Thrombin Receptor Activating Mutations

ALTERATION OF AN EXTRANCELLULAR AGONIST RECOGNITION DOMAIN CAUSES CONSTITUTIVE SIGNALING*

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Constitutively active thrombin receptors were generated while constructing chimeric receptors to identify the structural basis for thrombin receptor agonist specificity. Substitution of eight amino acids from the Xenopus receptor's second extracellular loop (XECL2B) for the cognate sequence in the human thrombin receptor was sufficient to confer robust constitutive activity. Smaller substitutions within the XECL2B site yielded less constitutive activation, and substitution of several unrelated sequences at this site caused no activation. Expression of the XECL2B receptor caused high basal 45Ca efflux in Xenopus oocytes and high basal phosphoinositide hydrolysis and reporter gene induction in COS cells. Of note, a mutant receptor in which all four of the Xenopus thrombin receptor's extracellular segments replaced the cognate human sequences showed much less constitutive activity than XECL2B and preserved responsiveness to agonist. This partial complementation of the XECL2B phenotype by addition of other Xenopus extracellular structures suggests that the XECL2B mutation causes constitutive activation by altering interactions among the human receptor's extracellular domains. Thus, a change in an extracellular loop of a G protein-coupled receptor can transmit information across the cell membrane to cause signaling, perhaps via a conformational change similar to that caused by agonist binding. Indeed, the site of the activating mutation in XECL2B coincides with a putative agonist-docking site, supporting the hypothesis that agonist interactions with the thrombin receptor's extracellular loops contribute to receptor activation.

A variety of naturally occurring and engineered mutations in G protein-coupled receptors have been found to cause constitutive activation. Their importance is underscored by the observation that activating mutations in G protein-coupled receptors underlie a variety of human diseases. The locations of activating mutations both within a single receptor and across receptors are widespread, with activating mutations reported in transmembrane domains 2, 3, and 7; in cytoplasmic loops 1 and 3; and at the junction of transmembrane domain 2 and extracellular loop 1 (reviewed in Refs. 1–4; see also Refs. 5–9). This diversity suggests that specific interactions maintain G protein-coupled receptors in their off-state(s) and that these interactions can be disrupted in a variety of ways. We recently generated a constitutively active thrombin receptor in the course of studying chimeric thrombin receptors designed to identify receptor domains that distinguish the human from Xenopus thrombin receptor agonist peptides. Building human thrombin receptor sequence into the Xenopus receptor (specifically small regions of the receptor's amino-terminal exodomain near transmembrane domain 1 and its second extracellular loop) conferred human receptor-like agonist specificity (10, 11). These same receptor regions, particularly receptor residues 260–268 in the second extracellular loop, were identified by a second approach that sought receptor mutations that complemented loss-of-function mutations in the agonist (11). In an attempt to confer Xenopus receptor-like specificity to the human receptor, we built Xenopus receptor sequences into the human receptor, the converse of the chimera experiments just described. Strikingly, several of the resulting chimeras were constitutively active. The substitution responsible for activation mapped to residues 259–268 in the receptor's second extracellular loop, the same region previously identified as responsible for agonist specificity. These studies clearly show that mutation of a G protein-coupled receptor's extracellular domain can cause transmembrane signaling. The observation that the site of the activating mutation is one previously shown to be involved in agonist recognition suggests that the activating mutation may cause a conformational change similar to that caused by agonist docking and supports the hypothesis that agonist interactions with extracellular loops can contribute to transmembrane signaling.

