Agonist Recognition by Proteinase-activated Receptor 2 and Thrombin Receptor

Thrombin receptor and proteinase-activated receptor 2 (PAR2) define a family of G protein-coupled receptors that are activated by a novel proteolytic mechanism. Specific cleavage of their amino-terminal exodomains unmasks a new amino terminus which then serves as a tethered ligand, docking intramolecularly to the body of the receptor to effect signaling. Identification of the docking interactions between tethered ligand domain and receptor is critical for understanding transmembrane signaling by these receptors. Synthetic “agonist peptides” that mimic the tethered ligand domains of thrombin receptor and PAR2 act as agonists at their respective receptors. Toward defining the docking interactions which mediate receptor activation, we determined the specificity of the thrombin receptor and PAR2 for their respective agonist peptides and used receptor chimeras to identify the receptor domains responsible for such specificity. PAR2 responded to both thrombin receptor and PAR2 agonist peptides. In contrast, thrombin receptor was selective for its own agonist peptide. Substitution of the extracellular face of PAR2, its amino-terminal exodomain and three extracellular loops, for the cognate thrombin receptor structures yielded a chimeric receptor with PAR2-like agonist specificity. Substitution of individual extracellular domains revealed that the primary determinant of agonist specificity was extracellular loop 2. Strikingly, substitution of either the amino-terminal exodomain or third extracellular loop alone caused marked loss of receptor function, but the double substitution yielded a functional receptor. Thus, the extracellular domains of these G protein-coupled receptors are more than simply passive links between transmembrane domains. They participate in agonist recognition and must interact, directly or indirectly, for proper receptor function.

The thrombin receptor is a G protein-coupled receptor that is activated by a novel proteolytic mechanism. Thrombin binds to and cleaves its receptor’s amino-terminal exodomain to unmask a new amino terminus. This then functions as a tethered peptide ligand, binding intramolecularly to the body of the receptor to effect signaling (1–3). Synthetic peptides that mimic the tethered ligand domains of the thrombin receptor behave as peptide agonists, activating the receptor independent of thrombin and receptor proteolysis (1, 4). Identification of the interactions by which the tethered ligand domain of the thrombin receptor causes transmembrane signaling is critical for understanding signaling by this receptor and perhaps other members of the vast G protein-coupled receptor family. Such information may also foster the development of drugs that block the thrombotic, inflammatory, and proliferative actions of thrombin (5, 6).

With the goal of identifying such docking interactions, we previously exploited the different agonist specificities of the human and Xenopus laevis thrombin receptors (7, 8). The tethered ligand sequences in these receptors are distinct: SFLLRN in human and TFRIFD in Xenopus. Synthetic “agonist peptides” mimicking these sequences are selective for their respective receptors. A detailed analysis of gain-of-function human-Xenopus thrombin receptor chimeras suggested that Arg⁵ in the human agonist peptide is recognized by the Glu₂⁶⁰ region in the second extracellular loop (ECL) of the receptor. Moreover, loss of function mutations at agonist position 5 could be complemented by mutations in the Glu₂⁶⁰ region of the receptor, and specific mutations in the Glu₂⁶⁰ region of the human receptor caused constitutive receptor activation (9). These observations suggest a probable role for this receptor domain in agonist recognition and receptor activation. However, these studies focused on identifying receptor domains which distinguished between the human and Xenopus agonist peptides and could not address where important structures that are conserved between these agonists might dock.

