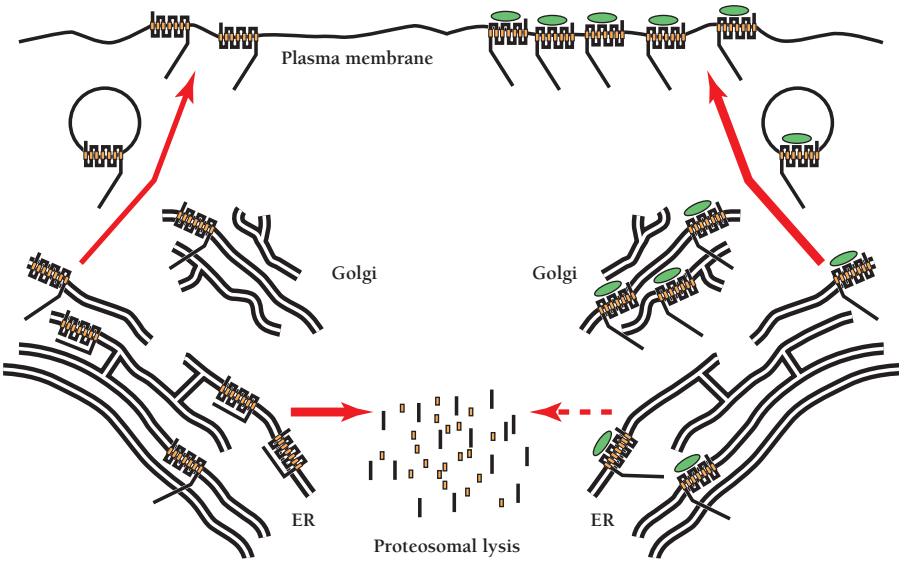

Figure 1. The fate of newly synthesized hδORs. On the left, receptors (represented by seven-transmembrane–spanning figures with yellow transmembrane domains) under endogenous conditions whereby only forty percent of the receptors reach the plasma membrane; the remaining sixty percent are retained in the ER, and are exported to the cytosol for proteasomal degradation. On the right, hδORs in the presence of a lipophilic hδOR ligand (represented by green-filled ovals) are processed through the ER more efficiently, so that fewer receptors are degraded and there is a concomitant increase in surface expression of receptor.

modulating receptor expression on the cell surface. Previously, it was not known why the chronic administration of opioid antagonists results in the greatly increased expression of the mu opioid receptor *(9–11)*. Petäjä-Repo and colleagues now hypothesize that increased surface expression results from the "pharmacological chaperone" properties of the lipophilic opioid antagonists.

The precise mechanism used by the ligands to rescue the precursor receptors is unknown; however, Petäjä-Repo and colleagues hypothesize that the cell-permeant ligands bind to the receptor precursors and stabilize conformations within the hydrophobic core of the protein. All cell-permeant ligands tested, which included opioid agonists and antagonists, were able to rescue hδOR, suggesting that active and inactive receptor conformations can be exported by the ER quality-control system.

Receptor heterodimerization in the ER is important for the formation of functional γ-amino butyric acid subtype B ($GABA_B$) receptors at the cell surface *(12)*. On the other hand, a mutant CCR5 chemokine receptor that is retained in the ER can inhibit the exit of the wild-type CCR5 receptor *(13)*. Opioid receptors exist as both homo- and heterodimers *(14)*. Thus, perhaps pharmacological chaperones work by enhancing dimer formation within the ER. Further studies will be required to investigate this possibility.

The signaling capacity of the rescued receptors at the cell surface remains to be investigated. A number of other questions also arise: Are the ligand-bound receptors able to signal within the ER itself? Are there ligands that can destabilize receptors and effect their degradation from the ER rather than from the plasma membrane? Can pharmacological chaperones be designed that facilitate receptor maturation without interfering with binding of endogenous ligand once the receptor had arrived at the surface? It will also be important to establish whether pharmacological chaperones will work in neurons. The delta opioid receptors are located on dendrites and also in dense core vesicles. Is it possible that dense core vesicles are the proteasomal equivalent (i.e., the destination of the receptors that do not go to the surface) in neurons? Furthermore, it is still an open question whether all G protein–coupled receptors (of which opioid receptors constitute only a small number) can be rescued (and expressed at the cell surface) with appropriate pharmacological chaperones.

