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Commentary

Protease-activated receptors start a family

Shaun R. Coughlin

Cardiovascular Research Institute and Department of Medicine, University of California, San Francisco, CA 94143-0524

Cells sense their environment by using specific receptors to detect extracellular ligands. Classically, information transfer occurs by the reversible binding of ligand to receptor. An exception to such reversible ligand–receptor interactions was uncovered with the cloning of a cell surface receptor that mediates responses to thrombin, a serine protease with a host of actions on platelets and other cells. The thrombin receptor is a seven-transmembrane-domain G protein-coupled receptor (1, 2), but it is activated by an unusual mechanism. Thrombin cleaves the amino-terminal exodomain of the receptor. This irreversible proteolytic event unmasks a new amino terminus that serves as a tethered peptide ligand, binding intramolecularly to other receptor domains to effect receptor activation (Fig. 1) (1, 3). It was hoped that the thrombin receptor might sire a family of receptors that use this mechanism, but until now it has remained solitary. This isolation now appears over. In this issue, Nystedt *et al.* (4) report the cloning of a new protease-activated receptor that is closely related to the thrombin receptor. In celebration of this new arrival, dubbed PAR-2 for proteinase-activated receptor 2, this commentary reviews the evidence for the mechanism of receptor activation that defines this fledgling family and notes some of the questions and opportunities raised by it.

The distinctive proteolytic mechanism by which thrombin-receptor activation proceeds is relatively well-established (Fig. 1) (1–3, 5, 6). Thrombin recognizes the amino-terminal exodomain of its receptor via two interactions: the LDPR amino acid sequence docks in the active center of thrombin, and the DKYEPF sequence binds the thrombin anion-binding exosite. Thrombin then cleaves the peptide bond between receptor residues Arg-41 and Ser-42 to unmask a new amino terminus beginning with the sequence SFLLRN. Mutation of the Arg-41/Ser-42 cleavage site to an "uncleavable" Arg/Pro site rendered the receptor unactivatable by thrombin (1). Replacement of the thrombin-cleavage recognition sequence LDPR/S with DDDDK/S, the recognition site for enteropeptidase, switched receptor specificity. Cells expressing this construct responded to enteropeptidase but did not respond to thrombin (5, 7). Thrombin cleavage of its receptor on intact cells was demonstrated by using antibodies to the activation peptide of the receptor (the fragment cleaved from the receptor by thrombin) vs. other receptor domains retained after cleavage (8). Mutation of the Arg-41/Ser-42 site to Arg-41/Pro-42 prevented receptor cleavage in these studies. The rates of receptor cleavage and second-messenger generation correlated well (8). Taken together, these data strongly suggest that cleavage of the Arg-41/Ser-42 peptide bond is not only necessary but sufficient for receptor activation by proteases.

The importance of the receptor's DKYEPF sequence for thrombin–receptor interaction has been demonstrated in functional studies with mutant receptors and in biochemical studies with receptor-based peptides (5, 6, 9). These studies identified receptor residues Tyr-52, Glu-53, and Phe-55 as key for interaction with thrombin and suggested that they might dock with the anion-binding exosite of thrombin in a manner similar to residues Phe-56, Glu-57, and Ile-59 of the leech anticoagulant hirudin (10) (Fig. 1). Recent x-ray crystallographic studies of cocrystals of thrombin with receptor-based peptides confirmed this analogy (11). The thrombin receptor has thus evolved at least two distinct domains (the LDPR and DKYEPF amino acid sequences) to mediate recognition by thrombin, presumably a device for promoting specificity.

How might proteolysis within the amino-terminal extension of a receptor activate that receptor? Synthetic peptides that mimic the new amino terminus created when thrombin cleaves its receptor were full agonists for receptor activation and bypassed the requirement for receptor proteolysis (1, 12, 13). This key observation suggested two possible models (1, 3): (*i*) the tethered-ligand mechanism described above, and (*ii*) the thrombin receptor is tonically constrained in an off state by the amino-terminal exodomain; receptor cleavage or competition by exogenous peptide then releases the receptor from this tonic inhibition. This second model was refuted by the observation that a mutant thrombin receptor lacking an amino-terminal exodomain was not constitutively active, as would be predicted by the release-from-inhibition hypothesis, and responded to synthetic peptide representing the tethered-ligand-domain peptide like the wild-type receptor. Other experiments confirmed that *intra* as opposed to *inter*molecular liganding is the predominant mode of thrombin-receptor activation (3).

The thrombin receptor can thus be viewed as a peptide receptor that contains its own agonist. This "agonist-peptide" or "tethered-ligand" domain is kept silent in the naive receptor to be unveiled by receptor cleavage. How is this accomplished? Structure–activity studies with synthetic peptides representing the tethered-ligand domain revealed the peptide's protonated amino group to be one of several features critical for agonist function (12–14). Creation of the corresponding protonated amino group in the receptor by thrombin cleavage of the Arg-41//Ser-42 peptide bond may thus be an important mechanism for "switching on" the intrinsic agonist peptide domain of the receptor. Release from steric interference provided by the activation peptide presumably also contributes.

