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Localization of Protein Kinases by Anchoring Proteins: A Theme in Signal Transduction

Daria Mochly-Rosen

A fundamental question in signal transduction is how stimulation of a specific protein kinase leads to phosphorylation of particular protein substrates throughout the cell. Recent studies indicate that specific anchoring proteins located at various sites in the cell compartmentalize the kinases to their sites of action. Inhibitors of the interactions between kinases and their anchoring proteins inhibit the functions mediated by the kinases. These data indicate that the location of these anchoring proteins provides some of the specificity of the responses mediated by each kinase and suggest that inhibitors of the interaction between the kinases and their anchoring proteins may be useful as therapeutic agents.

Stimulation of many signaling cascades results in activation of protein kinases, which in turn phosphorylate their respective substrates, leading to diverse physiological responses. These diverse effects result, at least in part, from compartmentalization of a number of signaling molecules including protein kinases. Components in tyrosine kinase–mediated signaling pathways are compartmentalized (*1*) as are several serine and threonine protein phosphatases and some protein kinases (*2*). This review summarizes data indicating that: (i) cell stimulation causes translocation of several serine and threonine protein kinases to new sites, presumably to alter their access to their substrates; (ii) compartmentalization of these kinases results from their binding to specific anchoring proteins at their respective sites; and (iii) compartmentalization is required for the physiological functions of these kinases.

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Adenosine 3′,5′-Monophosphate (cAMP)–Dependent Protein Kinase

The cAMP-dependent protein kinase (PKA) is composed of two regulatory and two catalytic subunits. There are several gene products for each of these subunits and multiple PKA isoforms. Both the catalytic subunit and the type II PKA holoenzyme can be differentially compartmentalized at specific subcellular locations both before and after cell stimulation. Type II PKA is found in the cell particulate fraction, often anchored through the regulatory domain (RII) near its protein substrates. For example, this enzyme associates with microtubules (*3*) near some of its known substrates, the microtubule-associated proteins (*4*). Binding of cAMP to the holoenzyme releases the catalytic subunits, enabling them to phosphorylate their substrates. Therefore, localization of the type II kinase near its substrates may ensure rapid phosphorylation of specific substrates in response to increases in the intracellular concentration of cAMP ($[cAMP]_i$) (*5*).

The dissociated catalytic subunits may translocate to new subcellular sites to phosphorylate other substrates. In Madin Darby bovine kidney cells, for example, the holoenzyme is localized on the Golgi complex (*6*). When $[cAMP]_i$ is increased, the distribution of the regulatory subunit remains unchanged, whereas the catalytic subunit is first found in the cytoplasm and then in the nucleus (*6*). Translocation of the catalytic subunit into the nucleus in response to increases in $[cAMP]_i$ has also been demonstrated by microinjection of fluorescently tagged recombinant regulatory and catalytic subunits (*7*). Before stimulation, both subunits are found in the cytosol (Fig. 1). When $[cAMP]_i$ increases after cells are treated with forskolin, some of the catalytic subunit begins to dissociate from the regulatory subunit and diffuses into the nucleus. Translocation of the catalytic subunit is transient (*6*); 1 hour after removal of forskolin, the catalytic subunit returns to the

cytosol and re-associates with the regulatory subunits (*7*). The nucleus is devoid of RII (*8*) and it is not clear whether a different binding protein inside the nucleus anchors the catalytic subunit to that site. However, an endogenous inhibitor of PKA enhances the rate of export of the catalytic subunit from the nucleus (9). Therefore, anchorage of the holoenzyme near its substrates may determine the substrate specificity of each PKA isoform (*5*, *10*), and transient translocation of the catalytic subunit into the nucleus may provide both access to new substrates and a temporal separation of responses to a single stimulus.

