Thrombin-induced Events in Non-Platelet Cells Are Mediated by the Unique Proteolytic Mechanism Established for the Cloned Platelet Thrombin Receptor

David T. Hung,*‡ Thien-Khai H. Vu,‡ Nicolas A. Nelken,‡§ and Shaun R. Coughlin‡‖

Departments of *Laboratory Medicine, ‡Cardiovascular Research Institute, §Surgery, and ‖Medicine, University of California at San Francisco, San Francisco, California 94143-0524

Abstract. We recently isolated a cDNA clone encoding a functional platelet thrombin receptor that defined a unique mechanism of receptor activation. Thrombin cleaves its receptor's extracellular amino terminal extension, unmasking a new amino terminus that functions as a tethered peptide ligand and activates the receptor. A novel peptide mimicking this new amino terminus was a full agonist for platelet secretion and aggregation, suggesting that this unusual mechanism accounts for platelet activation by thrombin. Does this mechanism also mediate thrombin's assorted actions on non-platelet cells? We now report that the novel thrombin receptor agonist peptide reproduces thrombin-induced events (specifically, phosphoinositide hydrolysis and mitogenesis) in CCL-39 hamster lung fibroblasts, a naturally thrombin-responsive cell line. Moreover, these thrombin-induced events could be recapitulated in CV-1 cells, normally poorly responsive to thrombin, after transfection with human platelet thrombin receptor cDNA. Our data show that important thrombin-induced cellular events are mediated by the same unusual mechanism of receptor activation in both platelets and fibroblasts, very likely via the same or very similar receptors.

We recently isolated a cDNA clone encoding a functional thrombin receptor by expressing cloning in *Xenopus* oocytes (23). The library used was made from Dami cells, a megakaryocyte-like cell line, and the clone was shown to be expressed by platelets. The clone encodes a seven transmembrane domain receptor with a one hundred-residue extracellular amino terminal extension. This extracellular extension contains a putative thrombin cleavage site (LDPR/S; / represents the point of cleavage) resembling the known thrombin cleavage site found in the thrombin-activated zymogen protein C (LDPR/I). Structure-activity studies with the cloned receptor (10, 22, 23) strongly suggest a unique mechanism of receptor activation. Thrombin cleaves its receptor at the LDPR/S cleavage site unmasking a new amino terminus beginning with the sequence SFLL . . .; this new amino terminus then functions as a tethered peptide ligand, binding to an as yet undefined site in the body of the thrombin receptor, effecting receptor activation. A peptide mimicking this new amino terminus was a full agonist for platelet secretion and aggregation, supporting the model of receptor activation proposed above and suggesting that this unusual mechanism accounts for platelet activation by thrombin (22).

In addition to its critical role in activating platelets to effect hemostasis and thrombosis (8), thrombin has potent actions on a variety of non-platelet cell types (reviewed in 16). For example, thrombin has potent chemotactic activities for monocytes (1) and is mitogenic for several cell types (3, 4), actions that may play important roles in inflammatory and proliferative processes in vivo. Are these assorted activities of thrombin also mediated by the unusual receptor activation mechanism described above? Do multiple receptors mediate thrombin's diverse actions, or does the cloned "platelet thrombin receptor" also account for thrombin-induced events in non-platelet cell types? To begin to address these questions, we took two approaches. First, the hamster lung fibroblast cell line CCL-39 is known to respond to thrombin with robust activation of the phosphoinositide turnover signaling pathway and with mitogenesis (11, 14, 15). We asked whether the novel thrombin receptor agonist peptide described above would elicit these two important thrombin-induced events in this naturally thrombin-responsive cell line. As implied above, the thrombin receptor is in essence a peptide receptor; the agonist peptide bypasses thrombin-mediated receptor proteolysis and activates the thrombin receptor independent of thrombin and its protease activity. Agonist peptide caused both phosphoinositide hydrolysis and mitogenesis in CCL-39 cells. We next transfected CV-1 cells, which are normally poorly responsive to thrombin, with the platelet thrombin receptor cDNA. Expression of the cloned platelet thrombin receptor in CV-1 cells reproduced the responses normally seen in CCL-39 cells, both thrombin-induced phosphoinositide hydrolysis and mitogenesis. In both cell types, thrombin- and agonist peptide-induced phosphoinositide hydrolysis was largely pertussis toxin insensitive. Thus activation of two important thrombin-induced

events in CCL-39 fibroblasts occurs via the same unusual activation mechanism used for platelet activation, and very likely via the same or a very similar receptor.