EXPERIMENTAL PROCEDURES

Construction of Mutant Receptor cDNAs and Their Characterization in a Xenopus Oocyte Expression System—cDNAs encoding human and Xenopus mutant thrombin receptors were generated by standard techniques (12) and confirmed by dideoxy sequencing (13). 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 (10, 14). To describe the various mutants, we use human thrombin receptor amino acid number with the start methionine as position 1 to designate both human residues and their cognate Xenopus receptor residues (10, 11). cDNAs encoding wild-type and mutant receptors were transcribed from cDNAs subcloned into pFROG (15). 25 ng of wild-type and mutant receptor cRNAs were injected per oocyte except as indicated. After culture for 24 h, receptor expression on the oocyte surface was measured as specific binding of antibody to the FLAG epitope. Surface expression of mutant receptors generally ranged from 50 to 150% of the wild type. Basal and agonist-stimulated 45Ca efflux, an index of phosphoinositide hydrolysis in the oocyte, was measured as described previously (15, 16). Briefly, oocytes were labeled with 45Ca for 3 h and then equilibrated with modified Barth’s solution with Hepes for 90 min. At this time, the oocytes were divided into groups of five, and

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constitutive activity when expressed in
mammalian cells as well as in the
Xenopus oocytes (Fig. 1). XECL2B failed to cause constitutive activation (Fig. 1). Thus, specific sequence must be substituted at the XECL2B site to effect constitutive activation. This result contrasts with that obtained with a well studied activating mutation in the α_{adrenergic} receptor. Mutation of Ala^{293} in the α_{adrenergic}-receptor's third cytoplasmic loop to any other amino acid caused constitutive activation, suggesting that the mechanism of mutational activation is disruption of an interaction that normally prevents the unliganded receptor from activating G proteins (21). By contrast, the observation that multiple and specific amino acid substitutions are required for the constitutive activation seen with XECL2B raises the possibility that the XECL2B activation of the mitogen-activated protein kinase and other intracellular signaling pathways (20, 22). Thrombin further stimulated phosphoinositide hydrolysis and luciferase expression in cells expressing XECL2B. The 20% increase in lucifer-

1 The abbreviations used are: DMEM, Dulbecco's modified Eagle's medium; CDTA, 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid.
expression caused by thrombin in cells expressing XECL2B was less than the 2-fold increase for thrombin-induced phosphoinositide hydrolysis presumably because luciferase accumulated between XECL2B transfection and stimulation with thrombin. The fact that the XECL2B receptor could indeed respond to thrombin raised the question, might the constitutive activity seen in cells expressing XECL2B depend in part on tonic activation by residual thrombin from serum or by some other protease? Ablation of the thrombin cleavage site in the XECL2B receptor chimera (XECL2B-S42P) ablated its ability to respond to added thrombin, but did not alter its constitutive activity (Fig. 2). The constitutive activity of XECL2B thus does not require unmasking of the receptor’s tethered ligand domain. This is consistent with the hypothesis that the XECL2B mutation increases the receptor’s probability of entering an active conformation even in the absence of agonist. Alternatively, one could postulate that unstimulated XECL2B and wild-type receptors enter the active conformation with the same frequency, with the XECL2B mutation interfering with receptor uncoupling and shutdown.

![Fig. 1. A, constitutive activity of chimeric thrombin receptors expressed in Xenopus oocytes. The wild-type human thrombin receptor (HTR) or chimeric thrombin receptors in which the Xenopus thrombin receptor’s amino-terminal exodomain (XenAT) or extracellular loops (XECL1, XECL2, or XECL2) were substituted for the cognate human sequences were expressed in Xenopus oocytes (see “Experimental Procedures”). Basal 45Ca release was measured as an index of each receptor’s constitutive activity, and maximally stimulated 45Ca release (10 nM thrombin) was measured as an index of receptor function. The level of surface expression for each chimera was determined by antibody binding. All chimeras used in these studies did express on the oocyte surface, in general at 50–150% of wild-type receptor levels. Surface expression of constitutively active chimeras was less than that of the wild-type receptor per unit of cRNA injected (see Fig. 3); thus, the high basal 45Ca release in oocytes expressing these chimeras was not due to high expression levels. B, effect of more limited substitutions within the second extracellular loop. The wild-type human thrombin receptor or chimeras in which the first or second half (XECL2A and XECL2B) of the second extracellular loop was replaced by cognate Xenopus receptor residues as well as chimeras with the indicated more limited substitutions in the XECL2B loop region were expressed in oocytes. Receptor expression and basal activity were determined as described for A. C, “control” substitutions. The wild-type or XECL2B chimeric receptors or mutant receptors in which other amino acids replaced those altered in XECL2B were expressed in oocytes (see D). Data in A–C are means ± S.D. (n = 3) and are expressed as (45Ca release per 10-min interval for each chimera)/(45Ca release per 10-min interval for the wild type). All are representative of at least three replicate experiments. D, location and sequence of activating and control substitutions. Substitution of Xenopus thrombin receptor sequence for the cognate human sequence between residues 259 and 268 of the human thrombin receptor yielded a chimeric receptor with constitutive activity. Additional chimeras with control substitutions at these sites (SGA, Conservative, and Random) did not show constitutive activity and mediated maximal responses to thrombin comparable to that elicited by the wild-type receptor.}