The proteins that anchor PKA type II holoenzyme to the cell particulate fraction are termed AKAPs (for A kinase anchoring proteins). AKAPs are abundant proteins associated with the cell cytoskeleton and many of them are also PKA substrates. Several AKAPs have been identified and cloned (*10–12*). These proteins, which bind RII, include microtubule-associated protein 2, the proteins Ht31, Ht21, p85, and AKAP79. Anchoring of the calcium- and calmodulin-dependent protein phosphatase 2B (calcineurin) by an AKAP79 protein has been demonstrated by interaction cloning and partial co-localization in cultured hippocampal neurons (*13*). Binding of this phosphatase occurs through a site distinct from the RII-binding site and is also specific; two other phosphatases do not bind to AKAP79. It is estimated that only 5% of the total amount of calcineurin in lysates from bovine brain associates with this AKAP (*13*). In addition, AKAP79 binds inactive calcineurin and can inhibit the catalytic activity of both the calcium-dependent form and a constitutively active mutant. These findings are not consistent with activation-dependent dissociation of calcineurin from AKAP79 as is found with PKA type II. Nevertheless, compartmentalization of both a kinase and phosphatase to the same site may provide a coordinate activity of two enzymes with opposite catalytic activities. Whether both enzymes act on the same protein substrate at this site remains to be determined.

Overexpression of AKAP75 (a homolog of AKAP79) in a human embryonic kidney cell line (HEK293) increases the proportion of RII associated with the cytoskeletal fraction from ~10% in control cells to ~90% in cells overexpressing AKAP75 (*14*). An increase in the amount of the catalytic subunit in the cytoskeletal fraction also occurs. These data are consistent with a role for AKAP75 in compartmentalizing the PKA holoenzyme.

If the binding of PKA to AKAPs is required for its localization and function, then inhibitors of this binding should interfere with PKA-mediated signal transduction. The binding sites on several AKAPs for the RII subunit have been mapped. Although the specific amino acid sequences of the binding sites differ, the RII-binding region on each AKAP corresponds to an amphipathic helical structure with acidic residues at the hydrophilic face of the helix (*11*). Mutations that disrupt this secondary helical structure in one AKAP, Ht31 reduce the RII subunit binding in vitro (*11*), and a 24–amino acid synthetic peptide derived from this helical region of Ht31 inhibits the binding of the regulatory subunit to several AKAPs in vitro (*8*). Moreover, microinjection of the peptide into hippocampal neurons prevents PKA-mediated regulation of a specific glutamate receptor–gated ion channel; this inhibition can be overridden by injection of purified PKA catalytic subunit. Because this peptide does not affect the catalytic activity of PKA, these results support the hypothesis that compartmentalization of PKA with its substrate is required for its function. The results also suggest that the PKA holoenzyme dissociates from the AKAPs, enabling peptide binding. However, there is no evidence yet indicating reversible association of RII to AKAPs.

The β-Adrenergic Receptor Kinase (βARK)

On binding of hormones to heterotrimeric, guanine nucleotide binding protein (G protein)–coupled receptors, such as the β-adrenergic receptor, the receptor undergoes phosphorylation and desensitization (*15*, *16*). Phosphorylation of the agonist-occupied receptor, which turns this signaling pathway off, is mediated by a protein kinase termed βARK or GRK2 [for β-adrenergic receptor kinase or G protein–coupled receptor kinase, respectively (*16*, *17*)]. On agonist binding to the receptor, βARK translocates from the cytosol to the plasma membrane (*16*, *17*) and phosphorylates the agonist-occupied receptor. Within several minutes after adrenergic receptor stimulation, more than 70% of the βARK in a cell translocates from the cytosol to the membrane; a sevenfold increase in the amount of membrane-associated kinase is observed, and one mole of phosphate is incorporated per mole of β-adrenergic receptor (*16*, *17*). Translocation of βARK is transient; within 20 min, most of the βARK activity is found in the cytosol again (*16*, *17*). Therefore, translocation of βARK localizes it to the site of its specific substrate.