Pharmacological chaperones offer great advantages over chemical chaperones in terms of selectivity of action—that is, their inability to affect the folding and maturation of other proteins within the ER. For example, the treatment of pain may be improved by the co-administration of a lipophilic partial opioid agonist that operates as a pharmacological chaperone to increase the availability of receptors at the cell surface. Moreover, Petäjä-Repo et al. suggest that the quality-control system of the ER is amenable to regulation by pharmacological intervention, and in particular, the precise design of pharmacological chaperones may enable any receptor of interest to be targeted and retrieved from the ER *(7)*.

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Emerging Roles of TACE as a Key Protease in ErbB Ligand Shedding

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The epidermal growth factor (EGF) family and their receptors—the ErbB family of type I receptor tyrosine kinases—participate in development, differentiation, proliferation, and survival (*1, 2*). Hyperactivity of ErbB signaling has also been strongly implicated in human cancer (*3*), whereas targeted mutation of ErbB1/EGFR in mice results in embryonic and perinatal lethality with epithelial cells being most profoundly affected by receptor loss (*4–6*). All endogenous ErbB ligands are synthesized as transmembrane precursors that are proteolytically cleaved (see Table 1) to release biologically active soluble growth factors, which act in an autocrine or paracrine manner (*7*) (Figure 1). Although in vitro studies have indicated that membrane-anchored ErbB ligand precursors may also be biologically active at the cell surface in a juxtacrine manner (*8–11*), genetic studies in Drosophila (*12–14*) and blockade of shedding in mammalian cells (*15*) highlight a requirement for proteolytic release of ErbB ligands.

The regulation of ErbB ligand presentation and processing has been an area of intense investigation over the last decade and many questions have been raised. Why are ErbB ligands synthesized as transmembrane precursors? Do membrane-anchored ligand precursors and shed soluble ligands have different signaling capacities? How is the proteolytic release of ligands regulated, and what proteases are involved? Progress in regard to better defining the proteases involved in ErbB ligand release, highlighted by the recent publication of Sunnarborg et al., suggests a larger role for the tumor necrosis factor-α (TNFα) converting enzyme (TACE/ADAM17) (*16*).

TACE, a member of a large family of disintegrin-metalloproteinases (ADAMs), is capable of processing diverse substrates including pro-transforming growth factor-α (proTGFα), TNFα, and L-selectin (*17*). Mice that produce catalytically inactive TACE protein that cannot bind Zn^{2+} (*Tace* $^{\Delta Zn/\Delta Zn}$) display a severe phenotype similar to the embryonic and perinatal lethality observed in EGFR-deficient mice (*4–6*). The eye, hair, and skin phenotypes of newborn *Tace* $^{\Delta Zn/\Delta Zn}$ mice are identical to those