Materials and Methods

Peptides

Agonist peptide SFLLRNPNDKYEPF and control peptides LLRNPNDKYEPF and FSLLRNPNDKYEPF were synthesized by UCSF's Biomolecular Resource Facility (San Francisco, CA) or were a generous gift from Robert Scarborough, COR Therapeutics, Inc. (South San Francisco, CA). Peptides were purified by reverse phase HPLC before use.

Receptor-expressing Cell Lines

A cDNA encoding a functional human platelet thrombin receptor was subcloned into pBJ1, an expression vector derived from pcDL-SRα296 (19) (pBJ1 was a generous gift from Dr. Mark Davis). The expression construct was cotransfected with a neomycin selection marker (18) into CV-1 cells by lipofection (6). Cells were grown in DME with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stable transfectants were isolated using neomycin analogue G418 at concentrations of 0.8 mg/ml. Individual clones were selected and screened by dot blot for expression of thrombin receptor mRNA. The highest expressing clones were expanded and used in subsequent phosphoinositide turnover assays.

Phosphoinositide Hydrolysis Assays

Cells were seeded into 12-well plates at a density of 10^5 cells per well and grown to confluence. Wells were then rinsed once with 2 ml DME containing 25 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then incubated with 0.5 ml of DME containing 25 mM Hepes buffer, pH 7.4, 100 U/ml penicillin, and 100 μg/ml streptomycin, and 2 μCi/ml [^{3}H]myoinositol for 20–24 h at 37°C. Cells were treated with LiCl (20 mM final concentration) 1 min before addition of agonist. After 15 min of agonist treatment, all wells were rinsed once with 1 ml cold PBS, and cells were then extracted with 750 μl 20 mM formic acid for 30 min at 4°C. In all experiments involving the used of Bordetella pertussis toxin islet-activating protein (List Biological Laboratories, Campbell, CA), cells were incubated at 37°C for 5 h before agonist addition in the presence of pertussis toxin at a final concentration of 100 ng/ml.

Cell extracts were loaded onto 1 ml packed volume columns of anion-exchange gel resin AG1X8, formate form, 100–200 mesh size (Bio-Rad Laboratories, Cambridge, MA) after columns were prepared with sequencial washes of 2 ml 2 M ammonium formate/0.1 M formic acid, 2 ml water, and 4 ml 20 mM NH_4OH, pH 9.0. Immediately after loading, columns were washed with 3 ml 40 mM NH_4OH, pH 9.0, followed by 4 ml 40 mM ammonium formate. Columns were then eluted with 4 ml 2 M ammonium formate/0.1 M formic acid into scintillation vials containing 10 ml of scintillation cocktail and vials were counted in a scintillation counter (Beckman Instruments, Palo Alto, CA) for 10 min each. This procedure collects inositol mono, bis, and tris phosphates (11).

Mitogenesis Assays

CCL-39 cells and CV-1 cells were grown to confluence in 96 well plates (Costar, Cambridge, MA) in 100 μl DME (Gibco Laboratories, Grand Island, NY) containing 10% FCS, 25 mM Hepes buffer, pH 7.4, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then rinsed in DME and incubated in 100 μl DME containing 25 mM Hepes buffer, pH 7.4, 100 U/ml penicillin, and 100 μg/ml streptomycin for 48 h. Agonist was then added with or without 1 μg/ml insulin, 5 μg/ml transferrin, and 0.5 mg/ml BSA (see Fig. 2) and the incubation continued for 12 h. At this time [^{3}H]thymidine was added at 1.0 μCi/well (see Fig. 2) or 5.0 μCi/well. TCA-insoluble radioactivity was determined after an additional 24-h incubation.

Transfected and untransfected CV-1 cells (see Fig. 5) were treated with [^{3}H]thymidine (0.5 μCi/well) and agonists simultaneously and the plates incubated for an additional 48 h at 37°C. [^{3}H]thymidine incorporation into TCA-insoluble material was determined as previously described (5). [^{3}H]thymidine uptake results were confirmed by determining percent positive nuclei by [^{3}H]thymidine autoradiography as previously described (5).

