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Cytokine and Growth Factor Receptors in the Nucleus: What's up With That?

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Abstract Signaling via cell surface receptors that are anchored by a single transmembrane domain is a well-established paradigm. Ligand binding to the extracellular domain of the receptor facilitates receptor dimerization, which juxtaposes the intracellular domains, typically activating intrinsic or associated kinases. Two large families of tyrosine kinase activating receptors have been particularly well characterized: the receptor-type protein tyrosine kinases and the receptors for the α-helical cytokines, which activate non-covalently bound JAK family tyrosine kinases. Despite the well-established function of these receptors at the cell surface, both intact and cleaved receptors belonging to these families have been repeatedly detected in the nucleus. Furthermore, there is evidence that some of these receptors or receptor fragments function directly in modulating gene transcription. In this essay, I examine how close we are to demonstrating that direct translocation of receptors, or receptor fragments, from the cell surface to the nucleus is a physiologically relevant means of intracellular signaling that can supplant or complement canonical signaling cascades. J. Cell. Biochem. 95: 478–487, 2005.

Key words: receptor; tyrosine kinase; cytokine; growth factor; nuclear translocation; intramembrane proteolysis; transcription

CANONICAL TYROSINE KINASE SIGNALING PARADIGMS

Cell surface receptors that activate tyrosine kinases play a critical role in propagating intracellular signals that regulate cell proliferation, differentiation, and effector function. Members of one family, the receptor-type protein tyrosine kinases (RTKs), are composed of an amino-terminal extracellular domain (ECD) that binds ligand, a single transmembrane domain (TMD), and an intracellular domain (ICD) containing a tyrosine kinase catalytic unit. There are over 60 receptors in the human version of this family, which is divided into >20 subfamilies [Manning et al., 2002]. Among the most prominent subfamilies are those related to the epidermal growth factor (EGF) receptor (erbB1-4) and those belonging to the fibroblast growth factor subfamily (FGFR1-4). Signaling [Hunter, 2000; Schlessinger, 2000; Pawson, 2004] begins with ligand binding, which stabilizes receptor dimerization, thereby juxtaposing the tyrosine kinase domains of the RTKs on the cytosolic face of the membrane. These kinase domains cross-activate each other by phosphorylating critical residues within the catalytic domain, and then further phosphorylate critical tyrosines outside the catalytic domain, which in turn function as docking sites for proteins containing SH2 or PTB domains. These latter proteins act either as adaptors to recruit other proteins to the signaling complex or harbor catalytic domains capable of propagating the initial signal further. For example, the SH2-adaptor Grb2 recruits a component of the Ras signaling complex, which in turn activates the MAP kinase cascade. Another example is the SH2 domain containing enzyme phospholipase Cγ, which is recruited to the phosphorylated receptor to provide access to its phospholipid substrate in the plasma membrane. One of the phospholipase Cγ hydrolysis products activates

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protein kinase C. Eventually, a complex network of positive and negative regulators are activated, thereby precisely modulating the transcription of a specific set of genes.

The second receptor family, the helical cytokine receptors (HCR), all contain fibronectin-like domains that bind cytokine ligands which have a distinctive structure marked by multiple α-helices [Bazan, 1990]. The HCR family consists of >45 members, divided into two broad classes [Boulay et al., 2003; Renauld, 2003]. Examples include receptors for the interferons, many of the interleukins, and a number of polypeptide hormones (growth hormone, prolactin, erythropoietin). Many of the active receptor complexes consist of heterodimeric combinations of two HCRs. Some HCRs are shared among different ligand binding complexes. HCRs have an ECD–TMD–ICD structure similar to the RTKs, with the key exception that the ICD does not possess catalytic activity. Instead, JAK family tyrosine kinases are noncovalently associated with the ICD and the HCR–JAK complex functions in a manner that is analogous to the RTK: ligand binding induces dimerization and activation of the associated JAK kinases [Leonard and O'Shea, 1998; Stark et al., 1998]. The kinases phosphorylate tyrosine residues that, in turn, act as docking sites for STAT family transcription factors, which contain SH2 domains [Levy and Darnell, 2002]. The docked STATs are tyrosine phosphorylated and then dimerize, translocate to the nucleus, and modulate expression of a large cohort of genes.

