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Molecular Cloning of a Functional Thrombin Receptor Reveals a Novel Proteolytic Mechanism of Receptor Activation

Thien-Khai H. Vu,* David T. Hung,*†‡
Virginia I. Wheaton,* and Shaun R. Coughlin*†
*Cardiovascular Research Institute
†Department of Medicine
‡Cancer Research Institute
University of California, San Francisco
San Francisco, California 94143–0524

Summary

We isolated a cDNA encoding a functional human thrombin receptor by direct expression cloning in Xenopus oocytes. mRNA encoding this receptor was detected in human platelets and vascular endothelial cells. The deduced amino acid sequence revealed a new member of the seven transmembrane domain receptor family with a large amino-terminal extracellular extension containing a remarkable feature. A putative thrombin cleavage site (LDPR/S) resembling the activation cleavage site in the zymogen protein C (LDPR/I) was noted 41 amino acids carboxyl to the receptor's start methionine. A peptide mimicking the new amino terminus created by cleavage at R41 was a potent agonist for both thrombin receptor activation and platelet activation. "Uncleavable" mutant thrombin receptors failed to respond to thrombin but were responsive to the new amino-terminal peptide. These data reveal a novel signaling mechanism in which thrombin cleaves its receptor's amino-terminal extension to create a new receptor amino terminus that functions as a tethered ligand and activates the receptor.

Introduction

Elucidation of the mechanisms by which proteases activate cells has been a long-standing problem in cell biology. The serine protease thrombin is the most potent physiologic activator of platelet aggregation, and thrombin-induced platelet aggregation is critical for hemostasis and thrombosis (Berndt and Phillips, 1981; Hansen and Harker, 1988; Eidt et al., 1989). Thrombin is also a potent agonist for a number of biological responses that may mediate inflammatory and reparative responses to vascular injury (reviewed in Shuman, 1986). Thrombin is chemotactic for monocytes (Bar-Shavit et al., 1983), mitogenic for lymphocytes (Chen et al., 1976), and causes endothelial cells to express the neutrophil adhesive protein GMP-140 on their surface (Hattori et al., 1989). Thrombin also elicits platelet-derived growth factor production by the endothelium (Daniel et al., 1986) and is itself a potent and effective mitogen for mesenchymal cells (Chen and Buchanan, 1975). The mechanism by which thrombin activates platelets and other cells is unknown, and, despite considerable effort by a number of laboratories, the functional receptor(s) that mediates thrombin signaling has not been identified. Indeed, it is not known whether thrombin activates its receptor by a classical occupancy mechanism or by a more novel mechanism involving proteolytic cleavage of the receptor (reviewed in Berndt et al., 1985; Shuman, 1986).

Traditional ligand-binding approaches to identify a functional thrombin receptor have succeeded in identifying a number of thrombin-binding proteins (Gronke et al., 1987; Okamura et al., 1978) but have not led to identification of a signal transduction molecule. Moreover, because modified thrombins that function neither as thrombin agonists nor as thrombin antagonists do bind to platelets in a manner indistinguishable from wild-type thrombin binding (Davey and Luscher, 1967; Phillips, 1974; Martin et al., 1975; Tollefsen, 1974; Workman et al., 1977), it has not been possible to conclude that the sites identified in binding studies are related to the functional thrombin receptor. For this reason we adopted an expression cloning approach that followed thrombin-induced responses in Xenopus oocytes expressing exogenous mRNA to identify a functional human thrombin receptor cDNA. The thrombin receptor encoded by this cDNA clone was found to be expressed by both human platelets and endothelial cells. The deduced amino acid sequence of this clone predicted a novel member of the seven transmembrane domain receptor family. The extracellular amino-terminal extension of this receptor contained a putative thrombin cleavage site resembling the site cleaved when thrombin activates the zymogen protein C. We have demonstrated the importance of this site in receptor activation by mutagenesis experiments and have shown that a peptide mimicking the putative new amino terminus created by thrombin cleavage of its receptor is a potent agonist for the thrombin receptor expressed in oocytes and for platelet activation. Our results strongly suggest that the thrombin receptor is activated by an unprecedented mechanism in which thrombin-mediated proteolytic cleavage of the receptor in effect "unmasks" a ligand within the thrombin receptor's amino-terminal extension, thereby effecting receptor activation.

Results and Discussion

Development of an Assay for the mRNA Encoding the Thrombin Receptor

Stimulation of platelets by thrombin causes phosphoinositide hydrolysis and mobilization of intracellular Ca^{2+} (Rittenhouse-Simmons, 1979), signaling events thought to be critical in platelet activation. Receptors that signal via phosphoinositide hydrolysis and Ca^{2+} mobilization in mammalian cells have been expressed in Xenopus oocytes and found to confer receptor-dependent Ca^{2+} mobilization as measured by agonist-stimulated $^{45}Ca^{2+}$ release from labeled oocytes (Williams et al., 1988) or by agonist-induced inward Cl^- currents. The latter phenomenon has been exploited for the successful expression cloning of the substance K and serotonin 1c receptors (Masu et al., 1987; Julius et al., 1988). To determine if an analogous approach might be utilized to clone the functional thrombin receptor, we determined whether microinjection of Xeno-

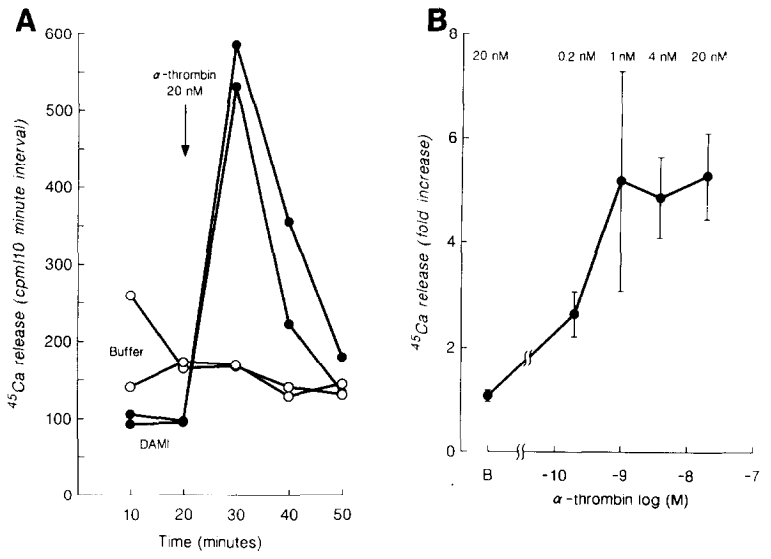

Figure 1. Thrombin-Induced $^{45}Ca^{2+}$ Release From Xenopus Oocytes Microinjected with Dami Cell mRNA

(A) Time course. Xenopus oocytes were microinjected with 50 nl of 10 mM HEPES (pH 7.0) (open circles) or with 50 nl of buffer containing 50 ng of unfractionated Dami cell mRNA (closed circles). The oocytes were maintained in culture for 48 hr and then loaded with $^{45}Ca^{2+}$. Agonist-induced Ca^{2+} release from pools of five oocytes was measured as described in Experimental Procedures. The data shown are the raw counts (cpm released per pool of 5 oocytes per 10 min interval; curves from duplicate pools of buffer-injected and Dami mRNA–injected oocytes are shown). Thrombin (20 nM) was added immediately following collection of the 20 min sample. Similar results have been obtained in over 20 experiments.