ADP Ribosylation

Membrane preparation and ADP ribosylation was performed essentially as previously described (2). Thrombin receptor-transfected CV-1 cells were grown to confluence in eight 100-mm plates. Four of these plates were incubated in the presence of pertussis toxin (100 ng/ml final concentration) for 5 h at 37°C. Cells were then harvested using calcium/magnesium-free PBS with 0.4% EDTA, pelleted by centrifugation at 1,000 rpm for 10 min, resuspended in 800 μl of lysis buffer (50 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM EGTA, 1 mM PMSF, 1 mM DTT, and 1 mM benzamidine-HCl) and lysed by freeze thawing and shearing through a 27-gauge needle. Cell nuclei were pelleted by centrifugation at 1,000 rpm for 5 min at 4°C and the membrane-containing supernatant was removed and centrifuged at 14,000 rpm for 20 min at 4°C. The membrane-containing pellet was resuspended in 200 μl lysis buffer supplemented with 10% glycerol and protein concentrations were determined using Bradford reagent. 50 μg of pertussis toxin-treated or -untreated membranes were suspended in 30 μl of 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1 mM $MgCl_2$ and to each sample was added 50 μl 2× reaction buffer (2 mM ATP, 100 mM Tris-HCl, pH 7.4, 20 mM thymidine, 40 mM arginine, 0.4 mg/ml BSA, 200 mM KPO_4, pH 7.5, 10 mM ADP-ribose, 20 mM $MgCl_2$, 2 mM EDTA, and 200 μM GTP) and 4 μl activated pertussis toxin (pertussis toxin was activated by incubating 20 μl pertussis toxin [1 mg/ml] with 20 μl 42 mM DTT, 20 mM Tris-HCl, pH 7.5 at 30°C for 20 min). Finally, 10 μl of 5 μM NAD containing 20 μCi [^{32}P]NAD was added to each sample and incubated at 30°C for 30 min. The reaction was stopped by adding 100 μl of NAD wash solution (5 mM NAD, 50 mM Tris-HCl, pH 7.5, 25 mM EDTA) to each sample. Membranes were pelleted by centrifugation at 14,000 rpm for 5 min, washed twice with 200 μl NAD wash solution, resuspended in 100 μl Laemmli sample buffer, boiled for 5 min, and analyzed by SDS-PAGE (12% acrylamide gel) and autoradiograph. Exposure time was 5 h.

Results and Discussion

Agonist peptide caused phosphoinositide hydrolysis in CCL-39 cells with an EC_{50} of ~10 μM (Fig. 1 *A*). The EC_{50} for thrombin-induced phosphoinositide hydrolysis in CCL-39 cells was ~30 pM (Fig. 1 *B*). The relative potencies of agonist peptide and thrombin in this system mimicked that seen for agonist-induced responses mediated by the cloned receptor in the oocyte system (22). Moreover, maximum responses elicited by saturating concentrations of agonist peptide (100 μM) or thrombin (1 nM) were similar (Fig. 1, *A* and *B*). Thrombin receptor agonist peptide also stimulated [^{3}H]thymidine uptake in CCL-39 fibroblasts by approximately 20-fold (Fig. 2), and was once again several logs less potent than thrombin. The lower potency of agonist peptide compared to thrombin is predictable given the current model of receptor activation. Thrombin acts enzymatically; the peptide acts by binding. Thrombin generates a tethered ligand; the peptide is free. The kinetic advantages for thrombin are apparent.

The specificity of the agonist peptide was confirmed in several ways. First, a control peptide missing the agonist peptide's first two amino acids (see Materials and Methods) did not stimulate mitogenesis or phosphoinositide hydrolysis in CCL-39 cells (data not shown), even at concentrations as high as 100 μM. Second, another control peptide in which the first two amino acids were switched in positions (SFLLRNPNDKYEPF to FSLLRNPNDKYEPF) had only minimal agonist activity (Fig. 2). Lastly, the actions of the agonist peptide were receptor dependent in the transfection studies discussed below.

