Mechanisms of Thrombin Receptor Agonist Specificity

CHIMERIC RECEPTORS AND COMPLEMENTARY MUTATIONS IDENTIFY AN AGONIST RECOGNITION SITE*

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Identification of the docking interactions by which peptide agonists activate their receptors is critical for understanding signal transduction at the molecular level. The human and Xenopus thrombin receptors respond selectively to their respective hexapeptide agonists, SFLLRN and TFRIFD. A systematic analysis of human/Xenopus thrombin receptor chimeras revealed that just two human-for-Xenopus amino acid substitutions, Phe for Asn87 in the Xenopus receptor's amino-terminal exodomain and Glu for Leu260 in the second extracellular loop, conferred human receptor-like specificity to the Xenopus receptor. This observation prompted complementation studies to test the possibility that Arg89 in the human agonist peptide might normally interact with Glu260 in the human receptor. The mutant agonist peptide SFLLEN was a poor agonist at the wild type human receptor but an effective agonist at a mutant human receptor in which Glu260 was converted to Arg. An “arginine scan” of the receptor’s extracellular surface revealed additional complementary mutations in the vicinity of position 260 and weak complementation at position 87 but not elsewhere in the receptor. Strikingly, a double alanine substitution that removed negative charge from the Glu260 region of the human receptor also effectively complemented the SFLLEN agonist. The functional complementation achieved with single Arg substitutions was thus due at least in part to neutralization of a negatively charged surface on the receptor and not necessarily to introduction of a new salt bridge. By contrast, charge neutralization did not account for the gain of responsiveness to SFLLRN seen in the human/Xenopus receptor chimeras. Thus two independent approaches, chimeric receptors and arginine scanning for complementary mutations, identified the Glu260 region and to a lesser degree Phe87 as important determinants of agonist specificity. These extracellular sites promote receptor responsiveness to the “correct” agonist and inhibit responsiveness to an “incorrect” agonist. They may participate directly in agonist binding or regulate agonist access to a nearby docking site.

Seven transmembrane domain G protein-coupled receptors respond to a structurally diverse set of ligands to regulate a host of biological processes. Catecholamines and certain other small ligands elicit responses by binding to their receptors’ transmembrane regions (1). The docking interactions by which peptide agonists activate their receptors are less well-characterized (2–7). The thrombin receptor can be viewed as a specialized peptide receptor that contains its own “tethered ligand.” Thrombin activates its receptor by cleaving the receptor’s amino-terminal exodomain. This limited proteolysis unMASKS a new amino terminus which then functions as a tethered peptide agonist, binding to the body of the receptor to cause signaling (8–10). Synthetic peptides which mimic the tethered ligand domain behave as peptide agonists, activating the receptor independent of thrombin. Identification of the interactions by which the thrombin receptor’s tethered ligand domain triggers transmembrane signaling is central to understanding signaling by this and perhaps other peptide receptors. Such information may also aid the development of novel pharmaceuticals for inhibiting thrombotic, inflammatory, and proliferative actions of thrombin (11, 12).

Three-dimensional structures for seven transmembrane domain G protein-coupled receptors are currently of low resolution, especially in the extramembranous regions (13). Thus structures that reveal the details of agonist docking, particularly for agonists that interact with the receptor’s extracellular loops, are unlikely to be available in the near future. Mutagenesis studies which examine functional end points remain a valuable approach that provides constraints for model building and analysis of future structural data.

We exploited the specificity of the human and Xenopus thrombin receptor homologues for their respective agonist peptides to identify the receptor domains which distinguish between these agonists (14). A chimeric receptor in which the Xenopus receptor’s extracellular surface (its amino-terminal exodomain and three extracellular loops) was replaced with that of the human receptor showed a remarkable gain of responsiveness to the human agonist and loss of responsiveness to the Xenopus agonist, resulting in human receptor-like agonist specificity (14). More limited replacement of Xenopus with human receptor sequence suggested that two regions accounted for this change in specificity: residues 244–268 in the second extracellular loop and residues 76–93 located in the amino-terminal exodomain near the start of transmembrane domain 1 (14). An antiserum which recognized the latter region blocked receptor activation by agonist peptide, consistent with a role for this region in agonist function (15, 16).