(B) Concentration dependence. Oocytes were microinjected with buffer (B) or with Dami mRNA, then cultured and loaded with $^{45}Ca^{2+}$. Thrombin was added at the indicated concentrations and the fold increase in $^{45}Ca^{2+}$ release elicited by thrombin determined (cpm released during the 10 min incubation with thrombin per cpm released during the preceding 10 min interval). The response of buffer-injected oocytes to 20 nM thrombin is indicated above (B); other points depict the response of Dami mRNA–injected oocytes to the indicated concentrations of thrombin. Data shown are means ± SEM for triplicate determinations; the results are representative of three separate experiments.

pus oocytes with mRNA from cells normally responsive to thrombin might result in oocyte expression of a functional thrombin receptor detectable by thrombin responsiveness in the oocytes.

Because platelets contain little mRNA, a number of cell lines were screened for thrombin responsiveness to identify cellular sources of mRNA encoding the functional thrombin receptor. Thrombin-induced increases in cytoplasmic Ca^{2+} levels were measured using the Ca^{2+}-sensitive fluor Indo 1 and flow cytometry (Grynkiewicz et al., 1985). Of the cell lines screened, HEL and Dami cells showed the most robust thrombin-induced increases in cytoplasmic Ca^{2+} (see Experimental Procedures). These megakaryocyte-like cell lines (Papayannopoulou et al., 1987; Greenberg et al., 1988) thus were chosen as potential sources for thrombin receptor mRNA.

Microinjection of Xenopus oocytes with poly(A)$^+$ RNA prepared from HEL and Dami cells conferred thrombin responsiveness as measured by thrombin-induced $^{45}Ca^{2+}$ release from radiolabeled oocytes (Figure 1A). Uninjected Xenopus oocytes and oocytes microinjected with buffer or with mRNAs encoding the serotonin 1c receptor or platelet-derived growth factor β-receptor showed no response to thrombin (Figure 1A and data not shown). To confirm that thrombin-induced increases in $^{45}Ca^{2+}$ efflux represented true increases in cytoplasmic Ca^{2+} levels that were reflected in other signaling events, thrombin-induced inward currents were measured in oocytes expressing Dami mRNA. Exposure of such oocytes to thrombin elicited an easily detected 100 nA current (Figure 2); oocytes microinjected with buffer showed no response.

Before using the oocyte system as a basis for expression cloning of the thrombin receptor, the pharmacology of thrombin-induced Ca^{2+} mobilization in Dami mRNA-expressing oocytes was examined and found to be similar to the pharmacology of thrombin-induced platelet activation: First, thrombin elicits platelet activation at nanomolar concentrations (Davey and Luscher, 1967; Martin et al, 1975). The EC_{50} for thrombin-induced $^{45}Ca^{2+}$ mobilization in the oocyte assay was approximately 0.2 nM (Figure 1B). Second, modification of thrombin with active site inhibitors such as the thrombin-specific serine protease inhibitor D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) (Kettner and Shaw, 1979) is known to ablate thrombin's ability to activate platelets. PPACK treatment also blocked thrombin's activity in the oocyte system (data not shown). Third, we have recently demonstrated that recombinant thrombins with active site substitutions are proteolytically inactive yet, unlike PPACK thrombin, retain a functional oxyanion hole/small substrate–binding pocket. These mutant thrombins failed to activate platelets (D. T. H., T. H. V., and S. R. C., unpublished data) and did not stimulate oocytes expressing Dami mRNA. Fourth, the natural anticoagulant hirudin and the hirudin-derived peptide hirugen bind thrombin and inhibit its ability to activate platelets (Rydel et al., 1990; Jakubowski and Maragonore, 1990). Both of these thrombin antagonists blocked thrombin-induced responses in the oocyte assay. Fifth, the profound homologous desensitization caused by thrombin in platelets (Shuman et al., 1979) could be reproduced in the oocyte system. Voltage-clamped oocytes expressing Dami mRNA responded to 10 nM thrombin with an easily detected inward current (Figure 2); however, subsequent exposure of these oocytes to thrombin elicited no or markedly decreased responses (data not shown). All of these results have been reproduced in oocytes expressing the receptor clone (Figures 3 and 7, Table 1, and data not shown).

During the course of this work, two groups reported that

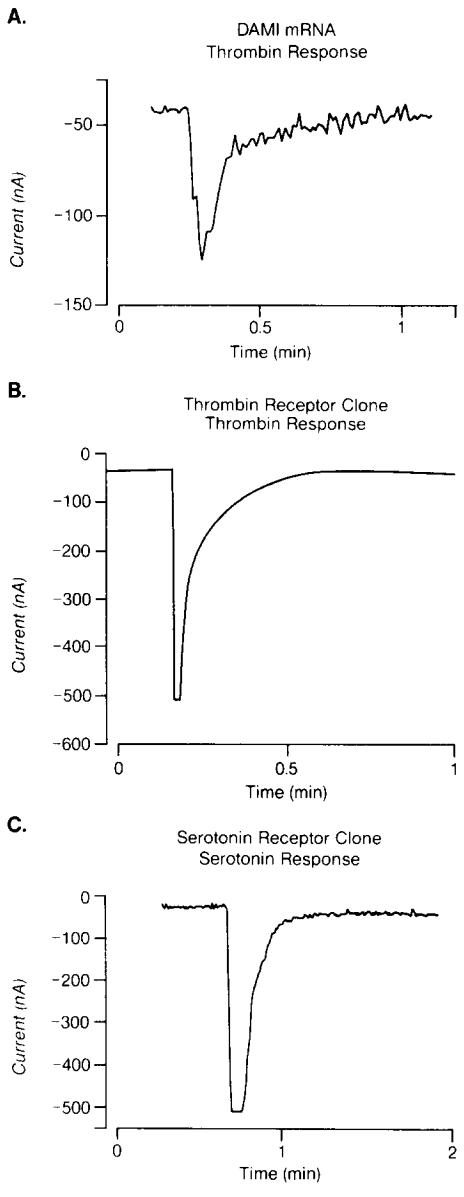

Figure 2. Thrombin-Induced Inward Current in Xenopus Oocytes Expressing Dami mRNA or Receptor cRNA

Xenopus oocytes were microinjected with buffer, unfractionated Dami mRNA, or cRNA transcribed from serotonin receptor or thrombin receptor cDNA clones. After 48 hr in culture, the oocytes were voltage clamped at −40 to −60 mV using the single electrode technique, then superfused with MBSH. The superfusion solution was switched to MBSH containing the appropriate agonist (1 μM serotonin for serotonin receptor cRNA–expressing oocytes; 10 nM thrombin for Dami mRNA– or thrombin receptor cRNA–expressing oocytes). Oocytes expressing Dami mRNA (A), thrombin receptor cRNA (B), or serotonin receptor cRNA (C) all responded rapidly to the appropriate agonist with an inward current. No response to either agonist was seen in oocytes microinjected with buffer (data not shown). This result is representative of those obtained in over 20 separate experiments.

microinjection of Xenopus oocytes with mRNA prepared from the thrombin-responsive hamster lung fibroblast cell line CCL39 (Van Obberghen-Schilling et al., 1990) or from human umbilical venous endothelial cells (Pipili-Synetos et al., 1990) conferred thrombin responsiveness upon the oocytes with appropriate pharmacology. Our results are consistent with these reports.

