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Research Overview

Proteinase-Activated Receptor Domains and Signaling

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ABSTRACT Given that the proteinase-activated receptors (PARs) are activated by a ''tethered ligand mechanism,'' an important question to answer is: Which other extracellular domains of the receptors are involved in this novel signaling mechanism? Further, as for other G-protein-coupled receptors (GPCRs), it is of importance to know about the intracellular receptor domains that are involved in coupling receptor activation to signal transduction. Studies summarized in this article have singled out the importance of extracellular loop-2 of PAR_1 and PAR_2 for tethered-ligand signaling. In human PAR_1, but not in PAR_2, a short sequence in the extracellular N-terminal domain (Q^{83} to G^{94}) is also important for receptor activation. As for other GPCRs, intracellular loops 2 and 3 mediate receptor-G-protein coupling, and the C-terminal sequence, with a putative ''palmitoylation'' site and target residues for kinase C phosphorylation, plays a role in receptor signaling and desensitization. This article provides an overview of the experiments leading to the current understanding of the PAR domains involved in signal transduction. Drug Dev. Res. 59:344–349, 2003. © 2003 Wiley-Liss, Inc.

RECEPTOR DOMAINS AND TETHERED-LIGAND ACTIVATION

As for other pharmacological receptors, PARs must be able to translate the binding of the ligand into a trans-membrane signal. In the case of the PARs, this process is complicated by the fact that the ligand remains attached to the receptor, so that a specialized "shut-off" mechanism must ensure that the receptor does not remain permanently activated. Also, like other receptors, the PARs must interact with and activate membrane-localized effectors (in this case, the G-proteins) to propagate a trans-membrane cellular signal. For this signaling purpose, and for the processes of ligand-mediated receptor activation and signal termination, specific receptor sequences playing key roles can be identified. To date, the majority of the "receptor structure-activity relationship" studies

Abbreviations used: Amino acids are abbreviated by their one-letter codes: A for alanine, S for serine, etc. AP = receptor-activating peptide (PAR-AP); ECL = extracellular receptor protein loop (ECL1,2,3); ERK = extracellular-regulated protein kinase (ERK1,2); GPCR = G-protein-coupled receptor; ICL = intracellular receptor protein loop (ICL1,2,3 and a putative transient icl4); MAPkinase = mitogen-activated protein kinase (also designated ERK); PAR = proteinase-activated receptor (PARs 1,2,3, and 4).

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undertaken to identify the receptor sequences responsible for function have been conducted using human PAR_1, with some work also reported for rat and human PAR_2. As yet, no studies of sufficient detail have been undertaken for PARs 3 and 4.

Figure 1 shows schematically the multiple domains of the PARs, comprising: (1) the N-terminal sequence that contains the cryptic tethered ligand (TL) and an N-terminal sequence (NT) important for PAR_1 signaling, (2) three extracellular loops (ECL1,2,3), (3) three main intracellular loops (ICL1,2,3) as well as a fourth putative loop, icl4, that can be formed transiently via reversible palmitoylation of a C-terminal cysteine residue, and (5) the C-terminal tail of the receptor involved in signal termination/desensitization and trafficking (see below). As well, Figure 1 depicts the potential sites of *N*-linked glycosylation (see Compton, this Special Issue of Drug Development Research 59(4):350). Information about the PAR domains involved in receptor activation has come mainly from studies of human /xenopus PAR_1 receptor chimeras, in which domains from human and *Xenopus laevis* PAR_1 have been exchanged [Gerszten et al., 1994] and from work with chimeras of human PAR_1, in which segments of the extracellular domains of murine PAR_2 were inserted into the sequence of PAR_1 [Lerner et al., 1996]. In such "domain-swap" experiments, it was found that substitution of the human PAR_1 ECL2 (see Fig. 1) into xenopus PAR_1 conferred on the chimeric receptor a sensitivity towards the human PAR_1AP, SFLLRN-amide, which was otherwise essentially inactive in the wild-type *Xenopus* PAR_1 [Gerszten et al., 1994; Nanevicz et al., 1995]. Also of interest was the finding that replacing hPAR_1 ECL2 with *Xenopus* ECL2 yielded a receptor that was constitutively active [Nanevicz et al., 1996]. An essential role for ECL2 in conferring agonist specificity and involvement in transmembrane signaling was therefore demonstrated.