With the long term goal of defining the agonist-receptor interactions which mediate receptor activation, we analyzed progressively finer chimeras to identify the specific amino acids in the human and Xenopus thrombin receptors which distinguish their cognate agonists. Ultimately, two single human for Xenopus amino acid substitutions in the Xenopus receptor, Phe for Asn87 in the amino-terminal exodomain and Glu for Leu260...
in the second extracellular loop, proved sufficient to confer robust responses to human agonist. The gain of function conferred by the Leu<sup>260</sup>Glu substitution prompted complementation studies to test the hypothesis that Arg<sup>5a</sup> in the human agonist peptide might normally interact with Glu<sup>260</sup> in the human receptor (Fig. 1). These studies again pointed to the receptor domains surrounding Glu<sup>260</sup> and to a lesser degree Phe<sup>87</sup> but revealed that these domains can contribute to receptor specificity by excluding activation by unwanted agonists instead of, or in addition to, providing direct docking interactions. Overall, our results strongly suggest that these extracellular receptor regions of the thrombin receptor participate in receptor activation by binding agonist directly or by regulating agonist access to a nearby docking site.

**MATERIALS AND METHODS**

Responses of wild type and mutant thrombin receptors to peptide agonists were determined in the Xenopus oocyte expression system using previously described procedures (8, 14). Briefly, cDNAs encoding mutant human and Xenopus thrombin receptors were generated by standard techniques (17, 18) and confirmed by dideoxy sequencing (19). All wild type and mutant receptors used in this study were epitope tagged with the FLAG sequence present at the amino terminus of the mature receptor protein (14, 20). To describe the various mutants, we use the amino acid number of the human thrombin receptor sequence with the start methionine designated as one to refer to both human and their cognate Xenopus receptor residues (8, 14); the relevant alignment is shown in Fig. 1 and Table I. cRNAs for the wild type and mutant receptors were transcribed from cDNAs subcloned into pFROG (8). 12.5 ng of wild type cRNA and 12.5-25 ng of mutant receptor cRNAs were injected/oocyte (8, 14). After culture for 24h, receptor expression on the oocyte surface was measured as specific binding of antibody to the FLAG epitope (14). The quantity of cRNA injected was adjusted to maintain surface expression of mutant receptors between 50 and 150% of wild type. Responses to wild type and mutant human and Xenopus agonist peptides were measured as agonist-induced <sup>45</sup>Ca efflux which reflects phosphoinositide hydrolysis in these cells (8, 14). For determining EC<sub>50</sub>s, at four to five different concentrations of each agonist (from sub EC<sub>50</sub> to maximally activating) were employed. All determinations of EC<sub>50</sub> values for mutant receptor activation included concentration response curves for the appropriate wild type receptor(s) in the same experiment. All experiments were replicated at least twice.

The peptide agonists used in these studies were synthesized as the carboxy amide forms and were purified by high performance liquid chromatography before use (14, 21).

**RESULTS AND DISCUSSION**

The tethered ligand domains of the human and Xenopus thrombin receptors are strikingly different: SFLRN for human and TFRFID for Xenopus (14) (Fig. 1). Synthetic peptides mimicking these domains show specificity for their respective receptors. Indeed, the human agonist peptide is approximately 1000-fold more potent at the human receptor than at the Xenopus receptor. Our previous studies showed that replacement of the Xenopus thrombin receptor’s amino-terminal exodomain and second extracellular loop with the corresponding human sequences resulted in a chimeric receptor with human receptor-like agonist specificity. Individually, these two substitutions resulted in substantial but less dramatic gains of responsiveness to the human agonist (14).

To identify the specific amino acids in the human and Xenopus thrombin receptors responsible for distinguishing between their cognate agonists, we constructed progressively finer chimeras and determined their responsiveness to human versus Xenopus agonist peptides (Fig. 1 and Table I). We first focused on the putative binding region in the receptor’s amino-terminal exodomain (14). Substitution of human receptor amino-terminal exodomain (HAT) residues 76–93 for the cognate sequence in the Xenopus receptor yielded some gain of responsiveness to the human agonist but an exaggerated loss of responsiveness to the Xenopus agonist. More informative results were obtained by substituting smaller overlapping segments (76–86, 82–90, and 87–94). The HAT78–90 chimera exhibited a substantial gain of responsiveness to the human agonist. Indeed, the human agonist is approximately 1000-fold more potent at the human receptor than at the Xenopus receptor. Within this region, HAT78–90 also yielded a more than 10-fold lower EC<sub>50</sub> for the human agonist compared to the wild type Xenopus receptor. Without this region, HAT78–90 a 5-fold gain. HAT96 showed no gain, and Ile<sup>87</sup> is conserved in the two receptors.