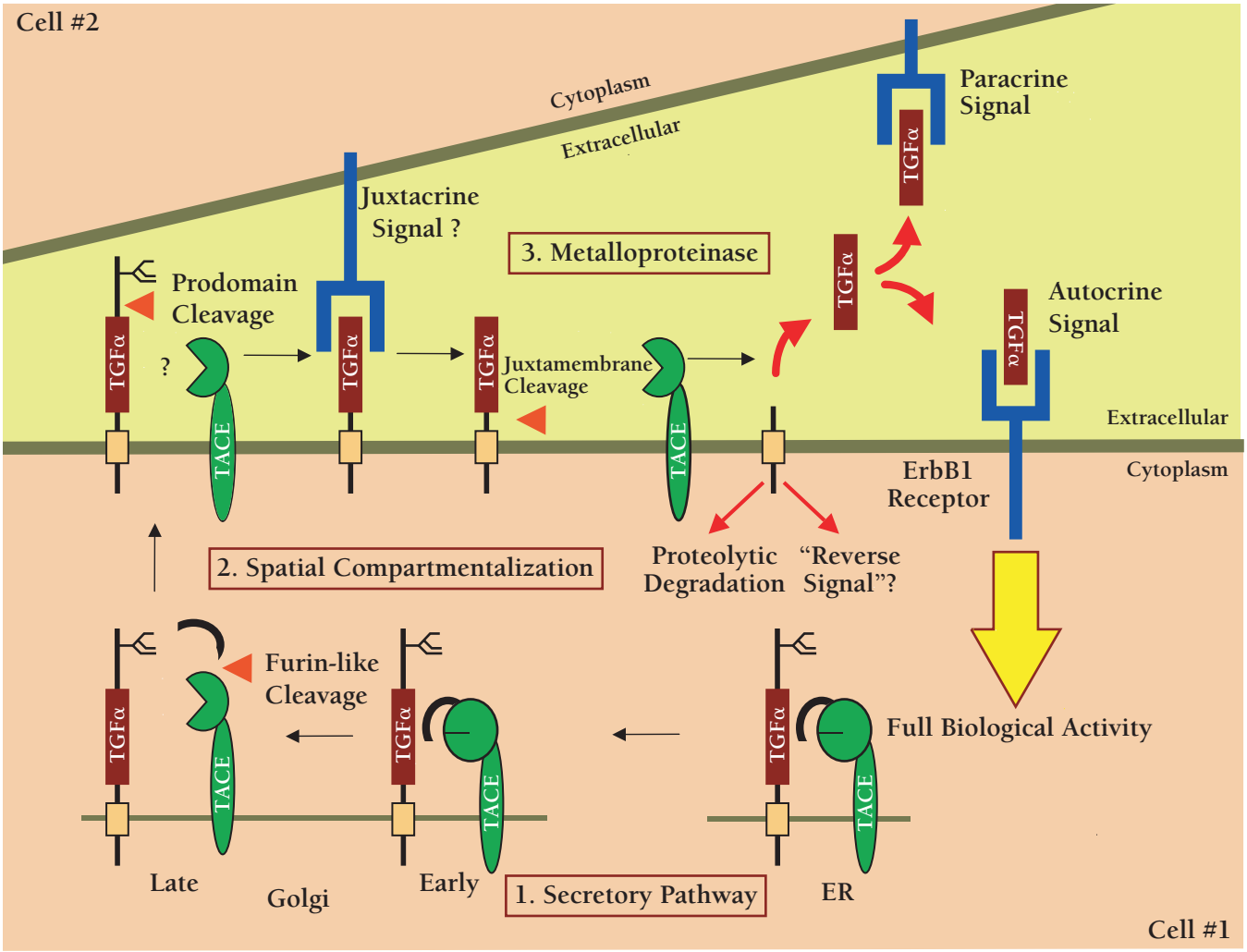

Figure 1. TACE-dependent proteolysis of the proTGFα extracellular domain as a regulatory event in ErbB1 receptor signaling. Genetic and biochemical data indicate that the release of soluble TGFα acting either as an autocrine or a paracrine signal is essential to achieve full biological activity in vivo. Data from Sunnarborg et al. suggest that TACE may also be involved in the N-terminal prodomain processing of proTGFα (*16*). Many questions still remain about the specificity and regulation of TACE as general ErbB ligand "sheddase." Some of these questions can be divided into three general areas. 1. Secretory Pathway. How is the biosynthesis and activation of TACE regulated and can active TACE cleave substrate during transit through the secretory pathway? 2. Spatial Compartmentalization. What is the trafficking of TACE, what membrane compartments is it localized to and how is access of TACE to substrate regulated? 3. Metalloproteinases. What is the specificity of TACE for other ErbB ligands? Is the catalytic activity of TACE regulated? What is the role of other metalloproteinase(s) in ErbB ligand shedding?

previously reported for TGFα-deficient mice (*18, 19*). Indeed, the involvement of TACE in proTGFα processing was confirmed by the reduced shedding of endogenous TGFα from *ras*- and *myc*-transformed embryonic fibroblasts derived from *Tace* $^{\Delta Zn/\Delta Zn}$ mice, and by the ability of recombinant TACE to correctly process a proTGFα juxtamembrane peptide in vitro. Mice lacking functional TACE have additional defects beyond those observed in TGFα–deficient mice, including defects in: epithelial maturation within multiple organs; formation of the lung vasculature; airway branching; and the spongiotrophoblast layer of the placenta (*17, 20*). Both the decreased size and the delayed cellular differentiation of TACE-deficient lungs are restored by the exogenous addition of soluble EGF in embryonic lung culture (*20*). Together, these genetic data further support the concept that TACE-dependent cleavage of proTGFα and other ErbB ligands is essential to development.