The association of βARK with the particulate fraction lasts many minutes. Therefore it is unlikely that it reflects binding of the active site of the enzyme to its substrate. Indeed, in vitro studies demonstrate that recombinant βARK binds directly to membrane-associated βγ subunits of G proteins through a site distinct from the active site, and that this binding increases the catalytic activity of βARK (*18*). Therefore, βARK appears to bind to βγ subunits released from the α subunit at an agonist-occupied receptor and to inhibit the response of the receptor to further stimulation. This prediction was confirmed using odorant-stimulated G protein–coupled receptors in olfactory cilia cells as an assay system. Inhibition of translocation of a βARK homolog, βARK2, by either a fragment of the enzyme or a 26–amino acid peptide that contains the βγ-binding site inhibits desensitization of odorant-occupied receptor, presumably because these peptides interfere with binding of βARK to βγ subunits (*19*). Therefore, translocation of βARK2 appears to be required for its function. Because there are multiple βγ subunits and several GRK isoforms (*20*), there may be isoform-specific translocation as well. Finally, because several GRK isoforms do not have the βγ subunit–binding region (*20*), it is possible that there may be other anchoring molecules for these kinases.

Raf

Raf-1 is a cytosolic protein kinase that is translocated to the plasma membrane in response to binding of growth factors to their receptors. Raf-1 is also found in the plasma membrane fraction in cells overex-

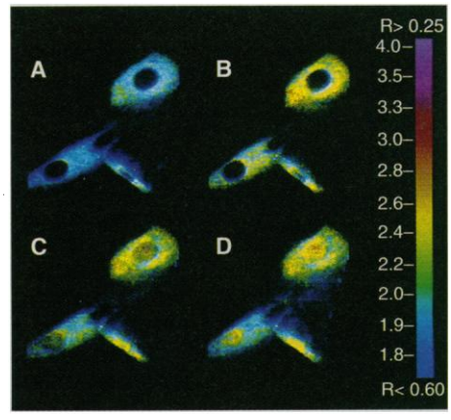

Fig. 1. Translocation of PKA catalytic subunit to the nucleus in response to changes in $[cAMP]_i$. Pseudo-color images of fluorescence emission ratio demonstrate the subcellular localization of the PKA subunits in three REF-52 fibroblasts before and during exposure to 50 μM forskolin. Increasing intensities denote an increase in the amount of free catalytic subunit of PKA. The spectrum on the right is the color scale from blue to red, where blue represents the lowest amounts of free catalytic subunit (under basal conditions). Black is found in areas were the PKA holoenzyme is not present (inside the nucleus before stimulation). Experimental details are described by Adams *et al.* (*7*). The figure was contributed by R. Tsien.

pressing the guanine nucleotide–binding protein Ras (*21*, *22*). Translocation is required and sufficient for Raf activation; targeting of Raf to the plasma membrane of COS cells by addition of a farnesyl group at a COOH-terminal CAAX sequence (in which C is cysteine, A means aliphatic amino acid, and X refers to any amino acid) results in partial activation of Raf (*22*, *23*) and subsequent downstream cellular responses (*23*). Farnesylation does not increase the catalytic activity of Raf in vitro (*24*). In addition, a single amino acid mutation in Raf that prevents Ras binding in vitro prevents also Ras-mediated activation of recombinant mutant Raf in vivo (*25*). Because Raf is found in the cell particulate fraction when Ras is overexpressed (*21*, *22*) and Ras physically interacts with Raf (*26*), it appears that Ras might be an anchoring molecule for Raf. Although there is evidence for Ras-independent pathways for Raf activation (*23*, *25*), Ras appears to have a role in anchoring of Raf near its activators.