The HAT87 human-for-Xenopus amino acid exchange disrupted a consensus sequence for N-linked glycosylation by substituting a Phe for Asn<sup>87</sup> (NIT, Fig. 1). Substitution of Ala for Asn<sup>87</sup> in the Xenopus receptor produced no gain of responsiveness to human agonist peptide (HAT87, I), thus the gain of responsiveness to human agonist seen with HAT87 was due to introduction of the phenyl side chain and not to ablation of an N-linked glycosylation site. Taken together, the data presented above show that human thrombin receptor amino-terminal exodomain residues 87–90 play an important role in defining agonist specificity with Phe<sup>87</sup> being particularly important.

We used a similar approach to identify the amino acids in the receptor’s second extracellular loop that distinguish between the human and Xenopus agonists. This loop can be divided into two halves separated by a cysteine thought to participate in a disulfide bridge between the first two extracellular loops (8). HECL, 254–268, a chimera in which the “second half” of the human receptor’s second extracellular loop was substituted for the cognate Xenopus residues, showed a remarkable gain of responsiveness toward the human peptide (Fig. 1, Table I). By contrast, a chimera containing the “first half” of this loop (HECL2, 244–254) had Xenopus receptor-like agonist specificity (Fig. 1, Table I). Because residues 254–258 are conserved in

![FIG. 1. Comparison of human and Xenopus thrombin receptor agonists and the domains that distinguish between them. The cleavage site at which thrombin acts to unmask the tethered ligand or “agonist peptide” domain is indicated by the small arrow. After cleavage, this domain binds intramolecularly to the body of the receptor to cause signaling. Synthetic peptides mimicking this domain are full agonists at the thrombin receptor, bypassing the need for cleavage by thrombin. The human and Xenopus tethered ligand sequences are compared as are the receptor regions found to be important in distinguishing between these two agonists. The important Phe<sup>87</sup> and Glu<sup>260</sup> regions are indicated in bold. Bold arrows indicate possible complementary changes.](Image 306x570 to 555x744)
Thrombin Receptor Agonist Specificity

Analysis of human/Xenopus receptor chimeras to identify the receptor sites that distinguish between their respective agonists

Epitope-tagged wild type human or Xenopus thrombin receptors or chimeric receptors were expressed in Xenopus oocytes and EC₅₀ values for[^45]Ca release in response to human (SFLLRN) or Xenopus (TFRID) agonist peptides was determined ("Materials and Methods"). Expression levels and maximal responses were similar for the chimeras and wild type receptors tested. Maximal responses expressed as agonist-induced[^45]Ca release/basal[^45]Ca release (8) typically ranged from 30 to 40-fold. In the human amino-terminal exodomain (HAT) series, the human receptor sequences shown replaced the cognate Xenopus sequences as indicated. The same convention is used for the human extracellular loop two (HECL2) series. In HAT87A, HECL2,259−262AETL, HAT87A + HECL2,260A, and HECL2, K261A + K264A, alanine replaced the indicated Xenopus residue(s). EC₅₀ values shown are representative of those obtained in at least two separate experiments.

| Receptor | Agonist | EC₅₀ (μM) |
|----------|---------|-----------|
| Human wild type | S | 1 |
| Xenopus wild type | F | >300 |
| Analysis of amino-terminal exodomain | | |
| Human sequence substituted | | |
| HAT76–93 | SFLLRN | 80 |
| HAT76–86 | SFLLRN | 200 |
| HAT82–90 | SFLLRN | 8 |
| HAT87–94 | SFLLRN | 200 |
| HAT86–90 | SFLLRN | 30 |
| HAT89–90 | SFLLRN | 60 |
| HAT86 | SFLLRN | >300 |
| HAT87 | SFLLRN | 30 |
| HAT87A | SFLLRN | >300 |
| Native Xenopus sequence | SFLLRN | VSRSARKPIRRNITKEAEQ |
| Analysis of second extracellular loop | | |
| Human sequence substituted | | |
| HECL2, 244−268 | PGLNITTC | 10 |
| HECL2, 244−254 | PGLNITTC | 250 |
| HECL2, 254−268 | CHDVNETLLEGYYA | 30 |
| HECL2, 259−262 | CHDVNETLLEGYYA | 1 |
| HECL2, 261−265 | TLLEG | 100 |
| HECL2, 264−268 | TLLEG | 120 |
| HECL2, 259−262AETL | AETL | 15 |
| HECL2, 260 | E | 10 |
| Native Xenopus sequence | E | 2 |
| Double HAT87A + HECL2,260 | A | 100 |
| Double HAT87A + HECL2,260A | A | >300 |
| HECL2, K261A + K264A | A | 300 |