The studies by Sunnarborg et al. provide an excellent example of the key criteria needed to unambiguously define a role for TACE in processing of proTGFα. Previous genetic studies have relied on examining the loss of substrate shedding in *ras*- and *myc*-transformed embryonic fibroblasts from *Tace* $^{\Delta Zn/\Delta Zn}$ mice, however it is unclear how transformation and constitutively active Ras signaling may affect proteolytic cleavage. In contrast, Sunnarborg et al. demonstrate defective TGFα shedding in

Table 1. Members of the ErbB Ligand Family Shed by Metalloproteinases

ErbB Ligand Precursors	Ligand Binding Specificity	Candidate Metalloproteinase	Data Implicating Metalloproteinase(s) in ErbB Ligand Shedding	*References*
Amphiregulin	ErbB1	ADAM17/TACE	Loss of AR processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a] and restoration by TACE overexpression	(*16*)
TGFα	ErbB1	ADAM17/TACE	Loss of TGFα processing in *Tace* $^{\Delta Zn/\Delta Zn}$ keratinocytes and restoration by TACE overexpression	(*16*)
			In vitro cleavage of soluble proTGFα and TGFα peptides	
			Loss of TPA-induced TGFα processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a]	(*24*)
			Loss of TGFα processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a]	(*17*)
			In vitro cleavage of TGFα juxtamembrane peptide	
HB-EGF	ErbB1, ErbB4	ADAM9/Meltrinγ	Overexpression of ADAM9 and mutants in Vero cells	(*44*)
		ADAM10/Kuzbanian	Antisense inhibited the lipoteichoic acid- (LTA)-induced transactivation of ErbB1 in NCIH292 cells[b]	(*43*)
		ADAM12/Meltrinα	Overexpression of ADAM12 and inactive mutants[c] in HT1080 cells	(*42*)
		ADAM17/TACE	Loss of HB-EGF processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a] and restoration by TACE overexpression	(*16*)
			Loss of TPA-induced HB-EGF processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a]	(*24*)
		MMP-3	In vitro cleavage of HB-EGF-AP chimera with MMP-3	(*45*)
		MMP-7	In vitro cleavage of HB-EGF with recombinant MMP-7	(*46*)
Neuregulinα2c[d]	ErbB3, ErbB4	ADAM17/TACE	Loss of TPA-induced NRGα2c processing in *Tace* $^{\Delta Zn/\Delta Zn}$ fibroblasts[a] and restoration by TACE overexpression	(*25*)
Neuregulinβ1 and β4[c]	ErbB3, ErbB4	ADAM19	Overexpression of ADAM19 and inactive mutants[c]	(*47*)

AR, amphiregulin; MMP, matrix metallproteinase

[a] *ras*- and *myc*-transformed embryonic fibroblasts

[b] ADAM10 antisense strategy did not directly examine HB-EGF processing.

[c] protease-inactive.

[d] NRG-1 isoforms

primary cultures of normal *Tace* $^{\Delta Zn/\Delta Zn}$ keratinocytes, and partially restore shedding by re-expressing TACE in these cells.

Another major limitation in defining the participation of specific ADAMs in ErbB ligand processing has been the difficulty of demonstrating the direct cleavage of ligands, and verifying that the ligands have been processed at the correct sites of proteolysis. In the case of TACE, in vitro assays demonstrated the proteolytic cleavage of TNFα, L-selectin, and TGFα juxtamembrane peptides (*17, 21, 22*). Sunnarborg et al. have extended this approach to proTGFα and its N- and C-terminal cleavage site peptides, demonstrating that TACE can correctly cleave both N- and C-terminal cleavage sites. Interestingly, most TACE substrates are cleaved within a short stretch of amino acids located in the juxtamembrane region of the extracellular domain. The possibility that TACE may be involved in both N- and C-terminal proTGFα cleavage events raises important questions about how TACE, which is an integral transmembrane protein, can recognize and discriminate between cleavage sites that are located within the extracellular domain of the same protein.

The importance of ErbB ligand shedding has also led investigators to examine the regulation and specificity of TACE and other metalloproteinases in the processing of other ErbB ligands. Indeed, the sequential cleavage of the extracellular domain of membrane-anchored EGF-like growth factors is a highly regulated process that can be activated by a wide variety of pharmacological agents as well as physiological stimuli (*23–25*). Using different experimental approaches, studies have implicated matrix metalloproteinases as well as ADAM proteases in the proteolytic cleavage and shedding of ErbB ligands (Table 1). The work by Sunnarborg et al. adds two other ErbB ligands to the list of TACE substrates, with their demonstration that TACE restores cleavage of transfected amphiregulin and HB-EGF in *ras*- and *myc*-transformed embryonic fibroblasts from *Tace* $^{\Delta Zn/\Delta Zn}$ mice. However, as detailed in Table 1, data for none of the other ligands or proteases provide the ideal composite of data to demonstrate loss of function in null cells, restoration by the candidate protease, and evidence of direct cleavage. In fact, in the case of ADAM9 as a candidate protease for the cleavage of HB-EGF, constitutive and stimulated ectodomain shedding of HB-EGF is comparable in embryonic fibroblasts isolated from ADAM9$^{-/-}$ and from wild-type mice, arguing against an essential role of ADAM9 in HB-EGF shedding in these cells (*26*).