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Extracellular G Protein-coupled Receptor Activating Mutations
The major uncoupling mechanism for regulating G protein-coupled receptors is phosphorylation of activated receptors by G protein-coupled receptor kinases (23, 24). Previous studies showed that replacing serines and threonines in the thrombin receptor’s carboxyl-terminal tail with alanines prevented its agonist-dependent phosphorylation in Rat-1 and COS-7 cells and rendered it insensitive to inhibition by coexpressed β-adrenergic receptor kinase 2 in the Xenopus system (17). We compared signaling by this “desensitization-defective” mutant (C-tail Ser/Thr → Ala) with that of XECL2B in COS-7 cells. Unlike XECL2B, the C-tail Ser/Thr → Ala mutant did not cause constitutively high phosphoinositide hydrolysis in the absence of thrombin (Fig. 2A). In the presence of thrombin, the C-tail Ser/Thr → Ala mutant showed enhanced responses compared with wild-type and XECL2B receptors, consistent with the hypothesis that each activated C-tail Ser/Thr → Ala receptor coupled longer to phosphoinositide hydrolysis before its signaling was terminated (Fig. 2C) (14). The signaling behavior of XECL2B is thus distinct from that of a mutant receptor with defective desensitization. Overall, our data are most consistent with the hypothesis that the XECL2B mutation increases the probability of the receptor entering an active conformation.

The XECL2B mutation substitutes native sequence from the Xenopus thrombin receptor at the cognate position in the human thrombin receptor. The XECL2B sequence clearly does not cause constitutive activation in its native context in the Xenopus receptor. How is it that the wild-type human and Xenopus thrombin receptors do not show constitutive activity while the chimera does? A chimera in which the Xenopus thrombin receptor’s entire second extracellular loop was substituted for the cognate human loop displayed less constitutive activity than XECL2B, and a chimera in which the Xenopus thrombin receptor’s entire extracellular surface was substituted for that of the human receptor (XEC All) showed less still (Figs. 1 and 3). These chimeras showed robust signaling to thrombin, thus their lack of basal signaling was not due to a general loss of function. The partial complementation of the XECL2B phenotype by addition of other Xenopus extracellular structures implies a direct or indirect interaction of these structures and suggests two alternative mechanisms for XECL2B’s gain of function. The XECL2B mutation may effect constitutive activation by interacting with neighboring exodomain structures, disrupting normal interactions among the human receptor’s extracellular domains that help constrain the receptor in an off-state, and/or generating novel interactions that cause activation. Alternatively, the receptor’s extracellular loops may interact only indirectly by constraining the arrangement of the receptor’s transmembrane domains. In this model, the XECL2B mutation would cause activation via linkage of extracellular loop 2 to transmembrane domains. Explaining the complementation phenomenon described above with this second model is more cumbersome than with the first.