The best single amino acid substitutions identified in each of the two "specificity regions" defined above were combined to generate "HAT87 + HECL2,260." This chimera had human receptor-like agonist specificity despite bearing only two human-for-Xenopus amino acid substitutions (Fig. 2 and Table I). The corresponding double alanine substitution mutant had only a small relative gain of responsiveness to human agonist compared to the wild type Xenopus receptor. Taken together with our earlier studies (14), this completes a systematic analysis of human/Xenopus thrombin receptor chimeras to identify the specific receptor residues which distinguish the human from Xenopus agonists. The data raised the possibility that the agonist peptide might interact directly with the receptor's extracellular surface at or near positions 87 and 260 and suggested a candidate docking interaction. In the agonist peptide, position 5 changes from the aromatic Phe⁵ in Xenopus to the basic Arg⁵ in human. In the receptor, position 260 changes from the hydrophobic Leu⁵ in Xenopus to the acidic Glu⁵ in human. The remarkable gain of responsiveness to human agonist conferred by replacing Xenopus receptor's Leu⁵ with Glu thus suggested that Arg⁵ in the human agonist might dock with Glu⁵ in the human receptor (Fig. 1 and Table I).

This hypothesis was first tested by introducing potentially complementary amino acid substitutions at these positions in the human agonist and receptor. Replacement of Arg⁵ in the human agonist peptide with Glu caused a remarkable loss of function at the wild type human receptor. Strikingly, this was remedied by replacing Glu⁵ in the human receptor with Arg but not Ala (Fig. 3).

To determine the specificity of this complementation phenomenon, we first tested agonist peptides in which Glu or Asp replaced the native agonist residues at positions 2–5 for their ability to activate wild type versus Glu⁵Arg and Phe⁵Arg mutant human thrombin receptors. Only the agonists with substitutions at position five were effectively complemented by the Glu⁵Arg mutation (Fig. 4A). Asp and Glu substitutions behaved similarly, consistent with a role for the negatively charged side chain.
aspartates or glutamates introduced at agonist positions 2, 3, or 4 formed an intramolecular salt bridge with the Arg5a side chain in this series of peptides, making these side chains unavailable to participate in interactions with the receptor. To examine this explanation of the lack of complementation seen for agonists with substitutions at positions 2–4, we tested a second series of agonists (SFLLRNPKDK, SFLDLNPNDK, SFLDFNPNDK, and SFDLFNPNDK) that

![Graph](image1)

**Fig. 2.** Two single amino acid substitutions confer human receptor-like specificity to the Xenopus thrombin receptor. Human or Xenopus wild type thrombin receptors or the Xenopus receptor in which amino acids 87 and 260 were replaced by the cognate human residues (HAT87 + HEC260) were expressed in Xenopus oocytes and responses to the indicated concentrations of human (SFLLRN) or Xenopus (TFRFD) agonist peptides were determined. The specific amino acid substitutions are indicated in single letter code. Data (mean ± S.D. (n = 4)) are expressed as percent maximal agonist-induced 45Ca release and represent four replicate experiments. Expression levels were similar for the three receptors. Average maximal responses were also similar and ranged from 25- to 50-fold increases in agonist-induced 45Ca release in the various experiments. Arrows point out the >100-fold gain in responsiveness (decrease in EC50) to human agonist and 4-fold loss of responsiveness (increase in EC50) to Xenopus agonist caused by the two amino acid substitutions.