Once recruited to the plasma membrane, Raf becomes attached to the cytoskeleton whereas Ras does not (*22*). Therefore, another protein or proteins must mediate the prolonged association of Raf with the plasma membrane. In search of other Raf-binding proteins, several laboratories have used the two-hybrid system and demonstrated that two members of the 14-3-3 protein family bind Raf (*27*, *28*). Studies in yeast (*27*, *29*) and in *Xenopus* oocytes (*28*) demonstrate that overexpression of 14-3-3 activates Raf function. However, 14-3-3 binds inactive Raf in the cytosol and also co-localizes with activated Raf at the plasma membrane (*27*, *28*). Therefore, 14-3-3 is unlikely to be a Raf activator, but rather it may be an adapter molecule that enables association of Raf with other activators (*30*). The use of peptides that mimic the interaction sites between Raf and 14-3-3 proteins may elucidate the role of this interaction in Raf signaling. The specificity of members of the 14-3-3 family for Raf has not been directly examined. However, members of the 14-3-3 family associate also with other proto-oncogenes (*31*). Therefore, 14-3-3 proteins may have a more general role in protein compartmentalization.

Protein Kinase C

Protein kinase C (PKC) is a well-characterized example of a cellular kinase that is translocated and activated in response to hormone stimulation. The PKC family members are kinases whose activity depends on phospholipid, diacylglycerol (DG), and in some cases on calcium (*32*). Before stimulation, PKC is present in the cytosol, whereas its activators are hydrophobic and are present in the membrane (*33*). Stimulation of cells with the tumor promoter phorbol ester or hormones that increase intracellular concentrations of DG induces translocation of PKC from the soluble (cytosolic) fraction to the particulate fraction as measured by cell fractionation (*34*). Translocation of PKC has also been demonstrated by immunofluorescence studies (*35*) and by electron micrography (*36*) and phosphorylation of endogenous substrates increases upon PKC translocation (*37*, *38*).

Individual PKC isozymes appear to be restricted to particular cell sites before stimulation. For example, in nonstimulated cardiac myocytes, εPKC is found in the nucleus (Fig. 1E), whereas βIIPKC is associated with fibrillar structures (Fig. 1C), possibly intermediate filaments (*39*). In some cases, a small proportion of the enzyme is tethered near its substrate; for example, part of the cellular αPKC co-localizes with vinculin in focal contacts of REF-52 fibroblasts (*40*) and with the myristoylated, alanine-rich PKC substrate MARCKS in punctate structures of macrophages (*38*). Therefore, co-localization of some inactive PKC isozymes with their substrates may serve to ensure preferential and rapid phosphorylation of these substrates after PKC activation.

In cells stimulated with hormones or phorbol esters, most or all of the cellular PKC translocates to new subcellular sites, including the plasma membrane (*35*), cytoskeletal elements (*41*), nuclei (*42*, *43*), and others (*44*). Furthermore, within the same cell, various isozymes are each localized to different subcellular sites after cell stimulation (*43*, *45*, *46*). For example, in primary cardiac myocytes, stimulation of the α1-adrenergic receptor results in translocation of βIPKC from the cytosol into the nucleus (Fig. 2, A and B), βIIPKC from fibrillar structures outside the nucleus to the perinucleus and membrane structures (Fig. 2, C and D), and εPKC from inside the nucleus and in the cytosol to cross-striated structures, possibly the contractile elements in these cells (Fig. 2, E and F). Therefore, individual activated PKC isozymes are differentially compartmentalized, suggesting that they mediate distinct cellular functions.

More direct indication that differential localization of individual PKC isozymes results in distinct cellular functions has been obtained. In human promyelocytic leukemia cells (HL60), βIIPKC translocates to the nuclear envelope in cells treated with stimuli that cause proliferation but not in cells treated with stimuli that cause differentiation (*46*). Moreover, only βIIPKC phosphorylates nuclear envelope–bound lamin B, whereas both α and βIIPKC phosphorylate soluble lamin B (*47*). This difference appears to result from the action of a βIIPKC-specific lipid nuclear-activating factor [NMAF; (*47*)]. Because association of βIIPKC with the nuclear envelope correlates with proliferative responses (*46*), compartmentalization of activated PKC isozymes close to specific substrates appears to determine their functions.