Recently, a second protease-activated receptor (PAR2) that is 30% identical to the thrombin receptor in its amino acid sequence was described (10, 11). Like the thrombin receptor, PAR2 is activated by limited proteolysis of its amino-terminal exodomain, and the synthetic peptide SLIGRL that mimics the newly created amino terminus is an agonist for PAR2 (10). The human-Xenopus chimera studies described above identified a probable recognition site for position 5 in the human thrombin receptor agonist peptide. Position 5 in the PAR2 and human thrombin receptor agonist peptides, SLIGRL and SFLLRN, is a conserved arginine. Glu₂⁶⁰, the putative docking site for Arg⁵, is also conserved in these receptors. These observations suggested that PAR2-human thrombin receptor chimeras might be useful for identifying docking interactions not probed in the human-Xenopus chimera studies. We, therefore, investigated the specificity of the thrombin receptor and PAR2 for their respective agonists and exploited their different specificities with receptor chimeras. Our results suggest that thrombin
The amino acid sequence and positions of the thrombin receptor (T) and proteinase-activated receptor 2 (P) tethered ligand domains and the portions of amino-terminal exodomain and ECL exchanged in the receptor chimeras. Amino acid residues are numbered according to published sequences (1, 11). Conserved residues are underlined. Chimeric receptors were generated by replacing one or more of the four human thrombin receptor extracellular domains shown with the corresponding domains from mouse PAR2. Chimeric receptor cDNAs were designated by the PAR2 domains they contain, i.e. ATE designates a thrombin receptor in which the indicated portion of the amino-terminal exodomain was replaced with the cognate PAR2 sequence. ECL1, ECL2, or ECL3 designate a chimeric receptor in which the indicated ECL is replaced by the first, second, or third ECL of the thrombin receptor were replaced by the cognate PAR2 domains. In chimeras with multiple substitutions, loop substitutions are designated simply by loop number (e.g. ATE+3 is a thrombin receptor in which a portion of the amino-terminal exodomain and the third extracellular loop were replaced by the cognate PAR2 domains).

**RESULTS AND DISCUSSION**

Thrombin receptor (1, 4) and PAR2 (10, 11) can be activated by peptides that mimic their respective tethered ligand domains, SFLLRN and SLIGRL. To determine the specificity of these receptors for their respective "agonist peptides," thrombin receptor and PAR2 were expressed in Xenopus laevis oocytes, and their responses to SFLLRN and SLIGRL were determined. Marked differences in agonist specificities were noted. Oocytes expressing thrombin receptor showed robust responses to the thrombin receptor agonist peptide SFLLRN, but such oocytes were virtually unresponsive to the PAR2 agonist SLIGRL (Fig. 2a and Table I). By contrast, oocytes expressing PAR2 showed robust responses to both SLIGRL and SFLLRN (Fig. 2b and Table I). In addition to providing an approach for defining the structures responsible for agonist specificity, these results point out an important caution in interpreting responses to SFLLRN in cell lines or tissues. Such responses cannot be automatically attributed to thrombin receptor, given the ability of SFLLRN and SLIGRL to activate PAR2.

**EXPERIMENTAL PROCEDURES**

Generation of cDNAs Encoding Wild-Type and Chimeric Receptors—Murine PAR2 receptor cDNA was obtained by reverse transcription of mouse kidney mRNA followed by polymerase chain reaction amplification using primers based on the published cDNA sequence (11): primer N206, 5'-TTGAGCTTGGTCATCAGAGTGGCAGTTCGCGTTCTGCA; and primer C776, 5'-CATACGGAATTCCTACCATCTCAAGTACAGCT- CATGTA. The polymerase chain reaction product was subcloned into pBLOG (12), and a nucleotide sequence was obtained by dideoxy sequencing. Polymerase chain reaction-induced errors in the sequence were corrected to match the published sequence (11) using diogunucleotide-directed mutagenesis (13, 14). A cDNA encoding an epitope-tagged PAR2 was generated by introducing a sequence encoding the FLAG epitope (DYKDDDDK) after residue Thr295, i.e. just carboxyl to the putative signal peptide site (15).

Mutant receptor cDNAs were generated using diogunucleotide-directed mutagenesis (13, 14) and verified by dideoxy sequencing (16). Specific constructs are described in Fig. 1. In the native thrombin receptor cDNA and all chimeric receptor cDNAs, the native thrombin receptor signal peptide was replaced by a prolactin signal peptide sopeptide. The FLAG epitope (DYKDDDDK) sequence was inserted at the amino terminus of the mature receptor protein (17).