As described, these two families of receptors have been viewed as functioning strictly at the cell surface, thereby modulating gene transcription "at a distance" by eventually dispatching a messenger, which is often a transcription factor, to the nucleus. Indeed, there exist an array of molecular strategies for delivering such messenger proteins to the nucleus [Xu and Massague, 2004]. In many instances, phosphorylation regulates translocation of a transcription factor, either directly (STAT, Smad) or indirectly (Erk. NFκB, β-catenin). However, evidence has now been accumulating for almost a decade that some cell surface tyrosine kinase activating receptors move directly to the nucleus. Although this is an appealingly simple mechanism, it is also inherently less "regulatable" compared to the multi-protein signaling cascades described above. A number of investigators have suggested that the nuclear translocation of receptors (and/or ligands) might ensure signaling specificity, especially among a group of related receptors that trigger very similar molecular events in the cytoplasm. On the other hand, it is conceivable that the canonical signaling mechanisms that are triggered by cell surface RTKs and HCRs may well be entirely sufficient to account for the biological activities of the initiating ligands. Thus, although evidence in support of a nuclear role for cell surface receptors continues to build, the concept is certainly not yet a part of the modern "canon" of cellular and molecular biology. So, when will we know if the direct translocation of cell surface receptors is physiologically relevant—or not? I will try to address this question, focusing on how we can disentangle canonical signaling "at a distance" from signaling mediated by receptors or receptor fragments that translocate directly to the nucleoplasm.

MOVEMENT OF INTACT RECEPTORS TO THE NUCLEUS

Both intact full-length receptors, as well as fragments corresponding to the ICD, have been identified in the nuclei of various cells and tissues. A number of recent reviews have discussed the nuclear movement of intact receptors in considerable detail, summarizing the rather extensive descriptive data and suggesting possible mechanisms [Wells and Marti, 2002; Carpenter, 2003; Clevenger, 2003; Johnson et al., 2004]. In brief, there have been multiple reports of intact receptors, and in many cases the cognate ligands also, in the nucleoplasm. The best-described examples include the erbB-1, -2, and -3 family members; FGFR-1, the prolactin receptor; the IFNγ receptor subunit IFNgR1; and the growth hormone receptor. In no instance is the story complete for a given receptor (and in many cases the data for a particular receptor has been reported by only a single group). It appears that receptor mediated endocytosis is usually required and this has been incorporated into some proposed mechanisms (see below). In addition, the receptors seem to possess a nuclear localization signal (NLS) or a binding site for a chaperone-like protein. A classical polybasic NLS has only been demonstrated in the case of erbB-3 [Offterdinger et al., 2002]. Receptors lacking an NLS might "piggyback" to

the nucleus by employing either an NLS in the ligand (IFNγ) or a chaperone protein that binds either the receptor itself (FGFR-1) or the ligand (prolactin). In the case of the FGFR-1, it has been shown that importin-β, a protein that is part of the nuclear translocation machinery and can function in the absence of an NLS, associates with FGFR-1 [Reilly and Maher, 2001]. This interaction is only seen in ATP depleted cells (ATP depletion prevents movement of importin-β to the cytoplasm), but is a potentially encouraging lead for unraveling some parts of the mechanism. In the case of prolactin, the peptidyl prolyl isomerase cyclophilin B, which is present in serum, binds prolactin and is co-endocytosed following prolactin binding to its cognate receptor [Rycyzyn et al., 2000]. Cyclophilin B can also function in the nucleus, in conjunction with PRL and Stat5a to modulate gene transcription [Rycyzyn and Clevenger, 2002]. However, the fate of the prolactin receptor, which has been cited in the nucleus by others [Rao et al., 1995], was not monitored in these particular studies.