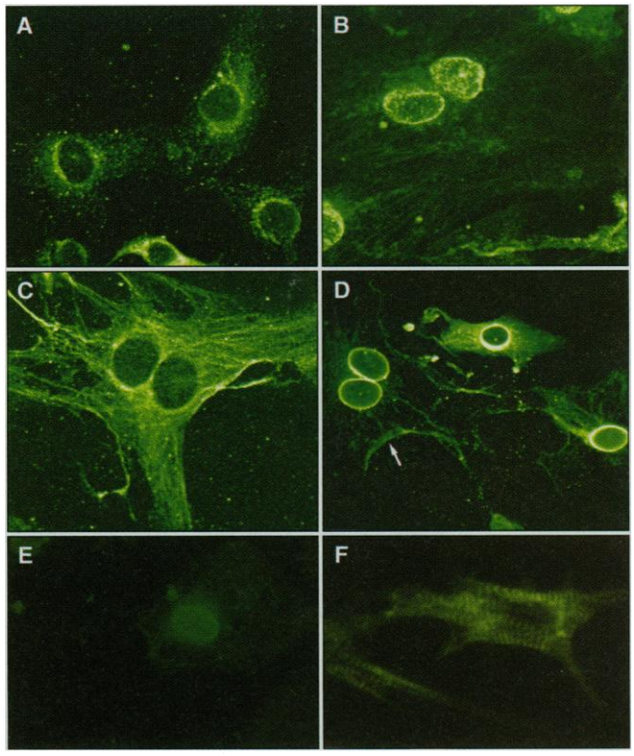

Fig. 2. Localization of PKC isozymes βI (**A** and **B**), βII (**C** and **D**) and εPKC (**E** and **F**) in primary cardiac myocytes before (A, C, and E) and after (B, D, and F) a 5-min treatment with 2 μM norepinephrine (*45*). Panels A through D are pseudo-color figures from fluorescence confocal microscopy, where black represents areas lacking isozyme immunostaining and green to green or white corresponds to areas with increased amounts of isozyme immunostaining. Some of these panels are reproduced from (*45*) with permission. Panels E and F are fluorescein isothiocyanate (FITC) true color photographs obtained with a conventional fluorescence microscope (*45*). Greater than 80% of the cardiac myocytes have the staining shown in each panel. Arrow in (D) indicates localization of βIIPKC to membrane structures.

The differential subcellular localization of activated PKC isozymes (*45*) and the observation that stable interaction of PKC with the plasma membrane in vitro is inhibited if the membranes are treated with trypsin (*48*) suggest that PKC binds to specific anchoring proteins located at the various subcellular sites. Several proteins that bind PKC have been identified and cloned, including several annexins, cytoskeletal proteins, and a nuclear protein (PICK1) (*49*, *50*). A group of proteins collectively termed RACKs, for receptors for activated C-kinase, have been also identified (*49*). One RACK, a homolog of the β subunit of G proteins (RACK1) has been cloned (*51*). So far, of all the PKC-binding proteins, only RACKs have been shown to be important for PKC-mediated functions.

RACKs of 30 to 36 kD were purified from the cell particulate fraction of rat heart and brain (*49*). These proteins bind activated PKC through a site distinct from the substrate binding site of PKC [Fig. 3 and (*49*)] suggesting that PKC binding to RACKs occurs after cell stimulation, to localize the active enzyme to the RACK site (*49*). This prediction has been supported by microinjection of *Xenopus* oocytes with purified RACKs. Microinjection of RACKs inhibits insulin-induced translocation of PKC and regulation of oocyte maturation (*52*), presumably because PKC binds to the soluble RACKs, and is thus prevented from binding to endogenous, anchored RACKs.