In a similar vein, substitution of the ECL2 sequence of murine PAR_2 into human PAR_1 conferred on the chimeric receptor sensitivity to the PAR_2AP, SLIGRL-amide, which does not activate human PAR_1 [Lerner et al., 1996]. Again, ECL2 was singled out for its importance in conferring agonist specificity; and the work demonstrated further that ECL3, as well as an extracellular N-terminal sequence (see below), were important for peptide-mediated receptor activation. Unfortunately, these domain-swap experiments did not examine in any depth the activity of the proteolytically revealed tethered ligand in addition to focusing on the activity of the PAR-activating agonist peptides. Further work using the PAR-activating peptides as probes singled out the importance of specific acidic residues in the ECL2 of PAR_1 and PAR_2 (ECL2, Fig. 1: E^{260} in human PAR_1 and $E^{232}E^{233}$ in rat PAR_2) for peptide-mediated receptor activation [Nanevicz et al., 1995; Al-Ani et al., 1999].

On the basis of these ECL2 substitution studies, it was suggested that there may be an electrostatic interaction between these acidic ECL2 residues and the basic arginine side chain at position 5 of the PAR-activating peptides [Nanevicz et al., 1995; Al-Ani et al., 1999]. However, as outlined elsewhere in this issue (see Hollenberg, this Special Issue of Drug Development Research 59(4):336), a comparable interaction between a basic residue at position 5 of the tethered ligand with an acidic residue in ECL2 probably does not account for activation of the PARs by the tethered ligand unmasked by trypsin or thrombin [Al-Ani et al., 2002]. Other acidic groups in the extracellular domain of human PAR_1, one in ECL2 (D^{256}) and another in ECL3 (E^{347}) have also been found to be important for receptor activation by PAR-APs (activating peptides), but not for the tethered ligand exposed by thrombin [Blackhart et al., 2000]. An important role for a non-acidic residue in ECL2 of human PAR_2 was revealed by the discovery of a polymorphic form of the receptor that has a phenylalanine to serine mutation at F^{240}. This polymorphic form of the receptor displays a reduced sensitivity to trypsin activation and a distinct profile for activation by PAR-activating peptides [Compton et al., 2000]. Using an alternate approach to assess receptor domain function, Bahou and colleagues generated monoclonal antibodies targeted to distinct sequences of the N-terminal domain of human PAR_1 [Bahou et al., 1994]. One of these monoclonals, targeted to a PAR_1 sequence comprising residues $Q^{83}$to G^{94}, was able to delineate an extracellular N-terminal stretch involved in activation of the receptor either by its tethered ligand (thrombin-unmasked) or by a PAR_1-activating peptide [Bahou et al., 1994].

In summary, much information singles out ECL2 (Fig. 1) as playing a key role for receptor activation of PARs 1 and 2 (and by extension, PAR_4) either by the proteolytically-revealed tethered ligand or by soluble receptor-activating peptides. That said, the direct interactions of the peptides and the tethered ligand with the extracellular receptor domains, so as to trigger receptor activation, would appear to differ, as outlined elsewhere in this issue (see Hollenberg, this Special Issue of Drug Development Research 59(4):336). Further, in human PAR_1, an essential role for receptor activation by both the tethered ligand and peptide agonists has been established for residues Q^{83} to G^{94} (designated NT in Fig. 1); the role for the com-parable sequence in PAR_2 has not been carefully exa-mined. In addition, it is clearly the case that the structure of the

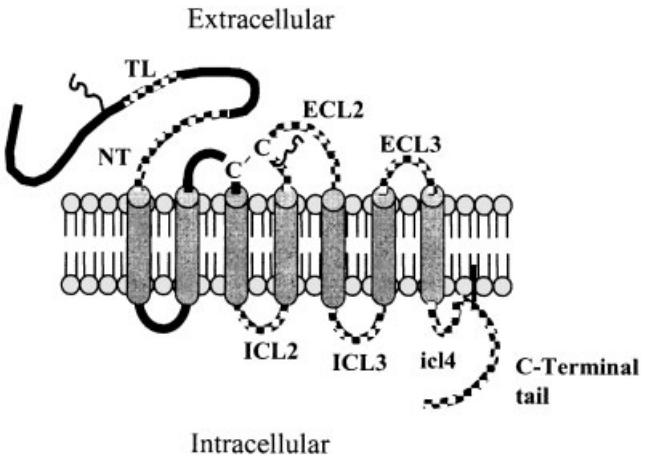

Fig. 1. Modelling of PAR functional domains. The cartoon illustrates the distinct regions (checkered sequences) of the receptor that are involved in the activation/signaling/desensitization/internalization process. The tethered ligand sequence (TL) is unmasked by proteolytic activation, resulting in the putative docking of the TL domain in approximation of extracellular loop-2 (ECL2). Residues in the N-terminal extracellular sequence (NT) play a role in the activation PAR_1 activation, as do residues in ECL3. G-protein coupling is mediated via intracellular loops 2 and 3 (ICL2, ICL3) with the possible participation of a putative fourth loop (designated in lower case letters, to indicate the potential transient nature of this loop: icl4) formed by a palmitoylated cysteine residue (solid line) in the C-terminal domain. The C-terminal tail sequence beyond the palmitoylated cysteine residue is thought to play an important role in receptor signaling kinetics, desensitization/internalization, and trafficking. Sites of PAR_2 glycosylation are also shown.

other ECLs of the PARs will contribute to receptor function; but the discrete sites of interaction between the proteolytically revealed tethered ligand and these extracellular domains remain to be established.