A major conceptual challenge has been identifying a mechanism for releasing an integral transmembrane protein into the cytoplasm and/or nucleoplasm. One mechanism, termed "retrotranslocation," has been proposed by a number of investigators. It is an alternative application of the endoplasmic reticulum (ER) associated degradation (ERAD) pathway [Tsai et al., 2002]. ERAD is a means for moving unfolded or misfolded proteins (typically viewed as nascent proteins which flunked a quality control test) from the ER lumen to the cytoplasm for ubiquitin-dependent degradation. ERAD employs the same protein channel (the sec61 complex) that it used by nascent polypeptides, which are destined for (normal) secretion or membrane insertion. The idea that ERAD can be "subverted" to facilitate the movement of cellular proteins from the cell surface to the cytoplasm (and perhaps on to the nucleus) is based on observations with toxins such as ricin. Following binding to cell surface glycoproteins, ricin travels via receptor endocytosis and subsequent movement from the endosome through the Golgi to the ER, where it is transported via the sec61 complex to the cytoplasm [Lord et al., 2003]. A critical aspect of ricin toxicity appears to be its ability to evade ubiquitination and thereby avoid the proteosomal degradation that is the usual fate of proteins moving through the ERAD pathway. It is far from clear at this point that normal cellular proteins can similarly use such a retrograde transport system in the manner of bacterial toxins. Furthermore, the movement of toxins through ERAD appears to be quite inefficient [Wesche, 2002]. Many questions remain. For instance, we do not know: (i) how integral membrane proteins that are in the ER membrane can be moved into and through the sec61 channel; (ii) how cellular proteins transiting this pathway avoid degradation; (iii) how proteins that do avoid degradation are unfolded in the ER and then re-folded upon reaching the cytoplasm; and (iv) how specific interactions with ligands, or other chaperones, can survive such a process of unfolding/refolding. In summary, we still lack a clearly defined, and therefore testable, mechanism leading from the plasma membrane to the nucleus and this deficiency will impede attempts to extend the mainly descriptive data that has accumulated thus far, concerning the nuclear translocation of intact receptors.

MOVEMENT OF ICDs TO THE NUCLEUS

In contrast to the enigmatic intact receptors, it is now quite clear how a receptor fragment, specifically the ICD of a receptor containing a single TM domain, can be delivered to the nucleus, via a process termed RIP, for regulated intramembrane (IM) proteolysis [Brown et al., 2000] (Fig. 1). From the viewpoint of the cellular biochemist, this is a fascinating signaling mechanism by virtue of its impressive conservation among all life forms (archaea, bacteria, and eukaryotes) as well as the fact that the key biochemical event is the liberation of the ICD from the intact receptor molecule via a proteolytic cleavage event that takes places, remarkably, within the lipid bilayer. There are three families of IM proteases [Wolfe and Kopan, 2004]: (i) the metalloproteases (exemplified by S2P); (ii) the aspartate proteases (exemplified by the presenilins (PSs)); and (iii) the serine proteases belonging to the rhomboid family [Freeman, 2004]. Substrates for the first two families of IM proteases are proteolytically cleaved in a two-step process: first at a juxtamembrane (JM) site within the ECD (or corresponding luminal domain in the case of substrates that are processed within intracellular membranes) and then at an IM site. The JM event, which is the initial cleavage and is