It is likely that specificity is determined in part by differential localization of isozyme-specific RACKs, providing anchorage of each isozyme close to its physiological substrates. RACKs specific, for example, for εPKC are expected to localize the enzyme to cross-striated structures, thus conferring a unique anchoring site and presumably unique function for this isozyme.

Inhibitors of PKC binding to RACKs can presumably mimic either the PKC-binding site on RACKs or the RACK-binding site on PKC (Fig. 3). To identify the PKC-binding site on RACKs, a 15–amino acid peptide (peptide I), representing a short similar sequence found in two PKC-binding proteins—annexin I and a member of the 14-3-3 family (*49*, *53*)—was synthesized. (This 14-3-3 binding protein is different from those identified as Raf-binding proteins). Peptide I binds PKC directly and inhibits PKC binding to RACKs (*54*). Furthermore, microinjection of peptide I into *Xenopus* oocytes inhibits insulin-induced translocation of PKC and oocyte maturation (*55*). Therefore, this peptide is a translocation inhibitor of PKC. Likewise, peptides that mimic the RACK-binding domain on the kinase may also act as translocation inhibitors in vivo (Fig. 3).

A peptide derived from a region of short

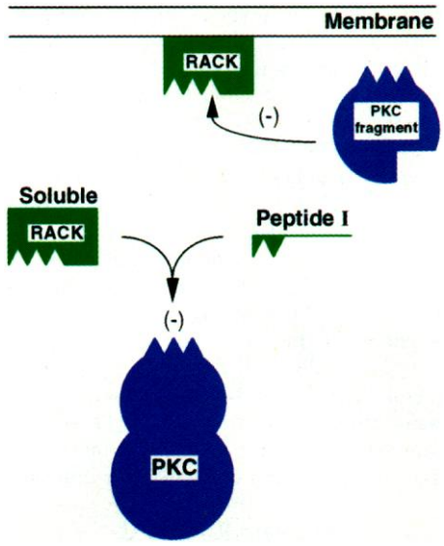

Fig. 3. Schematic representation of potential translocation inhibitors of PKC. See details in text; (−) denotes inhibition.

sequence similarity between βPKC and its anchoring protein RACK1 induces translocation of PKC and oocyte maturation when injected into *Xenopus* oocytes in the absence of insulin stimulation (*50*, *56*). Like the pseudosubstrate sequence in PKC that interacts with the substrate site of PKC and maintains it in an inactive state, this sequence may represent a pseudo-RACK site in the enzyme that binds to the RACK-binding site. It is possible that other kinases have similar pseudo-anchoring sites and that peptides that mimic these sites may act as translocation agonists. Such agonist peptides may be useful in evaluating the role of individual protein kinases in cell function. Moreover, translocation inhibitors and activators of protein kinases have potential therapeutic value.

A fundamental question in understanding the transfer of information from the outside to the inside of a cell is how kinases such as βARK and PKC that are present in one cell compartment are caused to translocate to another. The most likely explanation is that there is a weak interaction between these protein kinases and an anchoring protein in the target compartment. In the presence of the signal (for example free βγ subunits for βARK or increased concentrations of diacylglycerol for PKC), the binding affinity of the kinases for their anchoring molecules may increase, and the equilibrium between bound and free kinase may shift to favor the bound form. It is also possible that adapter proteins (such as 14-3-3 for Raf) mediate the increases in binding of these translocating kinases to their anchoring molecules.

In summary, a common theme in signal transduction is emerging. Several protein kinases bind to specific anchoring proteins at their site of action both before or after activation. The details of these complex events are only beginning to be uncovered. However, it is clear that localization of these serine-threonine protein kinases and other signaling molecules through protein-protein interactions is an essential component of signal transduction and provides an important means of regulation.