The complexities associated with both ligand and protease processing also contribute to the difficulties in unambiguously assigning a role for a particular protease to processing of an identified ligand. As depicted in Figure 1, ErbB ligands move through the secretory pathway where different modifications and cleavage events can take place. In Drosophila, the activity and

release of the soluble ErbB-like ligand, Spitz, is regulated by two other proteins, Star and Rhomboid (*13, 27, 28*). Star chaperones the Spitz precursor in the endoplasmic reticulum and directs its transport to the Golgi complex (*29*). Once in the Golgi apparatus, Rhomboid, a novel intramembrane serine protease, cleaves the Spitz pro-protein to initiate secretion (*30*). By analogy to the regulation of Spitz processing and release, any perturbation that interferes with processing and trafficking of ErbB ligands could appear to inhibit ligand cleavage and release. The overexpression of protease-inactive ADAM mutants may induce such perturbation. As pointed out by Sunnarborg et al., heterozygous *Tace* $^{+/\Delta Zn}$ mice display a wild type phenotype indicating that protease-inactive mutants do not function as dominant-negative proteins. In light of the complex and poorly understood biosynthesis and trafficking of ADAMs in the secretory pathway, the studies using overexpression of ADAM mutants should be viewed with caution, and other substrates and ADAM mutants should be used as controls. Thus, understanding the regulation of biosynthesis and trafficking of both ErbB ligands and their candidate proteases will be critical to defining the rate-limiting steps in proteolytic release of ErbB ligands.

Most ADAMs implicated in ErbB ligand processing are ubiquitously expressed, raising the possibility that distinct ADAM specificity for ligand processing may reflect variations in signaling and regulation of ADAM proteolytic activity in different cell types. Although multiple activators of ErbB ligand shedding have been identified (*23–25*), the proteases responsible for the shedding per se have not been completely characterized. For further progress to be made in understanding the mechanisms regulating these "sheddases," the experimental criteria required to identify candidate metalloproteinase(s) must be clearly defined. Sunnarborg et al. have begun to address the role and specificity of TACE in ErbB ligand processing, although many questions remain.

Data demonstrating a critical role for the proteolytic cleavage of ErbB ligands in ErbB signaling have created a fundamental paradigm shift in our understanding of the biology of ErbB ligand precursors. Numerous in vitro and in vivo studies support the concept that sequential processing of membrane-anchored ErbB ligands provides a mechanism for exquisite control over the temporal and spatial presentation of ligands to receptors (*31–39*). Thus, sequential processing can directly regulate the ability of a particular ligand to switch function from a potential juxtacrine mode to an autocrine or paracrine mode (*8–11*) (Figure 1). The studies by Sunnarborg et al. and others have established that the release of soluble ErbB ligands is a key upstream regulatory event in ErbB receptor signaling, and have thus identified protein processing as a target for therapeutic intervention (*15–17, 40–43*). For example, the use of either neutralizing antibodies to the ErbB1 receptor, or metalloproteinase inhibitors to effectively inhibit ErbB signaling, can inhibit the in vitro autocrine-dependent proliferation and migration of mammary epithelial cells (*15*). Additionally, in these mammary epithelial cells, autocrine presentation of a protease-cleavable ligand results in enhanced, persistent cell migration in a particular direction, whereas stimulation by constitutively secreted ligand results in a scattering response in these cells (*41*). Is there any physiological role for juxtacrine signaling by ErbB ligand precursors in vivo? Or is the primary role of the membrane-anchoring domains of these ligands the spatial regulation of release to appropriate cellular compartments? Answers to these questions will shape our paradigms of ErbB ligand signaling in the future.

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Emerging Roles for PKC Isoforms in Immune Cell Function

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The protein kinase C (PKC) family consists of thirteen members categorized as conventional, novel, atypical, or PKC-related isoforms, depending on whether diacylglycerol (DAG), calcium, or phosphatidylserine (PS) is required for their activation *(1, 2)*. Mice deficient in the conventional PKC isoform PKCβ are immunodeficient, exhibiting a loss of peritoneal B-1a B cells and reduced T-cell–independent antibody responses *(3)*. B cells from PKCβ-deficient mice also fail to respond to BCR stimulation, suggesting that PKCβ is a critical component of the BCR signaling machinery. Similarly, mature T lymphocytes in PKCθ-deficient mice exhibit defects in T cell antigen receptor- (TCR)-induced proliferation and reduced T-dependent responses *(4)*. These results demonstrate the critical role for specific PKCs in B and T cell receptor signaling and function.