Identification of a cDNA Clone

To enrich for the mRNA encoding the functional thrombin receptor and to determine its approximate size, Dami mRNA was subjected to size fractionation by centrifugation through sucrose gradients (Sumikawa et al., 1982) and assayed for thrombin receptor activity as described in Experimental Procedures. Most of the thrombin receptor activity was recovered in a gradient fraction containing mRNA of an average size of approximately 4 kb. The specific activity of the size-fractionated mRNA preparation was approximately 5-fold higher than that of unfractionated mRNA, suggesting that the functional thrombin receptor was probably encoded by a single mRNA species (data not shown). Accordingly, we proceeded with a direct expression cloning strategy that demanded that a single mRNA species be capable of conferring thrombin responsiveness.

A size-selected cDNA library was synthesized from the thrombin receptor mRNA-enriched 4 kb fraction using a cloning vector that allowed in vitro transcription of the cDNA insert (see Experimental Procedures). The library was plated in 50 pools of 20,000 clones per pool; capped cRNA transcribed from each pool was screened in the oocyte system for thrombin receptor activity. By progressive subdivision of positive pools (see Experimental Procedures), a single cDNA clone was identified that conferred both thrombin-induced $^{45}Ca^{2+}$ release (Figure 3) and thrombin-induced activation of the Ca^{2+}-dependent Cl^- current in Xenopus oocytes (Figure 2).

Pharmacologic Characterization of the Thrombin Receptor Clone

Expression of capped cRNA transcribed from the thrombin receptor clone conferred thrombin responsiveness upon Xenopus oocytes in an impressive manner. Figure 3A shows the dependence of the magnitude of thrombin responses on the amount of receptor cRNA microinjected per oocyte. The magnitude of thrombin-induced $^{45}Ca^{2+}$ release by oocytes microinjected with 50 ng of receptor cRNA was approximately 100-fold greater than that of oocytes microinjected with 50 ng of Dami mRNA (70,000 cpm versus 500 cpm; Figure 1A versus Figure 3A) and was comparable with agonist-induced $^{45}Ca^{2+}$ release by oocytes microinjected with cRNAs from other cloned receptors (serotonin receptor, platelet-derived growth factor receptor, and fibroblast growth factor receptor; data not shown). Similarly, the magnitude of thrombin-induced inward current in oocytes expressing the thrombin receptor clone was markedly greater than that of oocytes expressing Dami mRNA and was comparable to serotonin-induced responses in oocytes expressing the cloned serotonin receptor (Figure 2).

The EC_{50} for thrombin-induced $^{45}Ca^{2+}$ release in oocytes expressing the thrombin receptor clone was approximately 50 pM (Figure 3B). This leftward shift of the concentration response curve compared with that observed with

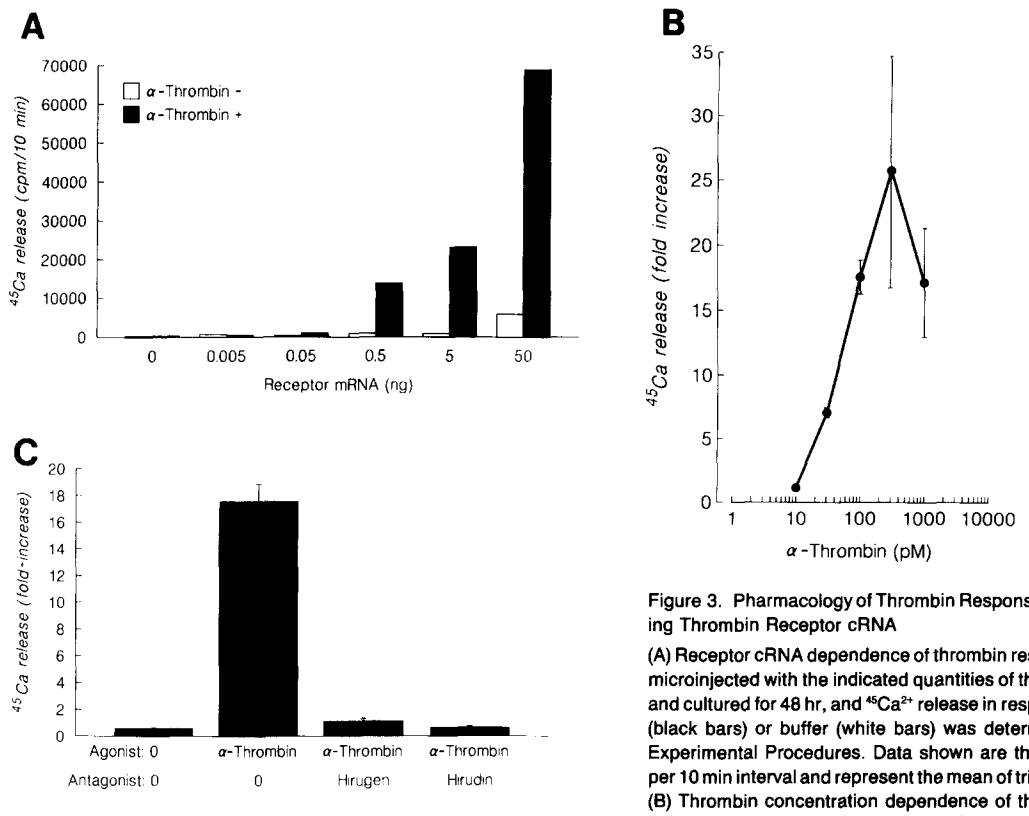

Figure 3. Pharmacology of Thrombin Responses in Oocytes Expressing Thrombin Receptor cRNA

(A) Receptor cRNA dependence of thrombin responses. Oocytes were microinjected with the indicated quantities of thrombin receptor cRNA and cultured for 48 hr, and $^{45}Ca^{2+}$ release in response to 1 nM thrombin (black bars) or buffer (white bars) was determined as described in Experimental Procedures. Data shown are the raw counts released per 10 min interval and represent the mean of triplicate determinations.

(B) Thrombin concentration dependence of thrombin-induced $^{45}Ca^{2+}$ release in receptor cRNA–expressing oocytes. Oocytes were microinjected with 5 ng of receptor cRNA and cultured for 48 hr, and $^{45}Ca^{2+}$ release in response to the indicated concentrations of thrombin was determined as described above. Data shown are the means ± SEM of three replicate determinations and are representative of results obtained in four replicate experiments.

(C) Hirudin and the anion exosite blocker hirugen block thrombin-induced responses in the oocyte system. Oocytes were microinjected with 2.5 ng of thrombin receptor cRNA and then cultured for 48 hr. Oocytes were then stimulated with buffer or buffer containing thrombin or thrombin plus hirudin or hirugen. The final concentrations in each case were thrombin, 30 pM; hirudin, 1 U/ml; hirugen, 100 μg/ml. The data shown are means ± SEM for triplicate determinations. This result was reproduced in three separate experiments.

Dami mRNA–injected oocytes (Figure 1B versus Figure 3B) was reproducible and likely due to a much higher level of receptor expression achieved in the cRNA-injected oocytes.