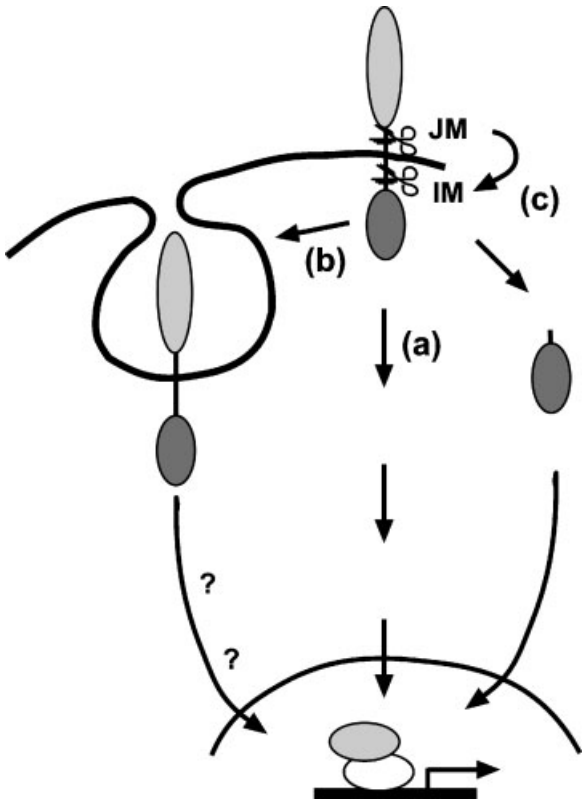

Fig. 1. Direct signaling to the nucleus by tyrosine kinase activating receptors. Three possible fates for signaling through tyrosine kinase activating receptors (i.e., either the receptor type tyrosine kinases or the helical cytokine receptors (HCR), which associate with JAK family tyrosine kinases) are schematized. The center of the figure (**a**) represents a canonical signaling cascade. Such pathways can regulate gene transcription and are well-described for both of these families of receptors (see text for a brief overview). The left side of the figure (**b**) illustrates the transit of intact receptors to the nucleus. Receptor-ligand induced endocytosis is apparently required, but the subsequent intracellular trafficking events are not well understood (signified by the question marks). The right side of the figure (**c**) illustrates the regulated intramembrane (IM) proteolysis (RIP) pathway for delivering the ICD of the receptor to the nucleus. An initial, regulated proteolytic cleavage event in the juxtamembrane (JM) region of the ectodomain is followed by constitutive cleavage within the plasma membrane by a specialized IM protease, such as one of the presenilins (PSs). The liberated ICD translocates to the nucleus and regulates gene transcription as part of a transcriptional complex. RIP signaling might also require an initial receptor-mediated endocytotic step [Cheng et al., 2003; Gupta-Rossi et al., 2004]. While it is clear that path (a) is critical for mediating most signals, it remains unknown what portion, if any, of a given signal flows through paths (b) and/or (c), for any of the tyrosine kinase activating receptors.

typically regulated, generates a membrane-bound "stub" which protrudes 8–20 amino acids from the bilayer. The stub is then processed in an apparently constitutive manner by the appropriate IM protease [Struhl and Greenwald, 2001; LaVoie and Selkoe, 2003].

The best studied of the S2P RIP pathway is the processing of cholesterol sensor SREBP2 [Horton et al., 2002]. The ICD that is liberated following sequential JM and IM cleavage is essentially a classical transcription factor, containing an NLS, a DNA binding domain (DBD), and a transcriptional activation domain (TAD). It translocates to the nucleus and regulates genes required for cholesterol biosynthesis. The best studied of the PS substrates are Notch and the Alzheimer's precursor protein (APP) [Selkoe and Kopan, 2003]. PS functions as part of a high molecular weight, integral membrane complex (γ-secretase) with three other proteins [Iwatsubo, 2004]. In both cases, JM cleavage is initiated by a cell surface metalloprotease belonging to the ADAM family. ADAM-17, better known as TNFα converting enzyme (TACE), plays a predominant, although not necessarily exclusive, role in the JM cleavage of Notch and APP [Brou et al., 2000; Allinson et al., 2003]. TACE has been directly implicated in the JM cleavage of many proteins, often in response to stimuli (such as phorbol ester) that activate various types of protein kinase C [Hooper et al., 1997; Black, 2002; Mohan et al., 2002; Weskamp et al., 2004]. In contrast to SREBP-2, the ICDs generated by PSs are not complete transcription factors. The Notch ICD, for example, appears to act as an adaptor linking a DNA binding protein (CSL) and a transcriptional activator (Mastermind) [Lubman et al., 2004]. The APP ICD also appears to act primarily as an adaptor within a transcriptional complex [Cao and Sudhof, 2001]. In all three of these cases (SREBP2, Notch, APP), RIP is believed to be the primary signaling mechanism and transcriptional modulation is a prominent function of the ICD. There is evidence that at least 10 other cell surface receptors signal primarily via RIP [Jung et al., 2003; Martoglio and Golde, 2003; Taniguchi et al., 2003] and additional RIP substrates are still being identified [Guay-Woodford, 2004].

Clearly, RIP provides a signaling mechanism for a number of receptors in higher eukaryotes that were previously "signaling challenged," but is it also utilized by those receptors already capable of tyrosine kinase based signaling? Thus far, two RTKs (erbB-4 and the CSF1-receptor (CSF1R)) and one HCR (the type I

interferon receptor subunit, IFNaR2) have been identified as candidates for RIP. The erbB-4 receptor has been characterized most thoroughly. Carpenter and colleagues [Ni et al., 2001] initially showed that endogenous erbB-4 ICD (e4-ICD) translocates to the nucleus and that γ-secretase inhibitors block this movement. Others have confirmed the role for PS [Lee et al., 2002] and the nuclear translocation of e4-ICD [Williams et al., 2004], albeit using over-expressed, tagged-constructs. Importantly, the latter studies also identified a functional NLS near the amino-terminal end of e4-ICD. The erbB-4 receptor is cleaved constitutively, in response to phorbol ester as well as following binding of the ligand heregulin [Ni et al., 2001]. TACE has been implicated in the JM cleavage [Rio et al., 2000] and the corresponding cleavage site has been sequenced and found to be eight amino acid residues from the TMD, in a stalk-like region of the ECD [Cheng et al., 2003]. Interestingly, the site is within an alternatively spliced erbB-4 exon; thus, there exists an erbB-4 isoform (Jm-b) which is refractory to cleavage and thus RIP signaling.