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Cytokine Signaling Through Nonreceptor Protein Tyrosine Kinases

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Cytokines are a family of soluble mediators of cell-to-cell communication that includes interleukins, interferons, and colony-stimulating factors. The characteristic features of cytokines lie in their functional redundancy and pleiotropy. Most of the cytokine receptors that constitute distinct superfamilies do not possess intrinsic protein tyrosine kinase (PTK) domains, yet receptor stimulation usually invokes rapid tyrosine phosphorylation of intracellular proteins, including the receptors themselves. It is now clear that these receptors are capable of recruiting or activating (or both) a variety of nonreceptor PTKs to induce downstream signaling pathways. Thus, the intracytoplasmic structure of cytokine receptors has evolved so as to allow the combined action of different PTK family members expressed in different cell types, which may ultimately determine the activity of cytokines.

Cell-to-cell communication is an essential aspect of many biological systems. Cytokines are a broad group of (mostly) soluble factors that mediate this communication and that have been extensively studied in the context of natural and acquired immunity, hematopoiesis, and inflammation (*1*). In addition, it is now widely appreciated that many cytokines act more broadly in many biological systems, including those regulating neural and embryonic development (*2*). Rapid progress has been made in the molecular characterization of cytokines and their receptors during the past 15 years, and the availability of recombinant cytokines has made it possible to study the function of each in its pure form. These studies have revealed two remarkable features of cytokines: their functional redundancy and extensive pleiotropy (*1*, *3*). In fact, a given biological function is often mediated by more than one cytokine, and each cytokine can display multiple functions. Typically, lymphocyte proliferation can be induced by a variety of interleukins in vitro, including interleukin-2 (IL-2), IL-4, IL-5, IL-6, IL-7, and IL-15 (*4*–*7*). Yet these interleukins can also induce distinct cellular responses such as lymphocyte differentiation and immunoglobulin class switching (*1*, *8*). Such diverse responses are mediated by a complex signaling system in which protein phosphorylation by nonreceptor PTKs appears to be critical. Here I summarize our current knowledge of the participation of such PTKs in cytokine signaling, primarily focusing on the IL-2–IL-2 receptor (IL-2R) system. Although the IL-2R has an unusual variety of intracellular signaling partners, all evidence suggests that a similarly heterogeneous array of signal transduction pathways can be invoked by other cytokine receptors.

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Structural and Functional Properties of Cytokine Receptors

Most of the cytokine receptors are type I membrane glycoproteins containing a single transmembrane domain and oriented with their NH_2-termini exterior to the plasma membrane. Cytokine receptors can be further divided into four subtypes, based on characteristic structural motifs found in their extracellular ligand binding domains (Fig. 1A). Regarding intracellular signal transduction, three important features of these receptors stand out. First, the COOH-terminal regions of these receptor molecules form cytoplasmic tails consisting of tens to hundreds of amino acids, yet they possess no motifs indicative of any known catalytic activity such as the PTK motifs that are the hallmark of many other growth factor receptors (*9*). Instead, two conserved motifs are found within the membrane-proximal regions of some receptors (referred to as box 1 and box 2 in Fig. 1A) (*6*, *10*). Second, many cytokine receptors consist of more than two subunits, although a few function as monomers. Third, one receptor subunit is often shared among different cytokine receptor complexes. For example, IL-2R consists of three subunits: the α, β, and γ chains (*11*), formally known as IL-2Rα, IL-2Rβc, and IL-2Rγc, respectively (Fig. 2). The small c denotes that these subunits are common to other cytokine receptors; IL-2Rβc is a subunit of IL-15R, whereas IL-2Rγc is a subunit of IL-4R, IL-7R, and IL-9R (*7*, *12*). Similarly, IL-3R shares its IL-3Rβc subunit with IL-5R and the granulocyte-macrophage colony-stimulating factor receptor (GM-CSFR), whereas the IL-6Rβc (gp130) subunit of IL-6R is also used by the leukemia inhibitory factor receptor (LIFR), the oncostatin M receptor (OSMR), and others (*3*). Thus, one envisages that the functional redundancy of certain cytokines may be partly explained by