![Graph](image2)

**Fig. 3.** Complementary mutations in agonist peptide and receptor. Wild type human thrombin receptor or mutant human receptors in which Glu260 was converted to arginine (E260R) or alanine (E260A) were expressed in Xenopus oocytes and 45Ca release in response to the indicated concentrations of wild type human agonist (SFLLRNPNDK, SFLLRN) or a mutant agonist peptide in which arginine in position five was changed to glutamate (SFLLENPNDK, SFLLEN). The longer agonist peptides were used because the SFLLEN hexapeptide was not sufficiently soluble. Data (mean ± S.D. (n = 4)) are expressed as percent maximal agonist-induced 45Ca release and represent four replicate experiments. Expression levels and maximal responses were similar for the three receptors. Maximal responses ranged from 35- to 45-fold increases in agonist-induced 45Ca release. Arrows point out the >50-fold decrease in EC50 for the glutamate bearing agonist and the 4-fold increase to native agonist caused by the Glu260Arg (E260R) substitution.

![Graph](image3)

**Fig. 4.** Specificity of complementary mutations. A, mutations in agonist suggest specificity for position 5. Synthetic peptides representing wild type sequence SFLLRNPNDK or peptides with the indicated substitutions were assayed for activity at the wild type human thrombin receptor (HWT, open bars), the Glu260Arg (E260R, hatched bars), and the Phe87Arg (F87R, closed bars) mutant receptors expressed in Xenopus oocytes. Peptides were added 100 μM and agonist-induced increases in 45Ca release determined. Data shown are mean ± S.D. (n = 2). This experiment was replicated once. Note the loss of function of the SFLLENPNDK and SFLLDNPNDK peptides at the wild type receptor which was remedied by the Glu260Arg mutation and to a lesser extent by the Phe87Arg mutation. B, arginine scan of receptor. Xenopus oocytes expressing wild type human thrombin receptor (HWT) or receptors bearing the indicated single arginine substitutions were assayed for responsiveness to SFLLENPNDK (100 μM) versus SFLLRNPNDK (10 μM). All mutant receptors were expressed at comparable levels on the oocyte surface. (*) indicates “loss of function” mutations; these mutant receptors yielded responses to both wild type and mutant agonists that were less than 15% of those seen with the wild type receptor. All other mutant receptors had responses to wild type agonist that were indistinguishable from wild type receptor. Accordingly, responses to the mutant peptide SFLLENPNDK were expressed as percent of the wild type receptor’s response to wild type agonist determined in parallel in each experiment. The data shown are the means of duplicate determinations. Similar results were obtained in two or more experiments with each mutant receptor. Receptors showing “positive” complementation were arbitrarily defined as those that conferred responses to maximal concentrations of SFLLENPNDK that were >50% of the maximal response of wild type receptor to wild type agonist. C, arginine scan
lacked Arg\(^\text{260}\). In this series, SFLLFNPNDK was an effective agonist at the wild type receptor, all aspartate substitutions caused loss of agonist function at the wild type receptor, and only SFLLDNPNKD was effectively complemented by the Glu\(^\text{260}\)Arg receptor mutation (data not shown). We cannot exclude the possibility that other intra-agonist interactions specific to positions 2–4 make negatively charged side chains introduced at these positions unavailable to the receptor. However, at face value, these data suggest that receptor position 260 looks primarily at agonist position 5.

Our second test of specificity was an “arginine scan” of the receptor’s extracellular surface, a search for mutant receptors that would respond to the SFLLEN mutant agonist (Fig. 4, B and C). Remarkably, complementary mutations were found only in the regions previously identified as important for receptor specificity by the chimera studies. Arg substitutions at Glu\(^\text{260}\) and Glu\(^\text{264}\) provided strong complementation and Arg substitutions at nearby residues 261, 263, 265, 268, and 269 caused lesser but reproducible gains in responsiveness to SFLLEN relative to SFLLEN (Fig. 4). Arg substitution at position 87, the other site identified in the chimera studies, provided weak complementation. By contrast, Arg substitutions in extracellular loops 1 and 3 and in the first half of loop 2, all sites found to be unimportant for agonist specificity in the chimera studies, failed to yield complementation. These findings again suggest that the Glu\(^\text{260}\) region and possibly Phe\(^\text{87}\) are positioned to interact with agonist position five.