The actions of the thrombin receptor agonist peptide described above strongly suggested that thrombin-induced phosphoinositide hydrolysis and mitogenesis in CCL-39

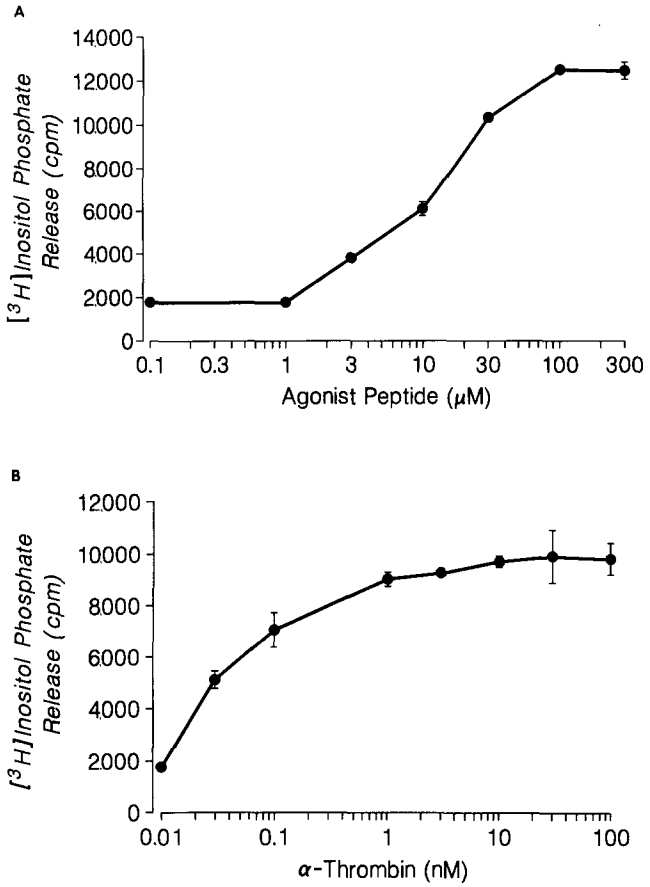

Figure 1. Thrombin-induced and thrombin receptor agonist peptide-induced phosphoinositide hydrolysis in CCL-39 fibroblasts. Quiesced CCL-39 fibroblasts loaded with [^{3}H]myoinositol (see Materials and Methods) were treated in the presence of 20 mM LiCl for 15 min at 37°C with thrombin receptor agonist peptide (*A*) or α-thrombin (*B*) at the concentrations indicated. Total [^{3}H]inositol phosphates were collected and quantitated as described in Materials and Methods. Each point represents the mean of three replicate determinations with standard deviations as shown. These results are representative of three replicate experiments.

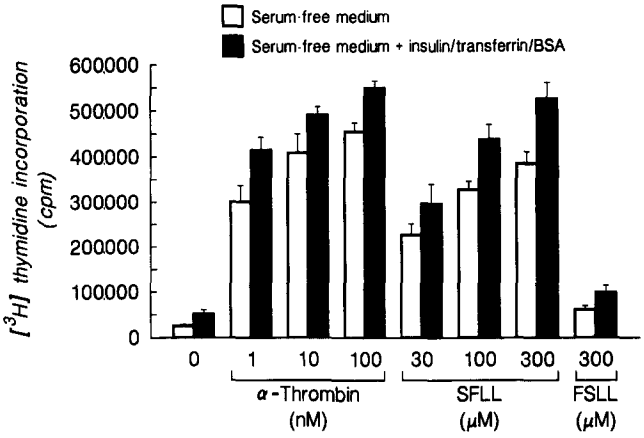

Figure 2. Thrombin and thrombin receptor agonist peptide cause mitogenesis in CCL-39 fibroblasts. CCL-39 fibroblasts were incubated in either serum-free DME (*open columns*) or serum-free DME with 1 μg/ml insulin, 5 μg/ml transferrin, and 0.5 mg/ml BSA (*closed columns*) for 48 h at 37°C. Cells were then stimulated with either α-thrombin, thrombin receptor agonist peptide (SFLLRNPNDKYEPF; *SFLL*), or control peptide (FSLLRNPNDKYEPF; *FSLL*) at the concentrations indicated for 12 h at 37°C, followed by addition of [^{3}H]thymidine in the continued presence of agonists for an additional 24 h at 37°C. [^{3}H]thymidine incorporation into TCA-insoluble material was determined as previously described (5). Each point represents the mean of three replicate determinations with standard deviations as shown. These results were replicated in three separate experiments. Similar results were obtained when the [^{3}H]thymidine concentration was increased fivefold, such that [^{3}H]thymidine could not be limiting.

cells are mediated, at least in part, by the same novel proteolytic mechanism of receptor activation seen in platelets, probably by the same or closely related receptors. Northern analysis of mRNA from CCL-39 fibroblasts suggested that the hamster homologue of the cloned human platelet thrombin receptor was expressed in this hamster fibroblast cell line (Fig. 3) as recently reported (12). Interestingly, Pouysségur and colleagues have reported that thrombin receptor agonist peptide elicited robust mitogenic effects on their subclone of CCL-39 cells only when FGF was included in the medium, while thrombin itself was mitogenic in the absence of FGF (21). We have not observed a requirement for additional mitogens for the action of agonist peptide on the CCL-39 cells used in our laboratory (Fig. 2). We do wish to state, however, that our data in no way preclude the existence of other thrombin receptors.