Thrombin-induced responses in receptor-expressing oocytes were blocked by the thrombin antagonist hirudin and the hirudin-derived peptide hirugen (Figure 3C). Hirugen is known to block thrombin-induced platelet activation by binding to and specifically blocking thrombin's anion exosite without interfering with thrombin's ability to cleave small substrate molecules (Jakubowski and Maragonore, 1990). The observation that hirugen blocked thrombin's ability to activate thrombin receptor–expressing oocytes (Figure 3C) suggested that the anion exosite of thrombin is required for activation of the cloned thrombin receptor just as it is for platelet activation (see below).

The specificity of responses in oocytes expressing the thrombin receptor clone was examined as described in Table 1. PPACK–thrombin and S205A mutant thrombin are incapable of activating platelets and failed to elicit responses in oocytes expressing the receptor clone, even when added at 40-fold the thrombin concentration required to elicit a maximum response. PPACK–thrombin is a chemically modified thrombin that is proteolytically inactive and has a blocked substrate-binding pocket/oxyanion hole. S205A–thrombin is a mutant thrombin that is proteolytically inactive yet retains the ability to bind the thrombin receptor (D. T. H., T. H. V., and S. R. C., unpublished data). The inability of these thrombins to activate the cloned receptor strongly suggests that proteolytic cleavage is required for receptor activation. In this regard, the serine protease trypsin did show agonist activity for

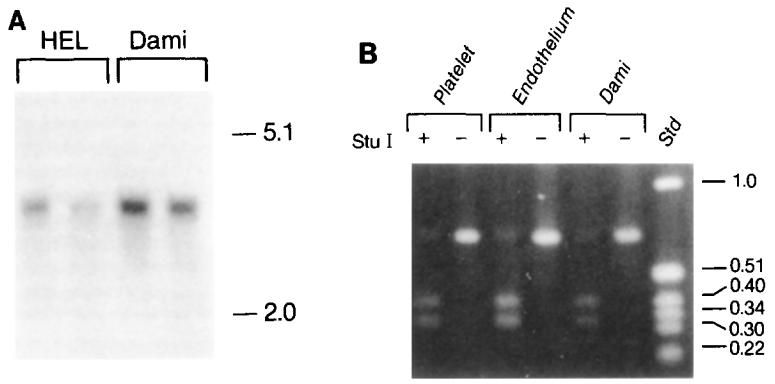

Figure 4. Detection of Thrombin Receptor mRNA in Megakaryocyte-like Cell Lines, Platelets, and Vascular Endothelial Cells

(A) Northern analysis of mRNA from HEL and Dami cells. Five micrograms of two independent preparations of poly(A)$^+$ RNA from HEL and Dami cells was denatured in glyoxal–DMSO and subjected to Northern analysis by standard techniques (Sambrook et al., 1989). The blot was hybridized at high stringency with random primer–generated ^{32}P-labeled probe representing the entire coding region of the functional thrombin receptor; hybridized probe was detected by autoradiography. The migration of 28S and 18S RNA is shown at right.

(B) PCR analysis of RNA from platelets and endothelial cells. Cytoplasmic RNA was prepared from human platelets or human umbilical venous endothelial cells as described in Experimental Procedures; approximately 5 μg of Dami cytoplasmic RNA was used as a positive control. The RNA was analyzed for thrombin receptor transcript by reverse transcription and PCR as described in Experimental Procedures. The identity of the PCR products with the appropriate portion of thrombin receptor sequence was confirmed by direct nucleotide sequencing.

the cloned thrombin receptor, while the highly specific protease enterokinase and the aromatic amino acid–specific protease chymotrypsin did not act as agonists for the cloned thrombin receptor. Trypsin is a lysine/arginine-specific protease, while thrombin cleaves proteins almost exclusively after arginines (Muszbek and Laki, 1984). These observations suggested that proteolytic cleavage after an arginine residue might be sufficient to activate the thrombin receptor, a conclusion supported by subsequent experiments (see below).

Detection of Thrombin Receptor mRNA in Megakaryocyte-like Cell Lines, Platelets, and Endothelial Cells

Expression of the functional thrombin receptor should be detectable in thrombin-responsive cells. Accordingly, Northern blot analysis for the thrombin receptor was performed on mRNA from HEL and Dami cells, megakaryocyte-like cell lines known to be thrombin responsive (see above). A single mRNA species of approximately 3.5 kb was detected in these cell lines (Figure 4A). Messenger RNA encoding the thrombin receptor was also detected in platelets and endothelial cells by reverse transcription of RNA derived from these cells followed by polymerase chain reaction (PCR) using primers derived from the receptor sequence. Analysis of the PCR products revealed a band of the predicted size (708 bp) and containing an appropriate internal StuI restriction site (Figure 4B). The bands were excised, subcloned into M13, and their nucleotide sequences determined. The nucleotide sequences of the platelet, endothelial, and Dami PCR products were all identical to the sequence of the corresponding region of the thrombin receptor clone. These data demonstrate that the thrombin receptor encoded by the cDNA clone we obtained is expressed in platelets and vascular endothelial cells; functional data obtained with a novel thrombin receptor agonist peptide described below confirm expression of the cloned thrombin receptor protein on the platelet surface (see below) and suggest that activation of this receptor may be sufficient for platelet activation.

Table 1. Specificity of Agonist-Induced $^{45}Ca^{2+}$ Mobilization in Oocytes Expressing the Thrombin Receptor Clone

Agonist	Concentration	Response Ratio (Receptor-Expressing Oocytes/Control Oocytes)
Thrombin	0.25 nM	37.0
PPACK–Thrombin	10 nM	0.9
S205A–Mutant Thrombin	10 nM	0.8
Enterokinase	10 U/ml	1.1
Trypsin	50 nM	1.3
Trypsin	500 nM	3.4
Trypsin	5000 nM	3.6
Chymotrypsin	5000 nM	1.0

Uninjected oocytes or oocytes microinjected with 5 ng of mRNA transcribed from the thrombin receptor clone were assayed for agonist-induced $^{45}Ca^{2+}$ release as described in the legend to Figure 1. Results are expressed as the ratio of the response obtained with receptor-expressing oocytes to that obtained with uninjected control oocytes and represent the mean of triplicate determinations. No agonist except trypsin caused $^{45}Ca^{2+}$ mobilization in uninjected oocytes. Trypsin at 500 and 5000 nM did elicit significant increases in $^{45}Ca^{2+}$ release in naive oocytes, but the response was reproducibly and markedly greater in oocytes expressing the thrombin receptor. These results are representative of four separate experiments.

Structure of the Thrombin Receptor cDNA and its Predicted Amino Acid Sequence

The functional thrombin receptor clone contained a 3.5 kb cDNA insert; the nucleotide sequence of this insert is shown in Figure 5. The cDNA sequence reveals a GC-rich 5′ untranslated region (bases 1–224), an open reading frame encoding a 425 amino acid protein (bases 225–1499), and a long 3′ untranslated region containing several polyadenylation signals and ending in a poly(A) tail (bases 1500–3480).