Previously identified activating mutations reside in putative transmembrane domains 2, 3, 6, and 7 and in the first and third...
extracellular loops (1–9). In addition, an activating missense mutation at the junction of transmembrane domain 2 and extracellular loop 1 has been uncovered in the melanocyte-stimulating hormone receptor (5). The location of the XECL2B mutation spans a putative asparagine-linked glycosylation site and is well within the thrombin receptor’s predicted second extracellular loop. This mutation clearly shows that changes in an extracellular domain of a G protein-coupled receptor can cause transmembrane signaling and receptor activation. As noted above, two independent approaches identified human thrombin receptor residues 259–268 as important determinants of the receptor’s agonist specificity. The XECL2B substitution involves this same region. The observation that the site of the XECL2B activating mutation overlaps a site shown to be important for agonist specificity, a putative agonist-docking site, is provocative. It suggests that the activating mutation may cause a conformational change similar to that caused by agonist docking and supports the hypothesis that agonist interactions with extracellular loops may be important for signal transduction.

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REFERENCES

1. Spiegel, A. M., Weinstein, L. S., and Shenker, A. (1993) J. Clin. Invest. 92, 1119–1125
2. Lefkowitz, R. J., Cotechia, S., Samama, P., and Costa, T. (1993) Trends Pharmacol. Sci. 14, 303–307
3. Lefkowitz, R. J., and Premont, R. T. (1993) J. Clin. Invest. 92, 2089
4. Coughlin, S. R. (1994) Curr. Opin. Cell Biol. 6, 191–197
5. Robbins, L. S., Nadeau, J. H., J ohnson, K. R., Kelly, M. A., Roselli-Rehfuss, L., Baed, E., and Mountjoy, K. G. (1993) Cell 72, 827–834
6. Kopp, P., van Sande, J., Parma, J., Duprez, L., Gerber, H., J ess, E., Jameson, J. L., Dumont, J. E., and Vassart, G. (1995) N. Engl. J. Med. 332, 150–154
7. Paschke, R., Tonacchera, M., van Sande, J., Parma, J., and Vassart, G. (1994) J. Clin. Endocrinol. Metab. 79, 1785–1789
8. Porcellini, A., Ciullo, I., Lavida, L., Amabile, G., Fenzl, G., and Avvedimento, V. E. (1994) J. Clin. Endocrinol. Metab. 79, 657–661
9. Schipani, E., Kruse, K., and Uppner, H. (1995) Science 268, 98–100
10. Gerdsen, R. E., Chen, J., Ishii, M., Ishii, K., Wang, L., Nanevicz, T., Turk, C. W., Vu, T.-H. K., and Coughlin, S. R. (1994) Nature 368, 648–651
11. 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
12. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) Methods Enzymol. 154, 367–382
13. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
14. Ishii, K., Hein, L., Kobilka, B., and Coughlin, S. R. (1993) J. Biol. Chem. 268, 9780–9786
15. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) Cell 64, 1057–1068
16. 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
17. Ishii, K., Chen, J., Ishii, M., Koch, W. J., Friedman, N. J., Lefkowitz, R. J., and Coughlin, S. R. (1994) J. Biol. Chem. 269, 1125–1130
18. Hung, D. T., Vu, T.-K. H., Nelen, N. A., and Coughlin, S. R. (1992) J. Biol. Chem. 267, 827–832
19. Paris, S., and Poussey, J. (1986) EMBO J. 5, 55–60
20. Chen, W. S., Lazar, C. S., Poenie, M., Tsien, R. Y., Gill, G. N., and Rosenfeld, M. G. (1987) Nature 328, 820–823
21. Kjelsberg, M. A., Cotechia, S., Ostrowski, J., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1430–1433
22. Hu, Q., Milfay, D., and Williams, L. T. (1995) Mol. Cell. Biol. 15, 1169–1174
23. Hausdorff, W. P., Caron, M. G., and Lefkowitz, R. J. (1990) FASEB J. 4, 2881–2889
24. Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1992) Annu. Rev. Biochem. 60, 651–668
25. Gliantz, S. A. (1992) Primer of Biostatistics, McGraw-Hill Book Co., New York