PAR-2 now arrives on the scene, cloned from a mouse genomic library with oligonucleotide probes based on the bovine substance K receptor. The open reading frame encoded a 395-amino acid protein with seven putative transmembrane domains and other features common to G protein-coupled receptors. Search of the European Molecular Biology Laboratory data base revealed PAR-2 to be most closely related to the thrombin receptor with ≈30% overall amino acid-sequence identity, 42% in the region bounded by transmembrane domains one and seven. This result raised the possibility that PAR-2 might be a protease-activated receptor, and indeed, trypsin proved to be a potent activator of PAR-2 expressed in *Xenopus* oocytes. Sequence alignment with the thrombin receptor (Fig. 1) suggested a possible cleavage site analogous to that used for activation of the thrombin receptor, and mutation of this site from Arg/Ser to Arg/Pro prevented PAR-2 activation by trypsin. As with the thrombin receptor, a synthetic hexapeptide peptide (SLIGRL) representing the first six amino acids following this putative PAR-2 cleavage

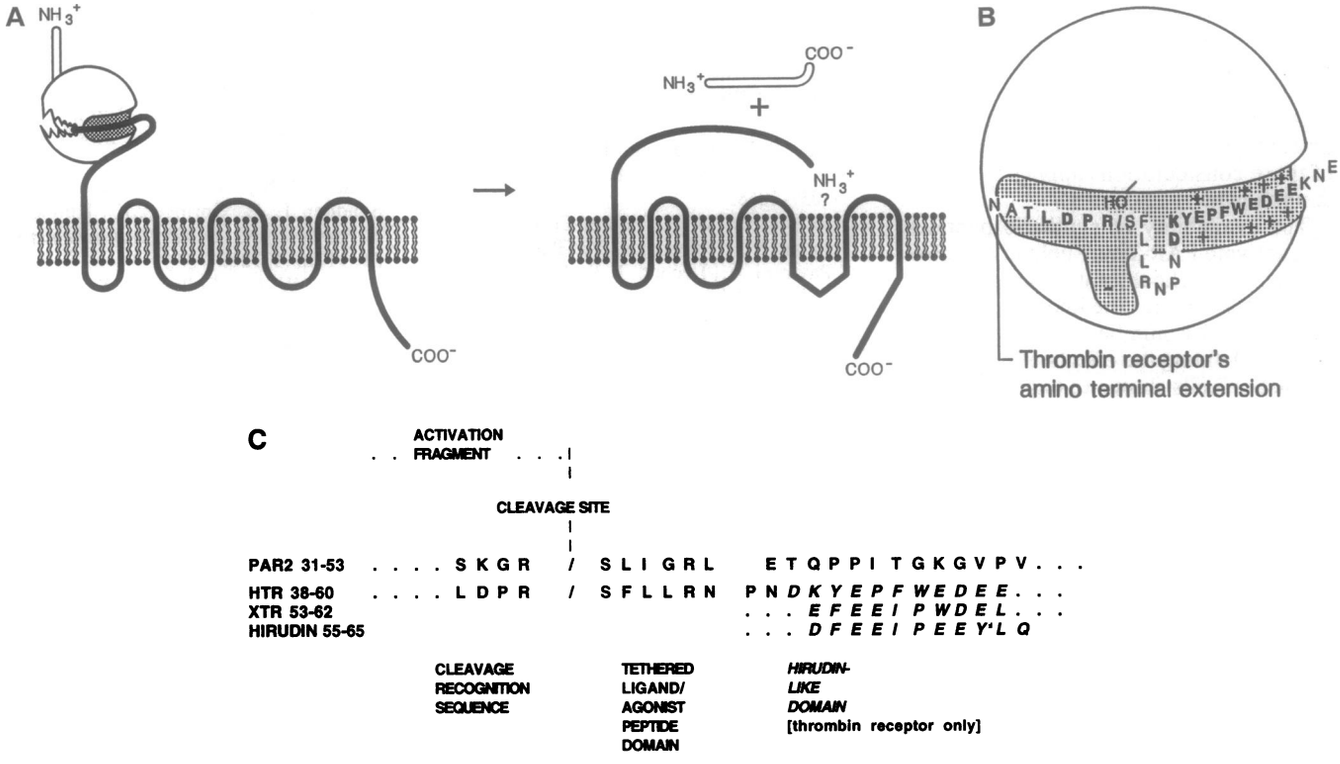

FIG. 1. Protease receptor activation. (*A*) Overall scheme. The thrombin receptor is depicted as a prototype. Thrombin, the sphere in this figure, recognizes the extracellular amino-terminal extension of its receptor (see *B* for detail). After binding to the amino-terminal extension, thrombin cleaves the receptor at the LDPR/S cleavage site (junction between open and filled receptor segments; see *C*), releasing an inactive fragment of the receptor amino terminus (open fragment) and exposing a new amino terminus. This newly unmasked amino terminus then functions as a tethered peptide ligand, binding within the body of the receptor to activate it. As shown, this binding event presumably translates into a conformational change in the cytoplasmic face of the receptor, effecting G protein activation. (*B*) Thrombin–receptor interaction. Thrombin has an extended substrate-binding surface (represented by the canyon running laterally) and can recognize residues both amino and carboxyl to the cleavage site of its substrate. Both receptor mutagenesis and crystallographic studies suggest that the thrombin receptor has evolved two separate domains to interact with thrombin in a bidentate fashion, conferring efficient recognition and cleavage. The hirudin-like domain (DKYEPF) of the receptor interacts with the anion-binding exosite of thrombin, whereas its cleavage site (LDPR/S) interacts with the active center of thrombin (see text). (*C*) Comparison of functional domains within the amino-terminal exodomains of PAR-2 and the human (HTR) and *Xenopus* (XTR) thrombin receptors. Spaces are added to separate putative functional domains. The cleavage site (/) and the activation peptide or fragment that would be cleaved from the receptor are indicated. The new amino termini that function as "tethered ligand" domains are aligned. Some conserved features are apparent. In the thrombin receptor, a domain rich in aromatic and acidic residues suggested a possible functional analogy to the carboxyl tail of the leech anticoagulant hirudin, known to bind the anion-binding exosite. This analogy is more apparent from the receptor sequence of *Xenopus* and has been supported experimentally (see text). The amino-terminal exodomain of PAR-2 is shorter than that of thrombin receptor, and the region of PAR-2 corresponding to the hirudin-like domain of thrombin receptor is rich in prolines and glycines. Whether this domain functions only as a hinge or tether or plays a role in protease–receptor interaction is unknown. *A* and *B* are reprinted with permission from ref. 5 (copyright Macmillan Magazines Limited).

site was an agonist for PAR-2 activation. At face value, PAR-2 certainly appears to be a second example of a receptor activated by proteolytic unmasking of a tethered peptide ligand.