The deduced amino acid sequence of the thrombin receptor is shown in Figure 5. A hydropathy plot of this sequence reveals the receptor to be a member of the seven transmembrane domain receptor family (Figure 6A). An unusual topological feature is the hydrophobic signal se-

Figure 5. Nucleotide and Deduced Amino Acid Sequence of Thrombin Receptor cDNA

The nucleotide sequence of the 3.5 kb functional thrombin receptor cDNA was determined as described in Experimental Procedures. The amino acid sequence encoded by the longest open reading frame is shown using the single letter amino acid code. Hydrophobic regions including a putative signal sequence and seven transmembrane spans are overlined. Possible asparagine-linked glycosylation sites are underlined. Consensus polyadenylation signals are in bold. The putative thrombin receptor cleavage site at position R41/S42 is indicated by carets.

quence with potential signal peptidase cleavage sites (von Heijne, 1984) located at T24 and A26 (single letter amino acid code; sequential numbering of amino acids beginning at the start methionine). A relatively long (approximately 75 amino acid) extracellular amino-terminal extension containing several consensus sites for asparagine-linked glycosylation precedes the first transmembrane domain. A disulfide bond linking cysteine 175 in the first extracellular loop with cysteine 254 in the second extracellular loop is proposed (Figure 9) by analogy with rhodopsin and β_2-adrenergic receptor (Dohlman et al., 1990; Dixon et al., 1988; Fraser, 1989; Karnik et al., 1988).

Alignment of the thrombin receptor's amino acid sequence with those of other members of the seven transmembrane domain receptor family suggests that the thrombin receptor may define a new subfamily that is most closely related to the peptide receptor (substance P, substance K, and neuromedin K) and glycoprotein hormone receptor (lutropin and thyrotropin) subfamilies (Figure 6B).

Inspection of the amino acid sequence of the thrombin receptor's amino-terminal extension revealed several informative features. Forty-one amino acids distal to the start methionine lies the sequence LDPRS, which strongly resembles the sequence cleaved by thrombin in the zymogen protein C (Figure 6C). Thrombin-mediated cleavage of the LDPRI site in the zymogen protein C is responsible for protein C activation (Stenflo and Fernlund, 1982). An additional 13 residues carboxyl to the receptor's putative thrombin cleavage site lies the highly acidic sequence EPFWEDEEKNES, reminiscent of an acidic region in the carboxyl tail sequence in hirudin, DFEEIPEE(Y-SO$^-_3$)L. The latter is known to bind thrombin's anion exosite (Rydel et

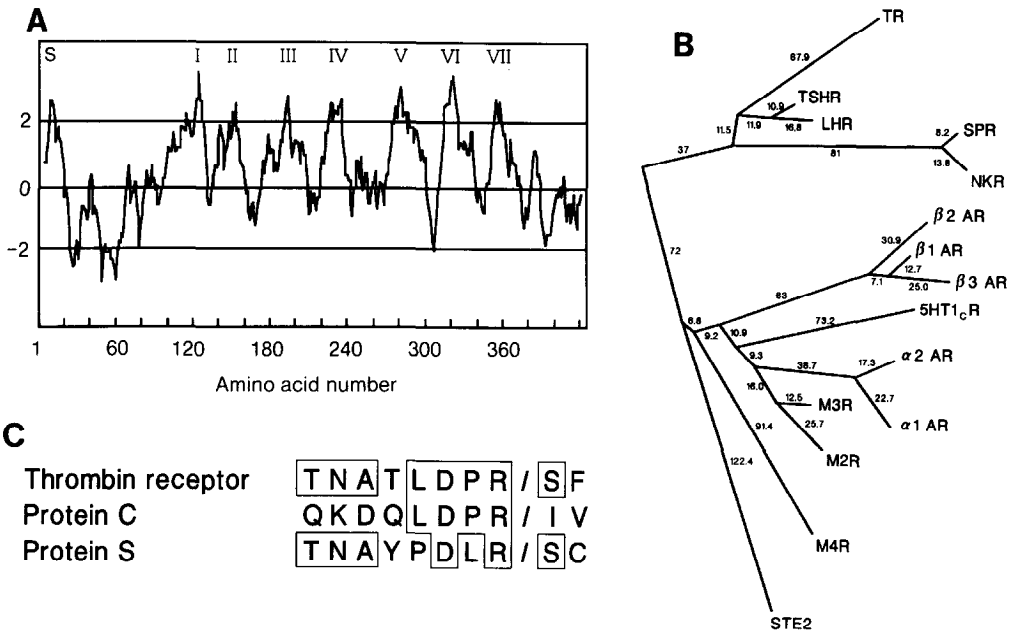

Figure 6. Analysis of the Thrombin Receptor's Amino Acid Sequence

(A) Hydropathy analysis. Eight hydrophobic domains including a putative signal sequence (S) and seven transmembrane spans (I–VII) were predicted.

(B) Relatedness of the thrombin receptor to other members of the seven transmembrane receptor family. A phylogenetic tree was constructed by progressive alignment (Feng and Doolittle, 1987) of amino acid sequences representing the cloned members of the seven transmembrane family of receptors shown (TR, thrombin receptor; TSHR, thyrotropin receptor; LHR, lutropin receptor; SPR, substance P receptor; NKR, neuromedin K receptor; AR, adrenergic receptor; $5HT1_cR$, serotonin 1c receptor; MR, muscarinic acetylcholine receptor; STE2, yeast α factor receptor). The numerical values labeling the tree indicate relative phylogenetic distance.

(C) Relatedness of the putative thrombin receptor cleavage site to known thrombin cleavage sites in natural thrombin substrates. The putative thrombin cleavage site in the receptor's amino-terminal extension is compared with known thrombin cleavage sites in protein C (Stenflo and Fernlund, 1982) and protein S (Dahlback et al., 1986). Note that the P1–P4 residues are identical in the thrombin receptor and protein C and that cleavage of R–S bonds is known to occur in natural thrombin substrates.

al., 1990), and a peptide mimicking the acidic region of hirudin, desulfato-hirugen (NGDFEEIPEEYL), is known to block thrombin-induced platelet activation (Jakubowski and Maragonore, 1990). As discussed above, this peptide also blocked thrombin's ability to activate the cloned receptor (Figure 3C), suggesting that thrombin's anion exosite may be important in mediating thrombin's interaction with its receptor. The location of a sequence resembling known thrombin cleavage sites in proximity to a site resembling a sequence known to interact with thrombin's anion exosite suggested that thrombin might bind to its receptor in part via the acidic region of the receptor's amino-terminal extension and activate its receptor by proteolysis of the arginine 41–serine 42 bond. We have begun to test this hypothesis as described below.

Site-Directed Mutagenesis of the Putative Thrombin Cleavage Site

Site-directed mutagenesis was employed to evaluate the importance of the putative thrombin cleavage site LDPRS in receptor activation. Arginine residues predicted to reside extracellularly were chosen for mutagenesis because of the specificity of the thrombin protease for arginine at the P1 position (Scheraga, 1977; Hogg and Blomback, 1978; Muszbek and Laki, 1984). The amino-terminal extension of the thrombin receptor contains 3 arginines: 1 within the LDPRS site at position 41, the others at positions 46 and 70. cDNAs encoding thrombin receptors with single amino acid substitutions of alanine for arginine at these potential thrombin cleavage sites were expressed in Xenopus oocytes. Oocytes expressing the R46A and R70A receptor mutants responded normally to thrombin (data not shown). By contrast, oocytes expressing the R41A receptor, the putative cleavage-site mutant, failed to respond to thrombin (Figure 7). Documentation of appropriate surface expression and function of the R41A receptor was obtained using a novel peptide agonist as described below (Figure 7). These data show that arginine 41 at the receptor's putative thrombin cleavage site is critical for receptor activation.

