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Extracellular Mutations of Protease-Activated Receptor-1 Result in Differential Activation by Thrombin and Thrombin Receptor Agonist Peptide

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ABSTRACT

The protease-activated thrombin receptor-1 (PAR-1) can be activated by both the tethered ligand exposed by thrombin cleavage and a synthetic peptide having the tethered ligand sequence (thrombin receptor agonist peptide or TRAP). We conducted a mutational analysis of extracellular residues of the receptor potentially involved in interaction with both the tethered ligand and the soluble peptide agonist. Agonist-stimulated calcium efflux in *X. laevis* oocytes or inositol phosphate accumulation in COS-7 cells was used to assess receptor activation. We have also examined the binding of a radiolabeled TRAP for the wild-type and mutant PAR-1 receptors. Our results indicated that most of the mutations strongly affected TRAP-induced responses without significantly altering thrombin-induced responses or TRAP binding. Several point mutations and deletion of extracellular domains (ΔEC3, ΔNH3) drastically altered the ability of mutant receptors to respond to TRAP, but not to thrombin, and did not affect the affinity for the radiolabeled TRAP by these mutant receptors. Only mutations that disrupted the putative disulfide bond or substitution of multiple acidic residues in the second extracellular loop by alanine had a significant effect on both ligand binding and thrombin activation. These results suggest that although both agonists can activate PAR-1, there are profound differences in the ability of thrombin and TRAP to activate PAR-1. In addition, we have found PAR-1 mutants with the ability to dissociate receptor-specific binding from functional activity.

G protein-coupled receptors (GPCRs) are a large family of integral plasma membrane proteins that interact with a variety of structurally diverse agonists to regulate distinct biological processes (Hamm, 1998; Ji et al., 1998). Different ligand recognition sites have been identified for the various GPCR subfamilies. Small ligands such as biogenic amines or neurotransmitters bind to a high-affinity pocket located in the transmembrane region of the receptor, whereas larger ligands such as peptides have high-affinity interactions with residues in the extracellular domains (Gudermann et al., 1997). The protease-activated receptors are members of the GPCR family of receptors that are activated by a novel mechanism. Thrombin binds to and cleaves protease-activated receptor-1 (PAR-1) within the extracellular amino terminus of the receptor, exposing a new amino terminal sequence that functions as a tethered ligand to activate the receptor (Vu et al., 1991). Synthetic peptides (thrombin receptor agonist peptides or TRAPs) of five or more amino acid residues with the identical sequence as the new amino terminus of PAR-1 are also able to fully activate the receptor (Scarborough et al., 1992; Coughlin, 1993). Subsequently three more members of the PAR family have been identified. PAR-3 and PAR-4 (Ishihara et al., 1997; Kahn et al., 1998; Xu et al., 1998) are also activated by thrombin, whereas trypsin and tryptase have been shown to activate PAR-2 (Nystedt et al., 1994, 1995; Molino et al., 1997). Similar to PAR-1, PAR-2 and PAR-4 are also activated by their respective tethered agonist peptide sequence, SLIGKVD (Nystedt et al., 1995) and GYPGKV (Kahn et al., 1998; Xu et al., 1998). PAR-3 does not appear to respond to its activation peptide (Ishihara et al., 1997), raising the possibility that there may be important differences in the activation mechanism within the protease-activated receptor family. There are also significant differences in the concentrations required for thrombin (picomolar) versus thrombin receptor agonist peptide (TRAP) (micromolar) to activate PAR-1 (Vu et al., 1991; Scarborough et al., 1992). This suggests profound differences in affinity, which are most likely due to the tethered mechanism. Previous studies have indicated that extracellular regions of the PAR-1 are involved in the activation of the receptor by thrombin and TRAP (Bahou et al., 1993; Gertzen et al., 1994; Nanevicz et al., 1995, 1996). These studies suggested that regions crucial

ABBREVIATIONS: GPCR, G protein-coupled receptor; PAR-1, protease-activated receptor-1; TRAP, thrombin receptor agonist peptide; BMS, Bristol-Myers Squibb; PI, phosphoinositide; ELISA, enzyme-linked immunosorbent assay; WT, wild-type; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; EC, extracellular; pFPhe, L-p-fluorophenylalanine; Har, L-homoarginine; Cha, L-cyclohexylalanine.