The data for CSF1R [Wilhelmsen and van der Geer, 2004] and IFNaR2 [Saleh et al., 2004] (from my own lab group) are more limited and the studies of each receptor have been reported by only a single lab. However, both reports demonstrate that cleavage occurs via a two-step process that involves both a phorbol ester sensitive protease (presumed, but not yet proven, to be TACE) and PS, culminating in the release of an ICD-sized fragment into the cytosol. As was the case with erbB-4, cleavage occurs constitutively, as well as in response to phorbol ester and cognate ligands. We have also shown that when the IFNaR2 ICD (I2-ICD) is fused to GFP and transfected into cells, it is found almost exclusively in the nucleus, suggesting that the I2-ICD possesses an NLS or binds to proteins that facilitate nuclear translocation.

Is RIP a common fate for RTKs and HCRs? It is probably too early to tell, but it seems likely that more examples will be discovered. On one hand, phorbol ester can induce ECD shedding of a number of other RTKs and HCRs [Arribas et al., 1996; Hooper et al., 1997; Carpenter, 2003; Schantl et al., 2004]. Based on observations with recombinant stubs [Schroeter et al., 1998; LaVoie and Selkoe, 2003], we can anticipate that many, if not all, of the stubs generated by ectodomain shedding events will be further processed by PS or other IM proteases and therefore represent candidates for RIP signaling. Furthermore, ICDs are often very labile [Schroeter et al., 1998; Brown et al., 2000]. Indeed, it has been proposed that rapid degradation is the primary mechanism for terminating RIP signals [Kopan, 1999; Brown et al., 2000]. Thus, ICDs may be frequently overlooked, accounting for the relatively few examples documented so far. Unfortunately, there are no obvious consensus sites for JM proteases-like TACE [Black et al., 2003; Hinkle et al., 2004] or for IM proteases such as the PSs [De Strooper et al., 1999; Okochi et al., 2002; Xia and Wolfe, 2003], precluding rapid identification of RIP candidates by simple informatics approaches. On the other hand, a combination of structural and cleavage data can sometimes be used to predict the length of the JM stalk, and therefore the likelihood of cleavage. Based on this type of analysis, it appears unlikely that other members of the erbB family (besides erbB-4) are RIP-ed by TACE and PS [Cheng et al., 2003]. Cleavage studies on CSF1R-insulin receptor chimeras also indicate that the insulin receptor does not undergo detectable RIP. Clearly, not all RTKs undergo RIP. Thus, while mechanistic considerations are the main stumbling block in determining if intact receptors function in the nucleus, I suspect that hunting down candidate ICDs may be the rate limiting step in making a persuasive case that RIP-ing RTKs and HCRs is physiologically relevant in a wide cellular context.

ACTIONS OF CELL SURFACE RECEPTORS IN THE NUCLEUS

The presence of intact and receptor fragments in the nucleus has fueled interest in the possibility that these proteins regulate gene transcription. Indeed, multiple studies have documented, to varying degrees, transcriptional effects. The transcriptional activity of two receptors believed to transit intact to the nucleus, erbB-1, and erbB-2 has been investigated by Hung and colleagues [Lin et al., 2001; Wang et al., 2004]. In these parallel studies, either cyclic amplification and selection of targets (CASTing) or chromatin immunoprecipitation (ChIP) based cloning was used, in conjunction with specific anti-receptor antibodies, to identify possible gene regulatory regions that bind the intact receptor. An A–T rich

element, found upstream of the cyclin D1 locus, was identified as a putative site for erbB-1 and a region upstream of the *Cox2* gene was identified for erbB-2. These genes were transcriptionally activated by EGF treatment or erbB-2 overexpression, respectively. Importantly, in both cases transcription correlated with chromatin binding of the receptor as revealed by ChIP analysis. These are intriguing discoveries; however, it has not yet been demonstrated that the expression of these genes is strictly dependent on nuclear transit of the corresponding receptors. The carboxyl terminal (CT) portion of the ICD (i.e., distal to the kinase domain) of both receptors stimulated expression of a Gal4 upstream activating sequence (Gal4UAS), when fused to the Gal4 DNA binding domain (Gal4DBD). Interestingly, in both cases the CT portion was far more active than the entire ICD; thus, it remains unclear what role the CT segment has in the context of intact receptor function in the nucleus.