The hypothesis that cleavage of the R41–S42 peptide bond mediates thrombin receptor activation was supported by an additional receptor mutant. Arginine–proline peptide bonds are known to be unusually resistant to proteolysis by thrombin. Accordingly, a mutant thrombin receptor containing the single amino acid substitution S42P was produced and expressed in the oocyte system. Like the R41A receptor, the S42P mutant receptor was not activa-

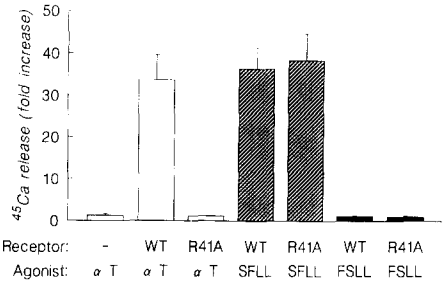

Figure 7. Agonist Activity of the "New Amino-Terminal Peptide" on Oocytes Expressing Wild-Type and Mutant Thrombin Receptor cRNA

Oocytes were microinjected with 5 ng of wild-type thrombin receptor cRNA (WT) or with 5 ng of cRNA encoding a mutant thrombin receptor with the amino acid substitution R41A (R41A). Uninjected oocytes or oocytes expressing thrombin receptor cRNAs were then cultured for 48 hr, and thrombin or peptide-induced $^{45}Ca^{2+}$ release was determined as described in Experimental Procedures. Agonists were added at saturating concentrations: thrombin at 250 pM and the new amino-terminal peptide SFLLRNPNDKYEPF (SFLL) at 25 μM. The control peptide FSLLRNPNDKYEPF (FSLL) was added at 100 μM. Data represent the means ± SEM of three replicate determinations; these results are representative of those obtained in four separate experiments. The agonist peptide has no activity on uninjected oocytes (data not shown). Qualitatively identical results were obtained when agonist-induced inward current in voltage-clamped oocytes was used as an endpoint rather than agonist-induced $^{45}Ca^{2+}$ release.

table by thrombin, but was fully activatable by the thrombin receptor agonist peptide (see below).

Evaluation of Peptides Mimicking the New Amino Terminus and Released Receptor Fragment for Agonist Function

Proteolysis at the R41 residue would release a fragment of the receptor's amino terminus and would reveal a newly created amino terminus beginning with S42, i.e., with the sequence SFLL. . . (carets, Figure 5). This suggested a number of possible mechanisms of receptor activation: First, the newly created amino terminus might function as a tethered ligand, binding to an as yet unidentified domain in the thrombin receptor and thereby mediating receptor activation (Figure 9). Second, the released receptor fragment peptide might function as a ligand for the thrombin receptor. Third, thrombin cleavage of the amino-terminal extension might release a tonic inhibitory action of the amino-terminal extension, thereby activating the receptor. The first hypothesis was favored by analogy with zymogen activation, a biological precedent for protease activation of other molecules. In the case of trypsinogen activation, proteolytic cleavage of trypsinogen to trypsin creates a new amino terminus that shifts its position and docks in a highly specific binding pocket within the trypsin molecule. A salt bridge formed between trypsin's new amino-terminal NH_3^+ (contributed by isoleucine 16) and the carboxylate of aspartate 194 mediates an additional conformational change that effects enzyme activation (Fehlhammer et al., 1977; Bode et al., 1978).

To begin to address the hypothesis that the thrombin receptor might be activated by an analogous mechanism, we produced synthetic peptides that mimicked the newly created amino terminus and the released receptor fragment peptide to evaluate their role in receptor activation. Oocytes expressing the wild-type and mutant thrombin receptors were treated with the synthetic peptide mimicking the new amino terminus created by thrombin's action (SFLLRNPNDKYEPF) or with control peptides (FSLLRNPNDKYEPF, LLRNPNDKYEPF, and the receptor fragment–related sequence PESKATNATLDPRSFLL). Strikingly, the new amino terminus peptide was a potent agonist for the wild-type thrombin receptor expressed in Xenopus oocytes (Figure 7). The "control" peptides listed above were devoid of activity on receptor-expressing oocytes, even at concentrations as high as 200 μM (Figure 7 and data not shown), and the new amino terminus agonist peptide had no activity on uninjected oocytes at concentrations as high as 200 μM (data not shown). The EC_{50} for agonist peptide–induced responses in oocytes expressing thrombin receptor cRNA was approximately 5 μM (data not shown). The maximal response to the agonist peptide in these oocytes was comparable to the maximal response to thrombin (Figure 7).

Oocytes expressing the R41A and S42P mutant thrombin receptors, which were not activated by thrombin itself, were effectively activated by the new amino terminus peptide (Figure 7); oocytes expressing these mutant receptors responded to peptide with an EC_{50} and maximum response that were not distinguishable from those obtained with oocytes expressing the wild-type thrombin receptor (data not shown). When agonist-induced inward current in voltage-clamped oocytes was used as an endpoint for thrombin- or peptide-induced responses, results qualitatively identical to those produced in the $^{45}Ca^{2+}$ release experiments shown in Figure 7 were obtained (data not shown).

Importantly, in addition to functioning as an agonist for oocytes expressing the cloned thrombin receptor, the new amino-terminal peptide was an effective agonist for platelet activation (Figure 8). The EC_{50} for platelet activation was approximately 30 μM (Figure 8); the slightly higher EC_{50} for platelets compared with receptor cRNA-expressing oocytes is probably due to a high receptor density ("spare receptors") achieved in the oocytes. In both the oocyte and platelet systems, the maximum response obtained with the peptide agonist was similar to the maximum response to saturating concentrations of thrombin (Figures 7 and 8); these data suggest that the new amino-terminal peptide is a full agonist for the thrombin receptor. The ability of the new amino-terminal peptide to activate oocytes expressing the receptor clone and platelets to the same extent as thrombin suggests that all information necessary for receptor activation may be provided by thrombin-mediated proteolysis of the R41–S42 peptide bond (see below).

Model of Thrombin Receptor Activation and its Implications

The studies described above suggest the following working model of thrombin receptor activation. Thrombin cleaves its receptor after arginine 41 in the amino-terminal extension domain, exposing a new amino terminus that is tethered to the receptor; this amino terminus then func-

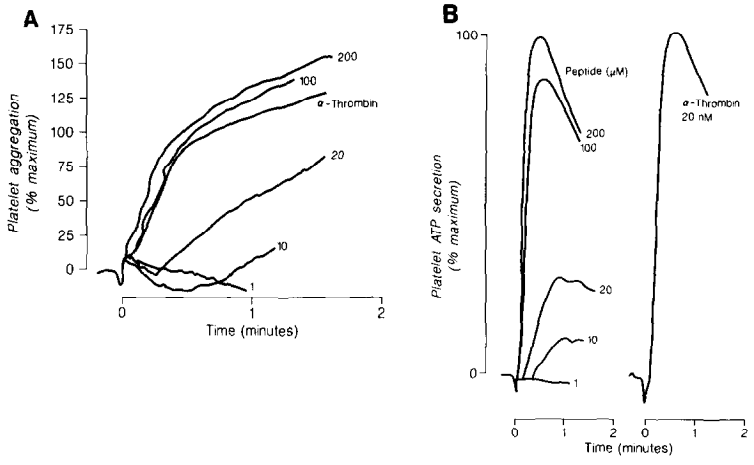

Figure 8. Agonist Function of the "New Amino-Terminal Peptide" for Platelet Secretion and Aggregation

Washed human platelets were prepared and agonist-induced responses assessed as described in Experimental Procedures.