Assay of Wild-type and Chimeric Receptor Expression and Signaling—The responses of wild-type and mutant receptors to agonist peptides were assayed in X. laevis oocytes as described (1, 7, 18). Briefly, cRNA was transcribed from wild-type or mutant receptors subcloned into pFROG (1). Mutant receptor cDNAs were microinjected into oocytes as described previously (1, 18). The agonist peptides SFLLRN and SLIGRL were synthesized in the carboxy amide form and purified by reverse-phase high performance liquid chromatography before use (4).

**RESULTS AND DISCUSSION**

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To identify the receptor domains responsible for the differing agonist specificities of the thrombin receptor and PAR2, we examined the ability of SLIGRL and SFLLRN to activate thrombin receptor-PAR2 chimeras. Because SLIGRL had little activity at the thrombin receptor, we built PAR2 domains into the thrombin receptor and looked for a gain of responsiveness to the PAR2 agonist. We first asked whether the differing specificities of PAR2 and thrombin receptor were a function of the exodomain or transmembrane segments of the receptor. A striking change in agonist specificity was conferred by replacing the ATE of the thrombin receptor and all three ECL with the cognate PAR2 sequences. This extracellular domain swap conferred more than a 35-fold increase in responsiveness to SLIGRL and an almost 8-fold decrease in responsiveness to SFLLRN (Fig. 2c and Table I). Thus, the extracellular faces of PAR2 and thrombin receptor appear to be responsible for distinguishing their respective agonists.

To identify which extracellular domains conferred this remarkable switch in agonist specificity, we generated four chimeric receptors. In each, a single extracellular domain of the thrombin receptor was replaced by the cognate segment of PAR2 (Fig. 2). Replacement of the second ECL of the thrombin receptor was sufficient to confer a marked change in agonist specificity: a more than 10-fold gain of responsiveness to SLI-
GRL and an approximately 7-fold loss of responsiveness to SFLLRN (Table I). Thus, most of the change in agonist specificity accomplished by substituting the entire extracellular domain of PAR2 for the cognate thrombin receptor structure was accounted for by ECL2. This result is analogous to that obtained using human-Xenopus thrombin receptor chimeras and suggests that PAR2 and thrombin receptor use similar strategies for agonist recognition. Indeed, at least one possible agonist-receptor interaction may operate in both systems. The human-Xenopus thrombin receptor chimera studies suggested a possible interaction between Arg^5^ in SFLLRN and Glu^260^ in the second ECL of the human thrombin receptor (8). These amino acids are conserved in PAR2; thus, an Arg^5^-Glu^260^ interaction might also operate in recognition of SLIGRL by PAR2. However, the different specificities of thrombin receptor and PAR2 suggest that other direct or indirect interactions between agonist and ECL2 must be responsible for distinguishing the PAR2 and thrombin receptor agonists.

Unlike the ECL2 substitution, the substitution of the ECL1 of PAR2 for the cognate thrombin receptor structure conferred...
A gain of responsiveness to both SLIGRL and SFLLRN (Table I). Thus, rather than changing agonist specificity, the ECL1 substitution caused a general change in signaling properties and was not informative.

Surprisingly, the substitution of either ECL3 or ATE individually yielded a remarkable loss of receptor function (Table I), despite the fact that substitution of all PAR2 exodomains for the cognate thrombin receptor domains yielded a functional receptor with PAR2-like specificity. This result suggested that ECL3 and ATE must interact, directly or indirectly, with other extracellular domains or with each other to permit proper receptor function. Toward identifying such interactions, we attempted to complement the loss of function caused by the ECL3 or ATE substitutions by making pairwise substitutions of ECL3 or ATE with the other PAR2 exodomains.