Of the receptors processed by RIP, there is evidence that IFNaR2 and erbB-4 modulate transcription. The data from our lab on the transcriptional effects of the I2-ICD has mainly involved testing the effects of Gal4DBD-ICD fusions on Gal4UAS activity [El Fiky et al., 2005]. The I2-ICD, when fused to Gal4DBD, can either activate or repress various reporters linked to a Gal4UAS, possibly dependent on the presence of other transcriptional regulatory elements in the reporter constructs. We also showed that the (unfused) I2-ICD represses transcription from a reporter containing an endogenous interferon stimulated gene response element (ISRE) [Saleh et al., 2004]. Stat2 binds the I2-ICD constitutively in a phosphotyrosine-independent manner [Nguyen et al., 2002; Saleh et al., 2002]. Since we have also shown that the Stat2 TAD mediates the transcriptional effects of the I2-ICD [El Fiky et al., 2005], it is conceivable that RIP-ed IFNaR2 might control some of the same genes regulated by the canonical JAK–STAT signaling pathway. A comprehensive survey of the genes regulated by the I2-ICD is required to test this hypothesis.

Two potential mechanisms of transcriptional regulation by the e4-ICD have been investigated. Similar to the observations cited above for erbB-1 and erbB-2, the CT domain of erbB-4 stimulates transcription in a Gal4 reporter assay whereas the effect of the entire e4-ICD is not detectable [Ni et al., 2001]. Subsequent studies showed that two isoforms of YAP, which contain either one or two WW domains, bind to PPXY motifs within the CT domain and enhance transcription of Gal4DBD fused to either the entire ICD or the CT portion [Komuro et al., 2003; Omerovic et al., 2004]. YAP activity is regulated by Akt phosphorylation and 14-3-3 sequestration [Komuro et al., 2003]. Thus, high levels of Akt kinase activity may effectively repress the transcriptional activity of full-length e4-ICD in some cell lines.

More recently, e4-ICD was found to activate the transcription of a reporter linked to the β-casein upstream region [Williams et al., 2004]. These authors had previously demonstrated that mice harboring a breast-specific erbB-4 knock-out displayed defects in the proliferation of mammary epithelium and, surprisingly, failed to activate Stat5A, despite intact prolactin signaling (prolactin also activates Stat5A) [Long et al., 2003]. The transcriptional regulation of β-casein is dependent on the erbB-4 NLS within the e4-ICD, the kinase activity of erbB-4, and the presence of a functional SH2 domain in Stat5A [Williams et al., 2004]. Moreover, e4-ICD and Stat5A are both detected in ChIP assays of regulatory elements in the β-casein promoter. This data suggests a model whereby ligand binding can activate the erbB-4 kinase, leading to the recruitment of Stat5A to a phosphorylated receptor tyrosine residue (Y694). RIP then liberates this complex, allowing the e4-ICD to chaperone Stat5A to the nucleus. Receptor-docked STATs are thought to facilitate STAT dimerization [Greenlund et al., 1995; Yan et al., 1996], so it is unclear why Stat5A is not "released" from erbB-4 to dimerize with itself (or other phosphorylated STATs) in this instance. Could this be due to changes in the receptor complex induced by concomitant proteolytic cleavage? It should also be noted that although these authors propose that the ICD binds DNA via Stat5A, this does not fit with current structural knowledge. Specifically, STATs bind DNA as dimers [Chen et al., 1998]. Stat5A binding to the e4-ICD via a reciprocal SH2-phosphotyrosine interaction would preclude dimerization of Stat5A and another STAT. Thus, an additional protein might be required for binding the e4-ICD to DNA. Similarly, the mechanism of DNA binding for the intact receptors erbB-1 and erbB-2 and the I2-ICD remains unknown, marking this as a major area of future investigation.

PHYSIOLOGICAL ROLES FOR RECEPTORS IN THE NUCLEUS—ARE WE THERE YET?