(A) "New amino-terminal peptide" stimulates platelet aggregation. Platelet aggregation in response to 1, 10, 20, 100, or 200 μM peptide SFLLRNPNDKYEPF or to 20 nM thrombin was measured in a lumiaggregometer.

(B) "New amino-terminal peptide" stimulates platelet secretion. Platelet ATP secretion in response to the indicated final concentrations of "new amino-terminal peptide" was followed by lumiaggregometry.

The data shown are raw tracings representative of aggregation or secretion responses obtained in triplicate for each agonist concentration and are representative of results obtained in more than five separate experiments. One hundred percent aggregation is arbitrarily defined as that occurring in response to a saturating concentration of thrombin at 1 min. One hundred percent secretion is arbitrarily defined as the maximal response occurring in response to a saturating concentration of thrombin. The control peptides FSLLRNPNDKYEPF and LLRNPNDKYEPF were both without activity at concentrations as high as 200 μM (data not shown).

tions as an agonist for the receptor, binding to an as yet undefined site and eliciting receptor activation (Figure 9). Direct biochemical demonstration of receptor cleavage awaits the availability of appropriate receptor antibodies.

The requirement for the native amino acid sequence at the amino terminus of the agonist peptide for receptor-activating function is interesting in light of the parallel with trypsinogen activation drawn above. Whether the details of thrombin receptor activation resemble those of trypsinogen activation, that is, whether receptor activation occurs via a conformational change caused by a salt bridge between the receptor's new amino terminus NH_3^+ and a carboxylate within the receptor's binding pocket, remains to be determined.

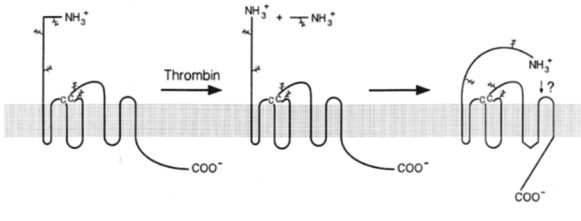

Figure 9. Mechanism of Thrombin Receptor Activation: Working Model

The amino-terminal extracellular extension of the intact and unactivated thrombin receptor is cleaved by thrombin, revealing a new amino terminus and releasing a short receptor fragment. The newly exposed amino terminus then functions as a receptor agonist, binding to an as yet undefined region of the thrombin receptor and activating it. The thrombin receptor is thus activated by a mechanism analogous to zymogen–enzyme conversion, as discussed in the text. The mechanism depicted above as well as the relatedness of the thrombin receptor to the seven transmembrane domain receptors (see Figure 6B) suggests that the thrombin receptor may be viewed as a peptide receptor that contains its own ligand, the sequence $S_{42}F_{43}$ . . . Whether the intramolecular rearrangement that mediates thrombin receptor activation represents an evolutionary specialization of earlier peptide receptors or a more primitive mechanistic heritage from which peptide receptors have evolved is unknown.

A corollary of the proteolytic mechanism of thrombin receptor activation is that the receptor is activated in a manner not reversible by dissociation of the agonist from the receptor. In the absence of a specific turn-off mechanism for the receptor, one might predict that thrombin-induced cell activation would proceed to completion independent of thrombin concentration (Martin et al., 1975). However, thrombin-induced cellular responses do show a threshold and a well-defined dose–response curve (Detwiler and Feinman, 1973a, 1973b; see also Figures 1B, 3B, and 8). This is compatible with the hypothesis that each activated thrombin receptor generates some quantum of a short-lived second messenger(s) before the receptor is inactivated by an as yet undefined mechanism. Indeed, the existence of such a mechanism has been suggested by the finding that thrombin treatment causes profound homologous desensitization to subsequent thrombin challenge both in platelets (Shuman et al., 1979) and in fibroblasts (Paris et al., 1988). In this regard it is interesting to note the large number of serine and threonine residues in the receptor's carboxyl tail, potential phosphorylation sites for a β-adrenergic receptor kinase–like enzyme (Lohse et al., 1989).

In summary, we have obtained a cDNA encoding a functional thrombin receptor by direct expression cloning in Xenopus oocytes. This receptor is expressed by both platelets and endothelial cells. The ability of a novel peptide agonist for this receptor to activate platelets strongly suggests a role for this receptor in mediating platelet activation. By providing a means of activating the thrombin receptor without utilizing thrombin itself, the agonist peptide makes possible direct studies of thrombin receptor activation independent of thrombin-induced fibrinogen clotting, protein C activation, and other effects of the thrombin protease. Assessing the role of this receptor in

other thrombin-induced cell activation phenomena becomes a readily approached problem.

Structurally, the thrombin receptor protein is a novel member of the seven transmembrane domain protein superfamily; whether the thrombin receptor will sire a subfamily of related protease receptors remains to be determined. The unusual mechanism utilized by the thrombin receptor for receptor activation (Figure 9) in essence requires a peptide receptor that contains its own ligand. This raises the interesting question of whether the intramolecular rearrangement utilized for thrombin receptor activation is an evolutionary specialization of earlier peptide receptors or is representative of a primitive heritage from which other peptide receptors may have evolved.

Experimental Procedures

Cell culture

HEL (Papayannopoulou et al., 1987) and Dami (Greenberg et al., 1988) cells were obtained from the ATCC (the latter generously provided through the ATCC patent collection by Drs. S. Greenberg and R. Handin, Harvard Medical School, Boston, MA) and maintained in suspension culture in RPMI with 10% calf serum. Before harvesting for mRNA preparation, cultures were incubated with phorbol 12-myristate 13-acetate (100 ng/ml for 24 hr) to increase the expression of platelet-specific genes (Papayannopoulou et al., 1987; Greenberg et al., 1988).

Cellular Ca^{2+} Mobilization

Dami, HEL, CCL-39, CHO, COS, CV-1, BALB/c, Swiss 3T3, Jurkat, IM-9, and U937 cell lines were loaded with Indo 1; relative increases in cytoplasmic Ca^{2+} levels caused by 10 nM thrombin were measured 30–60 s after the addition of agonist using a flow cytometer (Grynkiewicz et al., 1985).