What is the physical basis for the functional complementation seen in these studies? The most dramatic complementation was observed when Arg replaced Glu at positions 260 or 264. By replacing or neutralizing negative charges, Arg substitutions at these and nearby positions might be acting to eliminate repulsive electrostatic interactions with position five of the SFLLEN peptide rather than (or in addition to) providing a salt bridge for binding Glu\(^\text{260}\) in the mutant agonist (Fig. 5A). Arginine scanning of the Glu\(^\text{260}\) region did reveal an apparent helical periodicity, with positive complementation occurring every three to four residues (Fig. 4B), and it was possible that the local structure of this loop placed the Glu\(^\text{260}\) and Glu\(^\text{264}\) side chains in proximity. We therefore tested the double alanine substitution Glu\(^\text{260}\)Ala + Glu\(^\text{264}\)Ala for gain of responsiveness to the SFLLEN peptide. In contrast to single alanine substitutions which had little effect (Fig. 3 and data not shown), the double alanine substitution caused a gain of responsiveness to the SFLLEN peptide comparable to that seen with the Glu\(^\text{260}\)Arg substitutions (Fig. 5). This suggests that the Arg substitutions at positions 260 or 264 had complemented the SFLLEN peptide’s agonist function at least in part by neutralizing nearby negative charge at positions 264 or 260, respectively (Fig. 5).

Does neutralization of repulsive electrostatic interactions also account for the gain of responsiveness to the human agonist peptide achieved in the receptor chimera studies? The Leu\(^\text{260}\)Glu replaced a neutral side chain with an acidic one. Might this acidic side chain be acting by neutralizing nearby basic lysine side chains to decrease repulsive interactions with Arg\(^\text{260}\) in the human agonist (Fig. 5B)? Substitution of alanines

### FIG. 5. Charge neutralization in the arginine substitution mutants and chimeras.

Does removal of repulsive electrostatic interactions between agonist and receptor explain the gain of function seen in these studies? A, the sequence of the Glu\(^\text{260}\) region in the human wild type (HWT) and relevant mutant thrombin receptors. Residues that were altered in one or more of the mutants are shown in bold. The predicted net charge of this region at physiological pH is indicated at right. Note that the Glu\(^\text{260}\) region in the wild type human thrombin receptor (HWT) has a net charge of −2 and might repel the SFLLEN mutant agonist. Arginine substitutions in this region reduced this net charge, perhaps accounting for the observed gain of responsiveness to SFLLEN. A double alanine substitution mutant that would also neutralize the Glu\(^\text{260}\) region (E260A, E264A) was therefore tested for possible gain of responsiveness to the SFLLEN agonist (C). B, charged residues in the region of the Xenopus thrombin receptor corresponding to the human receptor’s Glu\(^\text{260}\) region. Two lysines that might interfere electrostatically with the action of the SFLLEN agonist are present at positions 261 and 264. The HECL2,260 substitution, which caused a gain of responsiveness to SFLLEN, did add a negatively charged side chain to this region. A double alanine substitution mutant that removed the potentially inhibitory lysines was tested for possible gain of responsiveness to SFLLN (see Table I). C, neutralization of negative charges in the Glu\(^\text{260}\) region causes gain of responsiveness to the SFLLEN mutant agonist. The wild type human receptor (HWT) or the human receptor in which the glutamates at positions 260 and 264 were converted to alanines (E260A + E264A) were expressed in Xenopus oocytes and responses to the indicated concentrations of SFLLRNPNKD (SFLLN) or SFLLDPNDK (SFLLD) were determined. Data shown are the means of duplicate determinations. Similar results were obtained in five separate experiments. ○, HWT SFLLRN; ○—○, E260A + E264A SFLLRN; ●, HWT SFLLRN; ○—○, E260A + E264A SFLLEN; ○—○, HWT SFLLEN.
appear to be due to removal of electrostatic interactions that interfere with the action of the human agonist on the Xenopus receptor.