Strikingly, a chimeric receptor 3ECL + ATE that contained both loss of function PAR2 extracellular domain substitutions was more responsive to agonist than the chimeras containing either substitution alone (Table I). Specifically, the chimeric receptor 3ECL + ATE had an EC50 for activation by SFLLRN that was more than 5-fold lower than the chimera containing only ECL3 and 4-fold lower than the chimera containing only ATE. Consistent with this and more striking still, chimeric receptors containing the second ECL of PAR2 and either ATE or ECL3 were virtually nonfunctional, but chimeras which contained the second ECL of PAR2 and both ECL3 and ATE of PAR2 yielded a receptor with signaling properties similar to those of PAR2 itself (Table I). These data strongly suggest that a direct or indirect interaction of the ATE and ECL3 domains is required for receptor structure or function. Regardless, these receptors somehow recognize an appropriate pairing of ATE and ECL3

### Table I

| Receptor | EC50 (μM) | Agonist peptide |
|----------|-----------|-----------------|
|          | SFLLRN    | SLIGRL          |
| TR       | 1.0       | >300            |
| PAR2     | 40        | 10              |
| ATE+1+2+3| 8         | 8               |
| ATE      | 240       | >300            |
| ECL3     | 0.1       | 150             |
| ECL2     | 7         | 30              |
| ECL3     | LOF       | LOF             |
| ATE+1    | 100       | >300            |
| ATE+2    | LOF       | LOF             |
| ATE+3    | 60        | >300            |
| 3+ATE    | 60        | >300            |
| 3+1      | 60        | >300            |
| 3+2      | >300      | >300            |
| 2+ATE+3  | 16        | 13              |
The three-dimensional structure of G protein-coupled receptors has not been solved at high resolution, and even the arrangement of the transmembrane helices with respect to one another is uncertain (19, 20). The results described above suggest that the ATE and third ECL of the thrombin receptor are in close proximity, which in turn suggests that transmembrane domain 1 must be near transmembrane domain 6 or 7. This arrangement is compatible with several current models of G protein-coupled receptor structure (Refs. 19 and 21; Fig. 3) and thus supports those models. Other recent experimental observations also support proximity of transmembrane domains 1 and 7 (22, 23).

In summary, these studies report the specificity of the related protease-activated receptors PAR2 and thrombin receptor for their respective agonist peptides. The observation that PAR2 responded not only to its own agonist SLIGRL but also to thrombin receptor agonist peptide SFLLRN points up an important caution in interpreting responses of cells or tissues to SFLLRN. Such responses cannot be automatically attributed to the thrombin receptor.

The observation that thrombin receptor failed to respond to the PAR2 agonist SLIGRL provided an opportunity to make gain-of-function chimeras to identify the receptor domains which distinguish the two agonists. These studies pointed to receptor ECL2 as a critical determinant of agonist specificity. This result is reminiscent of that obtained with human-Xenopus thrombin receptor chimeras and suggests that thrombin receptor and PAR2, perhaps not surprisingly, use a similar strategy for recognizing their respective agonist peptide domains. In this regard, the human-Xenopus chimeric receptor studies (7, 8) suggested a possible docking interaction between an arginine at position 5 in the human agonist peptide SFLLRN and negatively charged residues, especially Glu\(^{260}\), in the second ECL of the human receptor. These residues are conserved in human thrombin receptor and PAR2; thus, it is possible that an Arg\(^{5}\)-Glu\(^{260}\) interaction also occurs when SLIGRL docks with PAR2. However, given the gain of responsiveness to SLIGRL and decreased responsiveness to SFLLRN conferred by the substitution of the second ECL of PAR2 for the cognate thrombin receptor structure, the second ECL of these receptors must also specify distinct interactions with their respective agonists.