Determining the physiologic function of cell surface receptors in the nucleus requires disentangling the direct or "nuclear" pathway from the well-established canonical pathway acting via cytoplasmic mediators. The cleanest approach is to mutate a sequence in the receptor that is required for transit to the nucleus and demonstrate that there is a corresponding loss of signaling output. It is, of course, critical to ensure that such a mutation does not adversely effect receptor expression at the cell surface, ligand dependent dimerization, kinase activation, or other aspects of the canonical signaling cascade. In the case of the intact receptors, given our very limited understanding of the translocation mechanism, this remains a difficult task. Thus far, of the group of intact receptor reported to translocate to the nucleus, a proven NLS (or equivalent binding site for a nuclear chaperone) has been identified in only one case: erbB-3. It is not known if mutating this site interferes with receptor function at the cell surface. Since the intrinsic tyrosine kinase domain in HER3 is inactive, it will be necessary to ensure that the NLS-mutated erbB-3 receptor can heterodimerize with other erbB family members and activate the kinase activity of the dimer partner. Moreover, since nuclear localization of erbB-3 has been reported to be constitutive [Offterdinger et al., 2002], it is unclear what functional endpoints could be examined in this case. A possible NLS has been identified in erbB-1 [Lin et al., 2001]. Demonstrating that this sequence is required for nuclear transit and assessing the effect of disabling mutations in this sequence on transcriptional and phenotypic endpoints may be the best near-term bet for demonstrating the physiologic function for an intact receptor in the nucleus. It should be noted that a number of the putative NLSs in receptor proteins are co-incident with the poly-basic "stop-transfer" sequences, which are found immediately carboxyl-terminal to the TMD and are believed to be involved in normal positioning of the receptor in the plasma membrane. Thus, it will be important to ensure that mutating/eliminating such sites does not interfere with normal trafficking of the receptor from the ER to the cell surface.

In the case of putative RIP substrates, one approach to demonstrate function is to examine the effects of a particular ligand–receptor pair in the absence of components of the cleavage machinery, employing inhibitors, RNAi, or deficient cell lines to inactivate or eliminate the JM or IM cleavage enzymes. Employing this approach, Carpenter and colleagues demonstrated that heregulin dependent cell death of T47D cells was blocked by a γ-secretase inhibitor [Ni et al., 2001]. Similarly, heregulin induced maturation of oligodendrocytes was blocked by the same inhibitor [Lai and Feng, 2004]. A limitation of these studies is the possible effects of the missing enzyme (γ-secretase in these examples) on proteins that are transcriptionally induced by the e4-ICD. As noted above, a more targeted approach is to mutate the receptor. In this case, the RIP translocation mechanism dictates that two critical elements must be present in the receptor protein: a JM cleavage site and an IM cleavage site. The nature of these sites suggests that mutations which prevent cleavage are unlikely to interfere with other receptor functions such as dimerization. Assuming the ICD is a transcription modulator, it should also contain a NLS, TAD, and DBD or bind to other proteins which provide these functions. One can anticipate that discretely mutating these sites will be generally more difficult, but they represent additional targets for disentangling the cell surface and nuclear effects of RIP-ed receptors. For erbB-4, the TACE cleavage site is known [Cheng et al., 2003], a mutation which prevents PS cleavage has been tentatively identified [Williams et al., 2004], and the function of an ICD-specific NLS has been verified [Williams et al., 2004], setting the table for testing the physiologic role of RIP signaling vis-à-vis canonical signaling by mutating one or more of these sites. It should be noted that JM site mutants may not be entirely "clean," since it is conceivable that the shed ECD could have a signaling role itself [Han et al., 2001; Hardy et al., 2001]. Thus, mutating the IM site, or perhaps the NLS site, represents the preferred approach. Germline knock-out of erbB-4 is lethal, with key defects in cardiac and neural development and mammary gland proliferation having been established by transgenic rescue [Gassmann et al., 1995; Tidcombe et al., 2003]. Thus, the functional role of RIP deficient alleles of erbB-4 can be tested in one or more of the previously described mouse genetic systems [Long et al., 2003; Tidcombe et al., 2003; Anton

et al., 2004; Golub et al., 2004; Thuret et al., 2004]. Likewise, for IFNaR2 and CSF1R, once JM and/or IM cleavage sites are identified, it should be possible to determine if RIP is required for the anti-viral/anti-proliferative effects of type I interferons [Samuel, 2001] or for the diverse biological effects of CSF1R [Dai et al., 2002].

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