TRIGGERING AND QUENCHING TRANSMEMBRANE SIGNALING

As alluded to above, the activated receptor must not only couple to intracellular signaling mechanisms [outlined by Dery et al., 1998; Macfarlane et al., 2001; see also Macfarlane and Plevin, this Special Issue of Drug Development Research 59(4):367] but must also be subject to feedback inhibition, so that the signal can be turned off. Further, unlike other G-protein-coupled receptors, the PARs, once "used" by the proteolytic mechanism must be replaced at the cell surface without re-cycling, so as to allow the cell to respond again efficiently to proteinases. Built into the PARs are domains that deal specifically with each of these "challenges" faced by the receptor. In terms of generating intracellular signals, ICL2 and 3 as well as a portion of the C-terminal tail (Fig. 1) are believed to couple to the three principle G-proteins (G_q, G_i, $G_{12/13}$) that, in turn, regulate downstream PAR-stimulated processes. That said, very few detailed studies have been done [e.g., Verrall et al., 1997] to map the key PAR residues on ICL2, ICL3, and possibly in the putative fourth intracellular loop (icl4, Fig. 1) that are responsible for interacting with G-proteins [Swift et al., 2000]. An intriguing study employing short (10 to 19 residues) ICL3 sequences from PARs 1,2, and 4 that have been N-terminally palmitoylated, so as to act as cell-penetrating agonists (so-called pepducins), has identified specific sequences of the receptors that would appear to interact with and trigger G_q, resulting in a calcium signal [Covic et al., 2002a,b]. A potential role for the putative fourth intracellular loop of the PARs that might form upon palmitoylation of the C-terminal sequence (icl4, Fig. 1), in terms of signaling to downstream effectors [Qanbar and Bouvier, 2003], has yet to be evaluated for the PARs. Given the distinct abilities of the PARs to couple to different G-proteins [see Macfarlane et al., 2001; Hollenberg and Compton, 2002] (see also Macfarlane and Plevin, this Special Issue of Drug Development Research 59(4):367), even when the receptors are situated in the same cellular environment, it is evident that the distinct detailed structure-activity relationships for G-protein coupling by each of the ICLs of the different PAR family members will be of considerable interest.

Upon activation by their tethered ligands, the signal generated by PARs (e.g., elevation of intracellular calcium via stimulation of G_q) is transient, lasting a relatively short period of time. For instance, a PAR_1 or PAR_2 triggered elevation of the intracellular calcium concentration will return to baseline within 2 min [Kawabata et al., 1999]; and a PAR_1-mediated elevation of smooth muscle tension in a vascular or gastric tissue preparations returns to baseline within 5 to 10 min [Laniyonu and Hollenberg, 1995]. These transient

responses triggered by the PARs differ from more sustained responses to the activation of other G-protein-coupled receptors, like the alpha-adrenoceptor. For instance, in an endothelium-denuded rat aortic ring preparation that responds to thrombin-mediated PAR_1 activation with a transient increase in tension, activation of the alpha-adrenoceptor by phenylephrine causes a persistent elevation of tension that can last for at least 2 h. The very rapid desensitization of PAR responses is coupled to a receptor internalization process that is also linked to signaling events like the activation of MAPkinase/ERK1,2 [DeFea et al., 2000]. Like other G-protein-coupled receptors, both PAR_1 and PAR_2 can be modified by site-targeted phosphorylation in the C-terminal domain either by the so-called G-protein-coupled-receptor kinases (GRKs: [Krupnick and Benovic, 1998]) or by kinase C [Ishii et al., 1994; Böhm et al., 1996]. This phosphorylation in the C-terminal domain then plays a role in subsequent receptor interactions with beta-arrestin and dynamin [Dery et al., 1999; DeFea et al., 2000], leading to both signal down-regulation/desensitization and receptor internalization. In human PAR_2, the potential phosphorylation sites in the C-terminal domain at Serine-363 and threonine-366 have been found to play a key role not only in regulating the sensitivity and persistence of a signaling event, but also in changing *qualitatively* the manner in which MAPkinase/ERK1,2 is activated. This conclusion was reached by the findings: (1) that a $PAR_2S^{363}T^{366}/A^{363}A^{366}$ mutant lacking the potential C-terminal phosphorylation sites was more sensitive to trypsin activation, and generated a calcium signal that lasted 3-times longer than the signal caused by the wild-type receptor; and (2) that the same mutant activated ERK1,2 via distinct pathways [DeFea et al., 2000].