In contrast to the immunodeficiencies observed in PKCβ and PKCθ mice, two recent reports demonstrate that mice deficient in the novel PKC isoform PKCδ exhibit B cell hyperactivation, which leads to autoimmunity *(5, 6)*. Whereas early B cell development in the bone marrow of PKCδ-deficient mice is normal, exaggerated B cell expansion occurs in the spleen and other peripheral organs,

resulting in splenomegaly and lymphadenopathy. The presence of excessive serum autoantibodies specific for DNA and nuclear proteins led to immune-complex deposition and glomerulonephritis in PKCδ$^{-/-}$ mice. Furthermore, transplantation of PKCθ-deficient B cells into normal animals (i.e., adoptive transfer) indicated that this hyperproliferative phenotype was B cell specific (*5*), underscoring the essential role for PKCδ in negative regulation of B lymphocyte signaling.

The autoimmune phenotype of PKCδ$^{-/-}$ mice was associated with a loss of peripheral tolerance. For example, the B cells of mice that carry a BCR transgene that specifically recognizes hen egg lysozyme (IgHEL) will undergo negative selection (i.e., activation-induced cell death) in the periphery of transgenic mice that have been bred with transgenic mice expressing soluble hen egg lysozyme (sHEL) (*7*, *8*). However, negative selection observed in doubly transgenic IgHEL–sHEL mice was abrogated in the absence of PKCδ, suggesting a critical role for PKCδ in regulating peripheral B cell tolerance (*6*).

The mechanisms responsible for the breakdown of tolerance in PKCδ$^{-/-}$ B cells remain unclear. Peripheral B lymphocytes develop in a step-wise fashion from immature transitional 1 (T1) to transitional 2 (T2) to naïve, follicular mature B cells (*9*). T1 and T2 cells reside in distinct splenic microenvironments and exhibit differential responsiveness to BCR engagement whereby T1 B-cells might be the target for peripheral negative selection, and T2 B-cells might be the target for BCR-dependent positive selection (*10*). Evaluation of the signaling functions of PKCδ specifically within T1 vs. T2 peripheral B cell populations might therefore provide important insight into how PKCδ functions to regulate peripheral B cell tolerance.

Notably, the data from Miyamoto et al. suggest that PKCδ may regulate B cell activation by inhibiting interleukin (IL)-6 production. IL-6 expression and secretion are increased in PKCδ$^{-/-}$ splenic B cells (*5*). This is consistent with the exaggerated B cell expansion and plasmacytosis observed in IL-6 transgenic mice (*11*). IL-6 expression is dependent on the transcriptional activator NF-IL6, or the CCAAT/enhancer-binding protein (C/EBPβ). PKCs can phosphorylate NF-IL6 in vitro on inhibitory residue Ser^{240}, leading to a marked decrease of NF-IL6 DNA-binding activity (*12*). Lipopolysaccahride- (LPS)-induced NF-IL6 DNA binding activity was greatly increased in PKCγ$^{-/-}$ B cells (*5*), suggesting that PKCδ may regulate B cell tolerance, in part, by inhibiting IL-6 production through site-specific phosphorylation of NF-IL6.

Interestingly, PKCβ appears to possess a similar ability to phosphorylate critical inhibitory residues on Bruton's tyrosine kinase (Btk). PKCβ is activated upon BCR stimulation, in response to increased concentrations of DAG and Ca^{2+}. Sustained generation of these two second messengers is mediated by Bruton's tyrosine kinase (Btk) through the activation of PLC-γ isoforms (Figure 1) (*13*). Although PKCβ has a definite role in mediating the positive effects downstream of Btk, PKCβ (and possibly other conventional PKCs) can also inhibit Btk through a negative feedback loop (*14*). PKCβ specifically phosphorylates Btk on Ser^{180} within the Tec homology (TH) region, leading to the inhibition of Btk membrane translocation and activation (14). In addition, the atypical PKC isoform PKCμ (or PKD) also negatively regulates BCR signaling through the phosphorylation-dependent inhibition of the tyrosine kinase Syk (*15*). Therefore, the ability to negatively regulate lymphocyte signaling by site-specific phosphorylation at inhibitory residues may be a common characteristic of many PKC family members (Figure 1).