The connection between PAR-2 and thrombin receptor is buttressed by the observation that their tethered-ligand domains share several structural features (Fig. 1), suggesting potentially similar structure–activity relationships. The specificity of synthetic agonist peptides mimicking these domains for their respective receptors is unknown and may provide an opportunity to define agonist docking sites. Studies with thrombin receptor antibodies (15) and chimeras of thrombin receptors from different species (16) suggest that the extracellular surface of the receptor may contribute to agonist recognition. The striking conserved region in the second extracellular loops of PAR-2 and thrombin receptor is provocative in this regard (4).

The unknown specificity of the PAR-2 and thrombin-receptor agonist peptides raises another issue. Responses to the thrombin-receptor agonist peptide have been used to imply a role for the cloned thrombin receptor in various cellular responses with the implicit caveat that such peptides might also act at other as-yet-unidentified receptors. PAR-2 raises the possibility of a family of protease-activated receptors with related agonist peptide domains, lending weight to this caveat but also promising a richer pharmacology.

Which protease(s) is the physiologic activator of PAR-2 and what functions does PAR-2 serve? The cell types that express PAR-2 are not known. At the organ level, intestine, stomach, kidney, and eye yielded positive Northern blots, suggesting possible roles in regulating epithelial- or smooth muscle-cell function. Any of a host of known extracellular proteases might cleave the protease-recognition site of PAR-2; no equivalent to the hirudin-like domain of thrombin receptor speaks. Moreover, the existence of binding proteins that localize proteases to the surface of cells (17, 18), as well as the recent discovery of a cell-surface protease that sports a transmembrane domain (19), suggests a multitude of possible local autocrine and paracrine mechanisms for bringing proteases and receptors together. Proving a particular protease to be the physiologic activator for PAR-2 will thus take some doing.

The mechanism-based characterization of thrombin receptor and PAR-2 as specialized peptide receptors suggests the possibility that they evolved from preexisting peptide receptors. This hy-

pothesis implies that naturally occurring peptide agonists might exist for some PARs and that some PARs might be "artificially" activatable by protease without this feature having assumed physiologic significance. Whether either of these considerations applies to PAR-2 is unknown.

The availability of synthetic peptides that activate PAR-2 may help to decipher its functions. The thrombin-receptor agonist peptide mimics the known actions of thrombin on a number of cell types (1, 20–23). Defining the effects of the PAR-2 agonist on cells shown to express PAR-2 may provide clues to its physiological role. Similarly, antibodies that prevent thrombin-receptor cleavage block platelet thrombin responses and have recently been used to block platelet function *in vivo* (24). Alignment of PAR-2 and thrombin receptor points up peptides for raising analogous antibodies for blocking PAR-2 function and probing its role *in vivo*.

In addition to these physiological issues, many questions regarding the thrombin receptor and PAR-2 remain to be answered at the cell and molecular biological levels. Some questions are generic to all seven-transmembrane-domain receptors: Where does the agonist bind, and how does agonist binding transmit information across the cell membrane to effect receptor–G protein coupling? Other questions are more unique, prompted by the irreversibility of the proteolytic activation mechanism: Are special shut-off mechanisms required to terminate signaling by these irreversibly activated protease receptors? What is their fate after activation? How is the cell surface refreshed with new receptors? The discovery of PAR-2 provides an important new tool to address these questions, as well as to search for additional members of this unusual family.

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