To determine whether the cloned human platelet thrombin receptor could itself account for thrombin-induced cellular events in non-platelet cells, thrombin receptor cDNA was stably transfected into CV-1 cells and the ability of receptor-transfected cells to respond to both α-thrombin and throm-

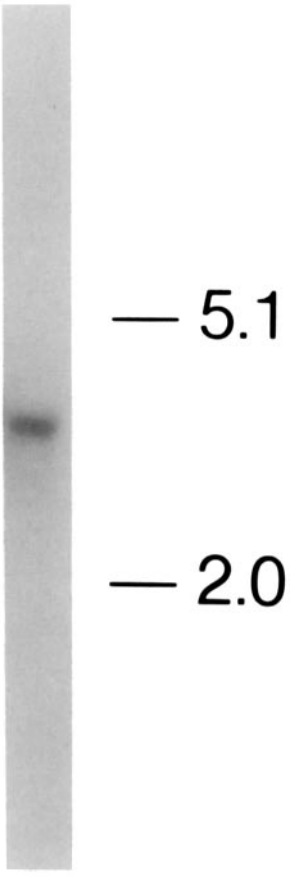

Figure 3. Detection of thrombin receptor mRNA in CCL-39 fibroblasts. Five micrograms of poly(A)+ RNA from CCL-39 fibroblasts was denatured in glyoxal-DMSO and subjected to Northern analysis using standard techniques (13). The blot was hybridized at high stringency with random primer-generated ^{32}P-labeled probe representing the entire coding region of the cloned human platelet thrombin receptor; hybridized probe was detected by autoradiography. The migration of 28S and 18S RNA is shown at right.