Oocytes

Xenopus oocytes were harvested from female Xenopus laevis and processed using published techniques (Coleman, 1984; Williams et al., 1988). To remove follicular cells, oocytes were incubated for 4 hr at room temperature with 1 mg/ml Sigma type II collagenase in modified Barth's solution (MBSH) without Ca^{2+}, then washed and incubated overnight at 18°C in MBSH II (MBSH containing 1 mg/ml bovine serum albumin, 1 mg/ml Ficoll, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml gentamicin). Dumont stage V oocytes were selected and microinjected with 50 nl of mRNA (1 μg/μl in 10 mM HEPES [pH 7.0]). cRNA (5 ng) transcribed from a cDNA encoding a secreted form of alkaline phosphatase (generously provided by Dr. S. Udenfriend) was coinjected with all mRNA or cRNA samples as an internal standard for selection of healthy oocytes (Tate et al., 1990). Microinjected oocytes were cultured for 48 hr at 18°C in MBSH II in individual wells in 96-well culture plates; the oocyte-conditioned medium was then assayed for alkaline phosphatase activity as described (Tate et al., 1990) and the "best expressing" oocytes were selected for functional assays.

mRNA Preparation and Size Fractionation

Cytoplasmic and poly(A)$^+$ RNA was prepared from HEL and Dami cells by standard techniques (Sambrook et al., 1989). Poly(A)$^+$ RNA was fractionated by size by centrifugation through a 10%–30% sucrose density gradient exactly as described (Sumikawa et al., 1982). Aliquots of each gradient fraction were analyzed for size by glyoxal gel electrophoresis. The remainder of each fraction was twice ethanol precipitated and RNA dissolved at 1 μg/μl in 10 mM HEPES (pH 7.0). Aliquots of each fraction were assayed in the oocyte system for thrombin receptor activity.

Assays

Agonist-induced increases in $^{45}Ca^{2+}$ release were assessed by published techniques (Williams et al., 1988). Briefly, intracellular Ca^{2+} pools were labeled by incubating groups of 30 oocytes in 300 μl of Ca^{2+}-free MBSH containing 50 μCi of $^{45}CaCl_2$ (10–40 mCi per mg of Ca^{2+}; Amersham) for 4 hr at room temperature. The labeled oocytes were washed and then incubated in MBSH II without antibiotics for 90 min. Groups of five oocytes were selected and placed in individual wells in a 24-well tissue culture plate (Falcon 3047) containing 0.5 ml per well of MBSH II without antibiotics. This medium was removed and replaced with fresh medium every 10 min; the harvested medium was analyzed by scintillation counting to determine $^{45}Ca^{2+}$ released by the oocytes during each 10 min incubation. The 10 min incubations were continued until a stable baseline of $^{45}Ca^{2+}$ release per unit time was achieved. At this point, two additional 10 min collections were obtained, then medium including agonist was added and agonist-induced $^{45}Ca^{2+}$ release determined (see Figure 1). Agonist-induced inward Cl currents were measured in voltage-clamped oocytes essentially as previously described (Julius et al., 1988), except that the single electrode voltage-clamp technique was employed.

cDNA Library Synthesis and Screening

A size-selected cDNA library was synthesized from the 4 kb mRNA fraction enriched for thrombin receptor activity using the method of Gubler and Hoffman (1983). After ligation to BstXI adapters (Aruffo and Seed, 1987), cDNAs of approximately 3.5 kb or greater were selected by acrylamide gel electrophoresis prior to ligation into the cloning vector pFROG. The pFROG vector was derived from pCDM6XL (a pH3M-derived vector; Aruffo and Seed, 1987; generously provided by C. Spencer Yost, University of California, San Francisco) by adding a linker inserting a restriction site for the rare cutter MluI next to the NotI site. pFROG placed the cDNA under the transcriptional control of the SP6 RNA polymerase promoter and directed the synthesis of a hybrid mRNA containing the 5′ untranslated region of Xenopus globin followed by message encoded by the cDNA insert. The E. coli strain MC1061 was transformed with the cDNA library by electroporation and plated in 50 pools of 20,000 clones per pool. MC1061 carrying a model clone, serotonin 1c receptor cDNA in pFROG, was included at 1 clone per 2000 as an internal standard. Plasmid DNA was prepared from each pool and made linear by digestion with NotI; capped cRNA was produced in vitro (Krieg and Melton, 1987) and assayed for thrombin receptor activity in the oocyte system as described above.

All pools were screened using both the voltage-clamp and $^{45}Ca^{2+}$ release assay. Of the first five pools screened, all showed some thrombin receptor activity; in the $^{45}Ca^{2+}$ release assay, thrombin-induced increases in $^{45}Ca^{2+}$ release ranged from 2- to 6-fold. The most active pool was replated at approximately 2000 clones per plate and rescreened in the oocyte system. Two of 10 pools screened were positive for thrombin receptor activity. The most active of these was replated at 300 clones per plate and the pools rescreened. By progressive selection and subdivision of active pools, a single clone was identified.

Characterization of the Clone

The 3480 nucleotide cDNA insert was subcloned into the XhoI site of pBluescript. Restriction fragments of the insert were subcloned into M13. The cDNA sequence was determined twice in each direction (three times for the coding region) by dideoxy sequencing (Sanger et al., 1977).

Detection of Thrombin Receptor mRNA in Platelets and Endothelial Cells by Reverse Transcription and PCR

Fresh platelets were prepared from 35 ml of blood by standard techniques (Baenzinger and Majerus, 1974; Charo et al., 1977); in harvesting the platelet-rich plasma, care was taken to avoid the buffy coat to lessen the probability of harvesting contaminating leukocytes. Cytoplasmic RNA was prepared from these platelets and from a single confluent 75 cm^2 tissue culture flask of human umbilical venous endothelial cells. cDNA was produced from these RNAs by reverse transcription (Gubler and Hoffman, 1983), and cDNA encoding the thrombin receptor was amplified by PCR (Saiki et al., 1988) using the two primers 5′-TGTGAACTGATCATGTTTATG-3′ and 5′-TTCGTAAGATAAGAGATATGT-3′; 30 cycles were performed (denaturing 97°C for 15 s, annealing 60°C for 30 s, extension 72°C for 60 s). Half of each sample was then digested with StuI. Specific amplification of thrombin receptor cDNA with these primers should yield a 708 bp product with an internal StuI site; StuI digestion of this product should yield restriction fragments of 324 and 384 bp. The actual products found were consistent with these predictions (Figure 4B). To confirm sequence identity

of these products with thrombin receptor cDNA, the 708 bp products were subcloned into M13 and their nucleotide sequence determined by dideoxy sequencing; all products had sequences identical to that of the appropriate region of thrombin receptor cDNA. Because of the extraordinary amplification that PCR affords, mRNA from leukocytes contaminating platelet preparations must always be considered as a potential origin for PCR products. The functional data showing platelet activation with the new amino-terminal agonist peptide (Figure 8), however, strongly suggest that the cloned thrombin receptor is expressed on platelets and is capable of mediating platelet activation.

Receptor Mutagenesis

cDNAs encoding mutant thrombin receptors were produced by oligonucleotide-directed mutagenesis (Kunkel et al., 1987). All mutations were confirmed by dideoxy sequencing. Mutant receptors were subcloned into pFROG for the production of cRNA for oocyte expression. Expression of functional receptor mutants on the oocyte surface was confirmed functionally using an agonist peptide (see above).

Materials

Purified human α-thrombin was a generous gift from Dr. John W. Fenton II, Albany Medical College of Union University, Albany, NY. Purified wild-type and mutant human α-thrombins were also produced from the corresponding prothrombin cDNAs expressed in Chinese hamster ovary fibroblasts (D. T. H., T. H. V., and S. R. C., unpublished data). Peptides were made by the University of California, San Francisco Biomolecular Resource Center or were generously provided by Dr. Robert Scarborough, COR Therapeutics, Inc., South San Francisco, CA; peptides were HPLC purified before use.

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