Potential phosphorylation sites in the C-terminal domain of human PAR_1 have also been linked to signaling. A human PAR_1 truncation mutant (Y397Z) lacking 7 of the potential 11 phosphorylation targets (beyond the putative reversible palmitoylation site forming icl4 shown in Fig. 1) was found to signal more robustly (inositide production) than the wild-type receptor and to remain persistently at the cell surface upon activation by a PAR-AP agonist peptide [Shapiro et al., 1996]. There are also distinct differences between members of the PAR family in terms of their signaling kinetics, presumably due to differences in phosphorylation of the C-terminal domain. For instance, the calcium signal generated by PAR_1 activation is shut off more rapidly than that generated by PAR_4 activation in a comparable host cell environment. Further, upon activation by its agonist peptide, PAR_4 remains at the cell surface for a longer time than does peptide-activated PAR_1. This increase in signal generation by PAR_4, associated with its persistent residence at the cell surface, can be correlated by a resistance of PAR_4 to phosphorylation, in comparison with PAR_1, upon activation of each receptor with its cognate agonist peptide [Shapiro et al., 2000]. Thus, differences in the C-terminal domains of the PARs appear to confer differences both qualitatively and quantitatively in their signaling kinetics.

The trafficking of PARs either from the cell surface to the cytoplasm (as alluded to above) or from the Golgi to the plasma membrane appears to differ from other members of the G-protein-coupled receptor superfamily. For instance, unlike the $beta_2$-adrenergic receptor, PAR_1 in selected cell types has an intracellular reservoir from which the cell surface receptor can be replenished via a mechanism regulated by the signaling/internalization process itself [Hein et al., 1994]. Thus, the PAR system is comparatively energy expensive compared to the $beta_2$-adrenergic receptor system, because the cell is required to synthesize new PAR receptors following each PAR signaling event. It is assumed that receptor phosphorylation, presumably on the C-terminal tail, may regulate this re-insertion mechanism. However, the observation that PAR-agonist peptide can initiate PAR signaling following proteolytic activation has led to the suggestion that a small proportion of "used" PAR is recycled back to the cell surface [Brass et al., 1994]. How exactly the cell prevents the tethered ligand in these "used" PARs from continually activating the receptor, whilst simultaneously allowing an exogenously added PAR agonist peptide to activate the receptor, is currently unclear. That said, correct folding and maturation of the receptor may be required for precise trafficking to the cell surface, since even a seemingly innocuous substitution of an alanine for a glutamine in the ECL2 of PAR_2 can lead to a substantial portion of the receptor sequestered intracellularly [Compton et al., 2002]. The precise receptor residues involved in this signal-mediated trafficking of PARs to the cell surface have yet to be defined.

Rather than being re-cycled to the plasma membrane, as are other G-protein-coupled receptors like the one for substance P, PARs 1 and 2, upon activation, are selectively targeted to the lysosome [Dery et al., 1999; Trejo and Coughlin, 1999]. The same is presumed to be the case for PAR_4; but as with the distinct activation dynamics that distinguish PAR_4 from PAR_1, there may also be differences for PAR_4 in the internalization and trafficking processes. The precise amino acid residues responsible for the selected targeting of PARs to the lysosomal

compartment remain to be identified. It is quite likely that amongst the PARs, the distinct C-terminal amino acid sequences that probably play key roles in this trafficking process will lead to distinct sites of receptor targeting upon activation.

CONCLUDING REMARKS

In summary, the distinct receptor domains in each of the PARs can be seen to play quite different roles in terms of (1) tethered ligand activation, (2) G-protein coupling, and (3) receptor desensitization/internalization and trafficking (both inward and outward). In general, ECL2 can be said to play a key role in docking of the PAR tethered ligand; but the precise site of this docking and the role(s) of the other extracellular domains in the receptor activation process have yet to be determined in detail for all of the PARs. G-protein coupling, no doubt, involves ICL2 and 3, with the possible participation of the C-terminal tail to be confirmed. Yet, the precise PAR amino acid residues that interact with the different G-proteins remain to be identified. Undoubtedly, the differences in ICL2 and ICL3 between PARs will be found to explain the ability of one member of the family (PAR_1) to couple both to G_q and G_i, [Hung et al., 1992; Swift et al., 2000], whereas another family member (PAR_4) couples to G_q but not G_i [Faruqi et al., 2000]. Finally, the intracellular C-terminal sequences of the PARs can be seen to affect signaling dynamics and trafficking. Again, distinct sequences in this C-terminal domain will confer distinct signaling and trafficking properties on the different members of the PAR family. Thus, much remains to be learned about the PAR structure-activity relationships for the different functions that the receptors perform. One can look forward with great interest to learning about the precise structural information that will define clearly the specific functional sites on all of the PARs.

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