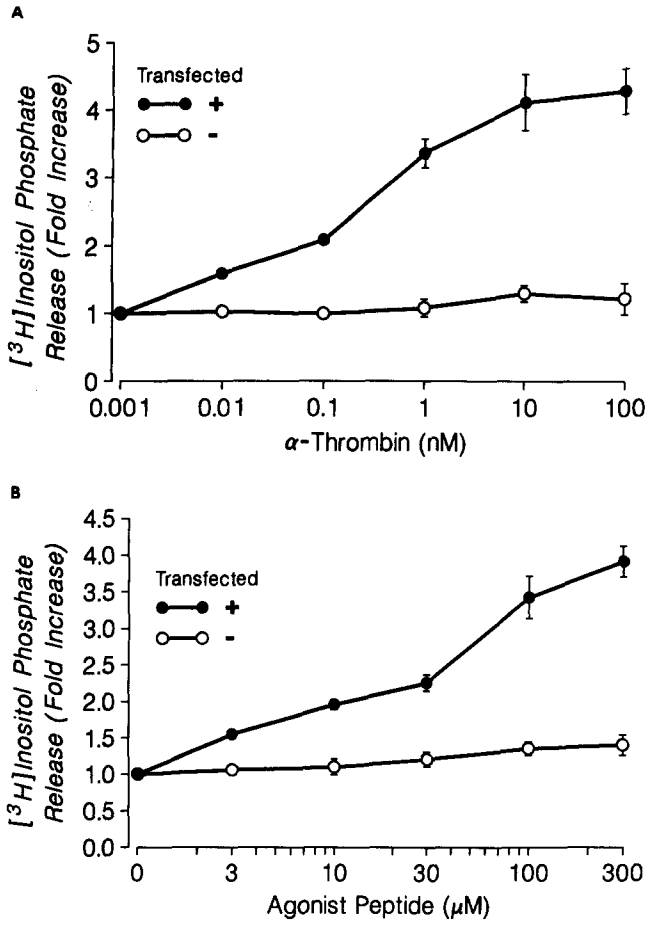

Figure 4. Transfection with platelet thrombin receptor cDNA confers thrombin-induced and thrombin receptor agonist peptide-induced phosphoinositide hydrolysis on CV-1 cells. Thrombin receptor-transfected (●) and untransfected (○) CV-1 cells labeled with [^{3}H]myoinositol (see Materials and Methods) were treated in the presence of 20 mM LiCl for 15 min at 37°C with α-thrombin (*A*) or thrombin receptor agonist peptide (*B*) at the concentrations indicated. Total [^{3}H]inositol phosphates were collected and quantitated as described in Materials and Methods. [^{3}H]inositol phosphate release induced by 0.001 nM α-thrombin was equivalent to baseline (i.e., did not stimulate inositol phosphate release). Each point represents the mean of three replicate determinations with standard deviations as shown. These results are representative of three replicate experiments.

bin receptor agonist peptide was examined. CV-1 cells transfected with the human platelet thrombin receptor responded with a 400% increase in inositol phosphate release when stimulated with saturating concentrations of either thrombin or agonist peptide (Fig. 4, *A* and *B*). The EC_{50}'s for thrombin and thrombin receptor agonist peptide were 100 pM and 10 μM, respectively, similar to the EC_{50}'s found in naturally thrombin-responsive CCL-39 cells and in oocytes expressing the platelet thrombin receptor cDNA (22). By contrast, untransfected CV-1 cells exhibited no significant increase in phosphoinositide hydrolysis when stimulated with either thrombin or thrombin receptor agonist peptide at concentrations as high as 100 nM and 300 μM, respectively (Fig. 4, *A* and *B*). Thrombin failed to activate phosphoinositide turnover in other clones expressing thrombin receptor mutants rendered unactivatable by cleavage site mutations (Hung,

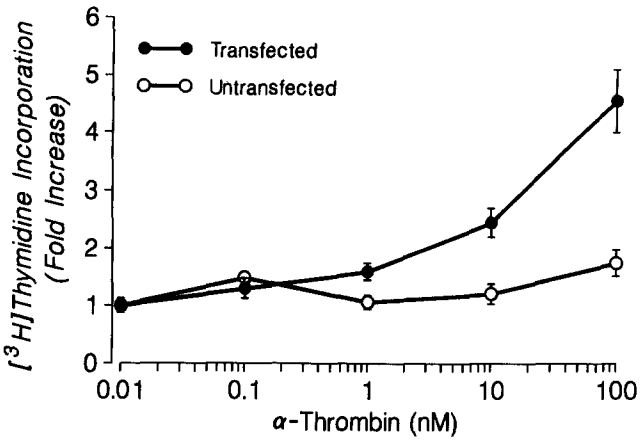

Figure 5. Transfection with platelet thrombin receptor cDNA confers thrombin-induced and thrombin receptor agonist peptide-induced mitogenesis on CV-1 cells. Quiesced thrombin receptor-transfected (●) and untransfected (○) CV-1 cells were stimulated with the indicated concentrations of α-thrombin for 48 h at 37°C and [^{3}H]thymidine incorporation into TCA-insoluble material was determined as previously described (5). Mean cpm per well of unstimulated receptor-transfected and -untransfected cells were similar (5,940 ± 738 [S.E.] and 6,566 ± 1,447 [S.E.], respectively).

D. T., T.-K. H. Vu, and S. R. Coughlin, manuscript in preparation). While anticipated from our previous studies (22), this is the first demonstration that the cloned thrombin receptor couples to phosphoinositide hydrolysis.