Alanine scanning of the Phe\textsuperscript{87} and Glu\textsuperscript{260} regions in the human receptor was undertaken to define the importance of individual side chains for responsiveness to agonist. Phe\textsuperscript{87} Ala caused a small loss of responsiveness to human agonist, a 13-fold increase in EC\textsubscript{50} Ala substitutions for the adjacent Ile\textsuperscript{88} and Ser\textsuperscript{89} caused 40- and 23-fold increases while substitutions at Glu\textsuperscript{89} and Asp\textsuperscript{91} had little effect (Table II). In the second extracellular loop, little or no loss of function occurred with alanine substitutions at residues 259–266. Thus in general, individual alanine substitutions at the residues that the chimera and complementation studies pointed to as important for maintaining receptor specificity caused little loss of responsiveness to agonist (Table II). However, larger deletions and substitutions in either region did cause substantial loss of receptor function (Table II and data not shown). The lack of dramatic loss of function from single alanine substitutions in the receptor’s specificity regions is perhaps not surprising. In the agonist itself, positions 3–6 presumably mediate receptor specificity (Fig. 1 and Ref. 14) Single alanine substitutions at these positions caused only partial loss of activity (14, 21), and each amino acid in the agonist probably interacts with several in the receptor.

In contrast to the individual alanine or arginine substitutions within the specificity regions of the receptor, single substitutions at aromatic or hydrophobic residues bounding these domains caused substantial loss of function (Table II and Fig. 4). Similarly, in the agonist peptide, in contrast to “specificity” positions 3–6, alanine substitution for the conserved Phe at agonist position 2 ablates agonist function (14, 21, 22). The finding of critical hydrophobic residues next to specificity residues in both receptor and agonist is tantalizing. Wells and colleagues (23) have recently found that the majority of the binding energy for growth hormone-receptor docking is contributed by hydrophobic interactions, with nearby charged and hydrophilic residues apparently helping to maintain specificity. Moreover, the docking interactions of thrombin’s anion-binding exosite with the thrombin receptor itself relies on the interactions of aromatic and hydrophobic residues that are surrounded by charged surfaces (9, 24). These analogies prompt the speculation that the critical Phe\textsuperscript{2a} in the thrombin receptor’s agonist peptide may dock in a hydrophobic pocket bound by the Glu\textsuperscript{260} and perhaps the Phe\textsuperscript{2b} specificity regions. Candidates for contributing to this putative hydrophobic pocket include the conserved aromatic cluster (266–271) at the carboxyl end of the thrombin receptor’s second extracellular loop and/or Ile\textsuperscript{88} in its amino-terminal exodomain; point mutations at these sites cause dramatic loss of function and both sequences are continuous with the specificity regions.

In summary, a systematic analysis of human-Xenopus chimeric thrombin receptors identified two small regions, residues 82–90 in the amino-terminal exodomain and 259–262 in the second extracellular loop, as important for distinguishing the human versus Xenopus agonist peptides. Within these domains, Phe\textsuperscript{87} and Glu\textsuperscript{260} made major contributions. These gain of function studies suggested the possibility of direct interactions between these regions and the human agonist peptide and raised the hypothesis that Arg\textsuperscript{5a} in the agonist might interact with Glu\textsuperscript{260}. This prompted a systematic arginine scan of the thrombin receptor’s extracellular surface in search of mutations which would complement an Arg\textsuperscript{5a}Glu mutant agonist peptide. Strikingly, this scan again identified the Glu\textsuperscript{260} region and to a lesser degree Phe\textsuperscript{87}, but not other sites in the receptor. The successful complementation of the Arg\textsuperscript{5a}Glu mutant agonist peptide by arginine substitutions in the receptor’s Glu\textsuperscript{260} region appeared to be due to removal of repulsive electrostatic interactions. By contrast, the gain of function to human agonist seen in the chimera studies could not be accounted for by such a mechanism.

Taken together, our data suggest two alternative models. The Phe\textsuperscript{87} and Glu\textsuperscript{260} regions of the receptor may simply play a gatekeeper role, regulating agonist access to a nearby binding site that is responsible for receptor activation. Alternatively, Arg\textsuperscript{5a} and perhaps nearby residues in the human agonist peptide may dock directly with the Glu\textsuperscript{260} region and/or Phe\textsuperscript{87} in the receptor and contribute to the conformational change that causes receptor activation. Our data show that these regions can both inhibit receptor activation by an “incorrect” agonist and promote receptor activation by the “correct” agonist, both features expected for a docking site. Moreover, our recent finding that mutations in the Glu\textsuperscript{260} region can cause constitutive activation of the human thrombin receptor support a direct role for this region in receptor activation.\textsuperscript{1}

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