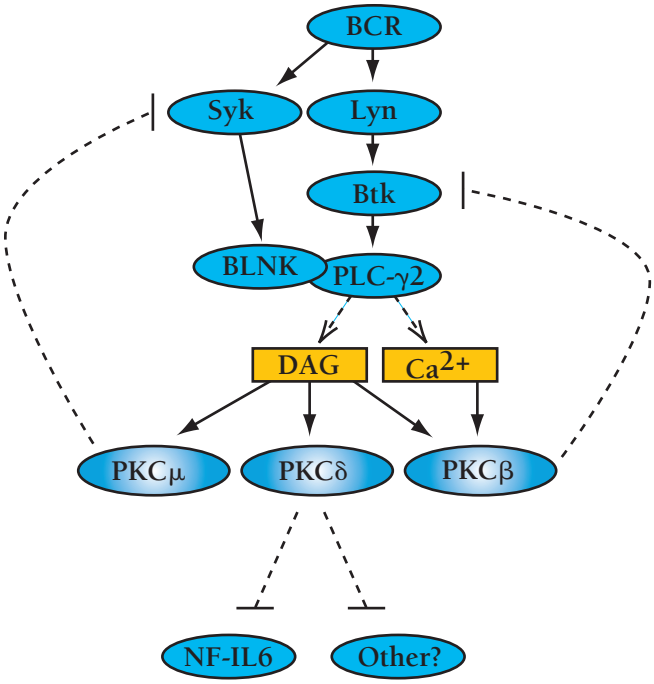

Figure 1. Negative roles for PKC isoforms in B lymphocyte signaling. B cell receptor (BCR) engagement leads to a cascade of signaling events, eventually leading to the activation of the B cell. Specific PKC isoforms that are activated by the BCR can inhibit B cell signaling. Although PKCβ and PKCμ function in negative feedback loops to inhibit the upstream activators Btk (itself activated bt the tyrosine kinase Lyn) and Syk, respectively, PKCδ may function to inhibit the NF-IL6 transcription factor. BLNK, B cell linker protein; Syk, a 72-kDa protein tyrosine kinase highly expressed in B cells; Btk, Bruton's tyrosine kinase; DAG, diacylglycerol; NF-IL6, nuclear factor for interleukin-6 expression.

In addition to the negative effects mediated by PKCδ on lymphocyte activation, an earlier study using chemical inhibitors suggested that PKCδ functions in a positive role in BCR dependent NF–κB activation (*16*). In contrast to these findings, the reports by Miyamoto et al. and Mecklenbräuker et al. demonstrate that NF–κB activation in B cells is completely intact in PKCδ-deficient mice (*5*, *6*). These differences in results emphasize that caution should be maintained when using inhibitors with partially overlapping specificity to assess the physiologically relevant function of proteins.

In contrast to the intact NF–κB function in PKCδ$^{-/-}$ mice,

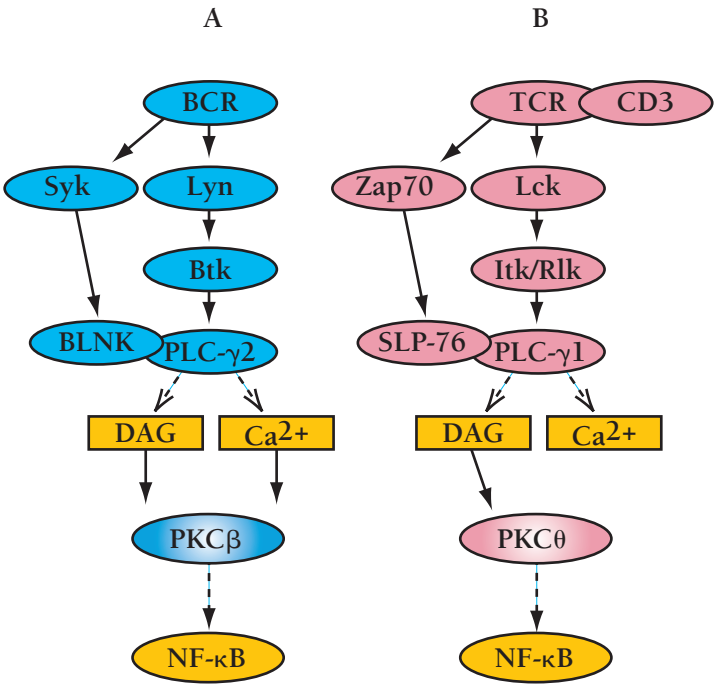

Figure 2. Positive roles for PKC isoforms in lymphocyte signaling. Engagement of the BCR **(A)** or the T cell antigen receptor (TCR) **(B)** activates a set of similarly acting signaling molecules. Specific Src (Lyn and Lck), Syk-Zap70, and Tec (Btk and Itk/Rlk) family kinases are activated. These events mediate increased activity of PLC-γ, leading to the generation of the second messengers DAG and calcium, which are required for the activation of most PKCs. Both PKCβ and PKCθ, respectively, function in controlling BCR and TCR dependent NF–κB activation. SLP-76, src homology (SH)2 domain–containing leukocyte protein of 76-kD.