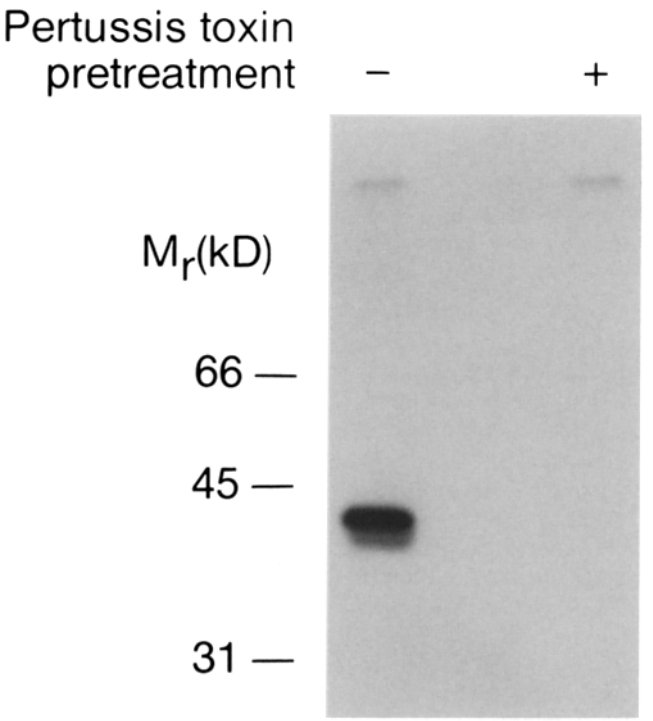

Figure 6. Demonstration of a lack of pertussis substrate in membranes from pertussis toxin-treated cells. Membranes from pertussis toxin-treated (+) or untreated (−) thrombin receptor-transfected CV-1 cells were analyzed for available pertussis substrates as described in Materials and Methods. Quantitation of radioactivity in the + lane band and in the corresponding region of the − lane revealed that at least 97% of pertussis substrate had been "removed" by pertussis toxin pretreatment.