Most interestingly, substitution of either ATE or third ECL of PAR2 for the cognate thrombin receptor domains caused loss of receptor function, but the corresponding double substitution permitted robust receptor function. This functional complementation was especially dramatic if the second ECL of PAR2 was included (see above). These observations demonstrate that the extracellular domains of these G protein-coupled receptors are more than simply passive links between transmembrane domains. They participate in agonist recognition and must interact, directly or indirectly, for proper receptor function.

Because thrombin-induced platelet activation and mesenchymal cell proliferation may play important roles in vascular disease (24, 25), the thrombin receptor has become a target for pharmaceutical development. The development of competitive antagonists for protease-activated receptors may prove difficult, given the irreversibility of the proteolytic activation mechanism and the proximity of tethered ligand and docking sites (17). However, the studies described above suggest that non-competitive antagonists might be developed as drugs that disrupt interactions among the extracellular domains of the receptor that are critical for receptor function.

**REFERENCES**

1. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
2. Vu, T.-K. H., Wheaton, V. I., Hung, D. T., and Coughlin, S. R. (1991) Nature 353, 674–677
3. Chen, J., Ishii, M., Wang, L., Ishii, K., and Coughlin, S. R. (1994) J. Biol. Chem. 269, 16041–16045
4. Scarborough, R. M., Naughton, M., Teng, W., Hung, D. T., Rose, J., Vu, T.-K. H., Wheaton, V. I., Turk, C. W., and Coughlin, S. R. (1992) J. Biol. Chem. 267, 13146–13149
5. Fenton, J. W., II (1988) Semin. Thromb. Hemostasis 14, 234–240
6. Coughlin, S. R., Vu, T.-K. H., Hung, D. T., and Wheaton, V. I. (1992) J. Clin. Invest. 89, 351–355
7. Ishii, M., Chen, J., Ishii, K., Wang, L., Nanevicz, T., Turk, C. W., Vu, T.-K. H., and Coughlin, S. R. (1994) Nature 368, 648–651
8. Nanevicz, T., Ishii, M., Wang, L., Chen, M., Chen, J., Turk, C. W., Cohen, F., and Coughlin, S. R. (1995) J. Biol. Chem. 270, 21619–21625
9. Nanevicz, T., Wang, L., Chen, M., Ishii, M., and Coughlin, S. R. (1996) J. Biol. Chem. 271, 702–706
10. Nystedt, S., Ensminger, K., Wahlestedt, C., and Sundelin, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9208–9212
11. Nystedt, S., Larsson, A. K., Aberg, H., and Sundelin, J. (1995) J. Biol. Chem. 270, 5950–5955
12. Chen, J., Bernstein, H. S., Chen, M., Wang, L., Ishii, M., Turk, C. W., and Coughlin, S. R. (1995) J. Biol. Chem. 270, 23398–23401
13. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. von Heijne, G. (1983) Eur. J. Biochem. 133, 17–21
16. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
17. Ishii, M., Hein, L., Koblika, B., and Coughlin, S. R. (1993) J. Biol. Chem. 268, 9780–9786
18. Williams, J. A., McChesney, D. J., Calayag, M. C., Lingappa, V. R., and Logsdon, C. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 4939–4943
19. Schertler, G. F., Villa, C., and Henderson, R. (1993) Nature 362, 770–772
20. Donnelly, D., and Findlay, J. B. C. (1994) Curr. Opin. Struct. Biol. 4, 582–589
21. Baldwin, J. M. (1993) EMBO J. 12, 1693–1703
22. Mizobe, T., Maes, M., Lam, V., Suryanarayana, S., and Koblika, B. (1996) J. Biol. Chem. 271, 2387–2395
23. Suryanarayana, S., von Zastrow, M., and Kobilka, B. K. (1992) J. Biol. Chem. 267, 21991–21994
24. Coughlin, S. R. (1993) Trends Cardiovasc. Med. 4, 77–83
25. Baykal, D., Schmedtje, J. J., and Runge, M. S. (1995) Am. J. Cardiol. 75, 828–878
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