recent studies demonstrate that PKCβ is the major PKC isoform required for BCR-dependent NF–κB activity. Splenic B cells from PKCβ-deficient mice exhibit severe defects in survival, Bcl-x_L induction, IκB degradation, and IκB kinase (IKK) activity in response to BCR engagement *(17)*. The mechanism of NF–κB regulation by PKCβ appears to be mediated through PKCβ-dependent recruitment of IKK into the membrane lipid rafts that are associated with the BCR signaling complex. In contrast, CD40-dependent NF–κB activity is fully intact in PKCβ$^{-/-}$ B cells, suggesting that this role for PKCβ is specific to BCR signaling. Similar to the role for PKCβ in B cells, TCR-dependent activation of NF–κB is abrogated in splenic T cells from PKCθ$^{-/-}$ mice *(4)*, suggesting an analogous function for PKCθ in regulating TCR-dependent recruitment of IKK into lipid rafts and NF–κB activation. Together, these findings suggest that PKCβ and PKCθ are responsible for cell-specific and receptor-specific activation of the NF–κB signaling pathway in B and T lymphocytes, respectively (Figure 2).

Implicit in the identification of specific roles for individual PKC isoforms in positive or negative regulation of lymphocyte signaling is that PKCs may represent useful potential drug targets for various immune disorders. For example, the autoimmune disease observed in PKCδ$^{-/-}$ mice suggests that negative regulatory functions by PKCs are crucial for maintaining proper immunological tolerance. It remains to be seen whether PKCδ function is reduced in certain autoimmune states, and whether specific activators of PKCδ can be developed to treat such conditions. Furthermore, because PKCδ and PKCβ seem to have negative and positive roles, respectively, in B lymphocytes, it will be interesting to test whether concomitant PKCβ-deficiency might abrogate the autoimmunity observed in PKCδ$^{-/-}$ mice.

Chemical activators such as TPA (12-*O*-tetradecanoylphorbol-13-acetate) are not specific for individual PKCs, nor are PKC isoform-specific activators available *(18, 19)*. In contrast, a number of inhibitors specific for individual PKCs do exist including antisense oligonucleotides and chemical inhibitors of catalytic activity *(18)*. The positive roles for PKCβ and PKCθ in lymphocyte function suggest that highly specific inhibitors of these PKC isoforms may have great potential in treating B-cell– or T-cell–specific disorders. Although no PKCθ-specific chemical inhibitors exist, several PKCβ-specific inhibitors are currently available, including the macrocyclic bis (indolyl) maleimides LY-333531, LY-379196 and LY-317615 *(20)*. These compounds are well tolerated systemically and can be administered orally *(21)*. The availability, safety, and efficacy of PKCβ-specific oral inhibitors suggest that they may also be useful in the treatment of certain B-cell immune disorders. For example, crossing Btk-deficient mice with lupus-prone (NZB x NZW)F_1 mice abrogates the systemic lupus erythematosus (SLE)-like symptoms in these animals *(22)*. Similar experiments involving the breeding of PKCβ$^{-/-}$ mice to (NZB/W)F_1, to PKCδ$^{-/-}$, or to other autoimmune-prone mice will be required to validate PKCβ as a drug target for B-cell–dependent autoimmune diseases. In addition, a clinically refractory subset of non-Hodgkin's diffuse large B cell lymphomas (DLBCLs) exhibit elevated PKCβ expression *(23)*. Recent studies in our laboratory demonstrate efficacy for PKCβ-inhibitors in specifically blocking the survival of PKCβ-expressing DLBCL tumor lines in vitro *(17)*.

Further studies will be needed to confirm the efficacy of PKCβ-specific inhibitors in treating B cell malignancies and autoimmune disease in vivo. The future development of PKCθ-specific inhibitors and PKCδ-specific activators may similarly lead to effective treatments for T cell lymphomas and various autoimmune conditions. Therefore, understanding the positive and negative regulation of PKC function in the immune system may lead to effective treatments for human disease in the near future.

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