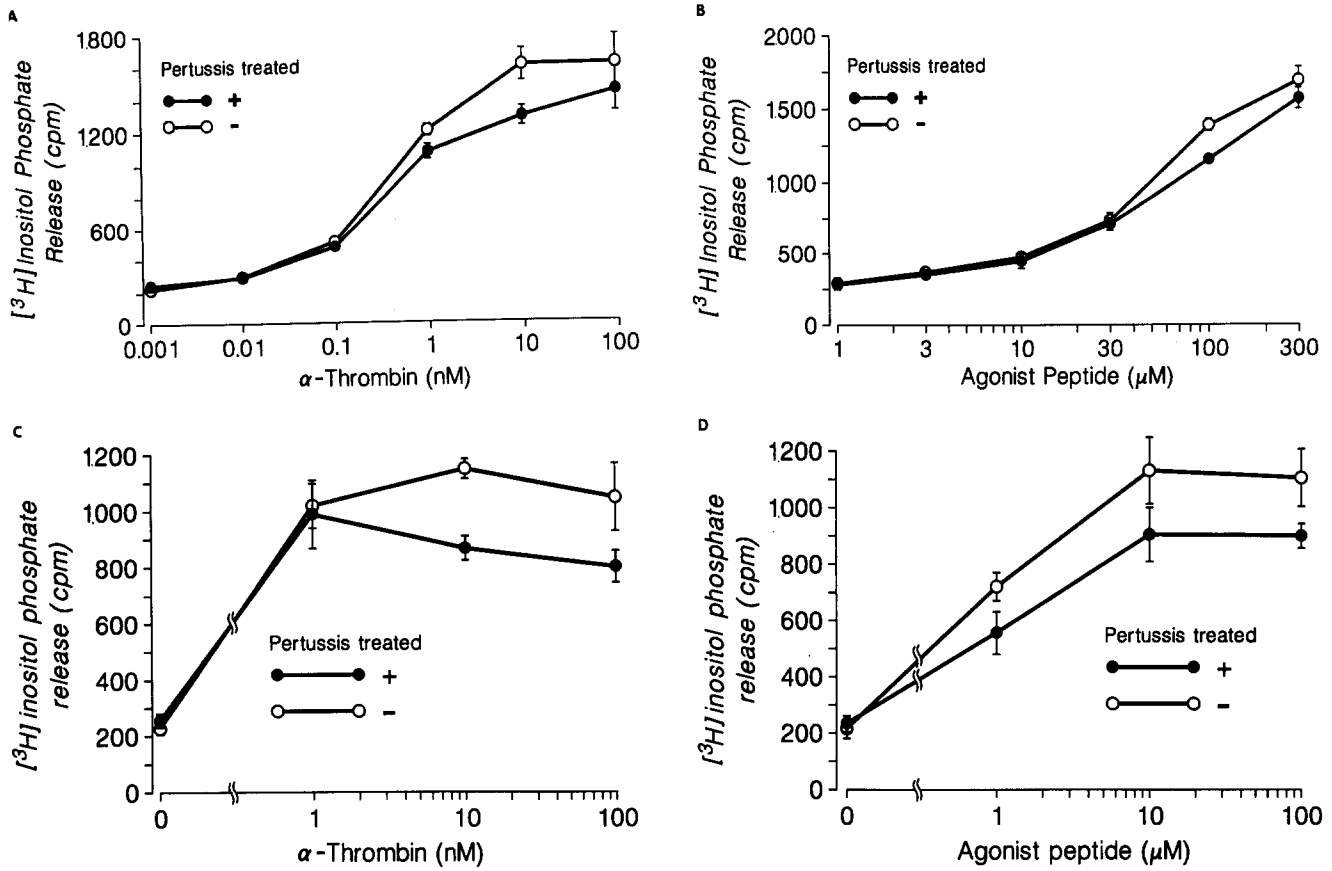

Figure 7. Thrombin-induced and thrombin receptor agonist peptide-induced phosphoinositide hydrolysis in thrombin receptor-transfected CV-1 cells and CCL-39 fibroblasts is largely pertussis toxin-insensitive. Quiesced thrombin receptor-transfected CV-1 cells (*A* and *B*) or CCL-39 fibroblasts (*C* and *D*) labeled with [^{3}H]myoinositol (see Materials and Methods) were incubated at 37°C for 5 h with either 10 μl of PBS (○) or an equal volume of PBS containing pertussis toxin to yield a final concentration 100 ng/ml (●). Cells were then treated for 15 minutes at 37°C with α-thrombin (*A* and *C*) and thrombin receptor agonist peptide (*B* and *D*) at the concentrations indicated in the presence of 20 mM LiCl. Total [^{3}H]inositol phosphates were collected and quantitated as described in Materials and Methods. Each point represents the mean of three replicate determinations with standard deviations as shown. These results are representative of three replicate experiments.

The cloned "platelet thrombin receptor" also conferred thrombin-induced mitogenesis to CV-1 cells. [^{3}H]thymidine uptake in CV-1 cells stably transfected with the cloned thrombin receptor cDNA increased ~500% in response to thrombin, while untransfected CV-1 cells showed minimal response (Fig. 5).

These studies show that transfection of CV-1 cells with the cloned platelet thrombin receptor cDNA conferred the thrombin responses seen in naturally thrombin-responsive CCL-39 cells. The similarities in the thrombin and peptide responses in native CCL-39 cells and receptor-transfected CV-1 cells extended to their pertussis toxin sensitivity. Pertussis toxin treatment sufficient to fully ADP-ribosylate available G-protein α subunits (Fig. 6) had little inhibitory effect on thrombin- or agonist peptide-stimulated phosphoinositide hydrolysis in thrombin receptor-transfected CV-1 cells (Fig. 7, *A* and *B*). In CCL-39 cells, pertussis toxin treatment caused only partial inhibition of thrombin- or agonist peptide-stimulated phosphoinositide hydrolysis (Figure 7, *C* and *D*). Published studies report both pertussis-sensitive and -insensitive thrombin–induced phosphoinositide hydrolysis in various cell lines (7, 9, 11, 14, 15), suggesting that the thrombin receptor may couple to phosphoinositide hydrolysis via different G-proteins determined by a cell's G-protein repertoire, or that different receptors may exist. Our studies demonstrate that the cloned thrombin receptor couples to phosphoinositide hydrolysis predominantly via a pertussis toxin-insensitive G-protein(s) in the cell lines we examined. The recently described G_q class of G-proteins, which lack a "pertussis site" (17) and couple to the β1 isozyme of phospholipase C (20), are candidates for mediating thrombin receptor-induced phosphoinositide hydrolysis.

In summary, these studies suggest that the unique mechanism of thrombin receptor activation described for platelet activation also mediates important thrombin-induced events (phosphoinositide hydrolysis and mitogenesis) in a naturally thrombin-responsive fibroblast cell line. Moreover, our studies show that the platelet thrombin receptor can confer these same responses to a cell line that is normally poorly responsive to thrombin. Our results strongly suggest that the hamster homologue of the cloned human "platelet thrombin receptor" accounts, at least in part, for thrombin responses in CCL-39 fibroblasts and that the cloned platelet thrombin receptor may mediate not only platelet activation but also mitogenesis and other thrombin-induced events in a variety of cell types. In this context, we have recently found thrombin receptor mRNA expressed in vascular endothelium and smooth muscle cells by PCR and Northern analysis (22, and

data not shown) and by in situ hybridization of human arteries (data not shown). Thus antagonists of the cloned thrombin receptor may be useful not only in inhibiting platelets and thrombosis, but also in blocking undesirable thrombin-induced inflammatory or proliferative responses in the blood vessel wall.

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