for ligand-receptor interactions could be localized to the amino-terminal domain proximal to the first transmembrane domain, the second extracellular loop, and possibly the third extracellular loop. The above-mentioned studies have been focused on identifying the domains or specific amino acid residues involved in the activation of the receptor by TRAP. In one study where the effects of PAR-1 amino-terminal mutations on both thrombin and TRAP activation were examined, similar attenuation of the responses was observed with the agonist peptide and thrombin (Bahou et al., 1994). We wished to explore whether this phenomenon was specific to the N-terminal region or would be found with mutations to other regions affecting the functional responses of the receptor. Therefore, we have conducted a comparative study of the effects of mutations of PAR-1 on the functional activation of the receptor by TRAP and thrombin. Concurrently, we have also examined the effect of the mutations on the affinity of a radiolabeled agonist peptide for the mutant PAR-1 receptors. To our knowledge, this is the first time that a radiolabeled TRAP has been used to correlate a ligand binding with its potency in stimulating the functional responses of mutant thrombin receptors.

Our results demonstrate that several extracellular mutations selectively ablate functional activation by TRAP but not by thrombin. These mutants however retain a high affinity for TRAP. This indicates that slight changes in the structure of PAR-1 can dramatically reduce the efficacy of TRAP without affecting the efficacy of the tethered ligand. The difference in the impact of the mutations on the activation of PAR-1 by thrombin and TRAP is a direct demonstration that these two agonists cannot be considered interchangeable. The results indicate that although TRAP is an agonist for PAR-1, it does not mimic the activation of the receptor by thrombin in all respects.

Experimental Procedures

Materials. All chemicals were from Sigma Chemical Co. (St. Louis, MO), unless otherwise specified. Tissue culture reagents were from Life Technologies (Grand Island, NY). *Taq* polymerase, restriction enzymes, protease inhibitors, and FuGene 6 were from Roche Molecular Biochemicals (Indianapolis, IN). α-Thrombin (13 mg/ml, 3000 U/mg, 350 μM) was from Hematologic Technologies (Essex, VT). [^{3}H]C721-40 [Ser-(pFPhe)-Har-Leu-Har-Lys-(^{3}H-Tyr)-NH_2, specific activity 46 Ci/mmol, custom synthesis] and myo-[^{3}H]inositol, specific activity 99 Ci/mmol, were from Amersham (Arlington Heights, IL). Dowex AG1x8 was from Bio-Rad (Hercules, CA). Peptides were synthesized as carboxy-amides as described previously (Scarborough et al., 1992). BMS 200261 [*trans*-cinnamoyl-(pFPhe)-(pGuanidino Phe)-Leu-Arg-Arg-NH_2] was synthesized as described in Bernatowicz et al. (1996). Thrombin receptor antibody 61-1 is a murine monoclonal antibody to a peptide containing amino acids 29 to 43 of the thrombin receptor that reacts primarily with residues N-terminal to the thrombin cleavage site and was generated as previously described (Norton et al., 1993)

Plasmid Construction and Site-Directed Mutagenesis. The wild-type (WT) PAR-1 construct was made by isolating the *Xho*I-*Eco*RI fragment from the expression construct described previously (Blackhart et al., 1996) and inserted into the *Xho*I and *Eco*RI sites of the mammalian expression vector pBJ5 or pSP72 (Promega, Madison, WI) for expression in oocytes. The PAR-1 construct was linearized with *Xba*I before use as in vitro transcription template. Mutations were introduced into the cDNA by first generating polymerase chain reaction fragments of the PAR-1 cDNA with the mutation and then replacing the same region of the wild-type receptor with the synthesized fragment. Introduction of the mutation was confirmed by DNA sequence analysis.

***X. laevis* Oocytes.** Isolation, injection, and analysis of expressed wild-type and mutant PAR-1 in *X. laevis* oocytes were performed as described previously (Blackhart et al., 1996). Functional responses to thrombin and TRAP were determined by measuring the amount of the agonist-stimulated [^{45}Ca] calcium efflux.

Phosphoinositide (PI) Hydrolysis in Mammalian Cells. COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 1 mM glutamine. Cells were plated at 10^5 cells in 35-mm six-well dishes and 0.75 μg of DNA was transfected using the FuGene 6 transfection reagent (Roche Molecular Biochemicals) according to the manufacturer instructions. PI determination was done as described in Nanevicz et al. (1996). Thirty-six hours after transfection, the cells were loaded with 2 μCi/ml myo-[^{3}H]inositol in serum and inositol-free Dulbecco's modified Eagle's medium and incubated for 16 h at 37°C. LiCl (10 mM) was added for 15 min at 37°C followed by addition of thrombin or TRAP at the required concentrations for 45 min. Cells were washed in cold PBS and extracted with 1 ml of 20 mM formic acid for 30 min at 4°C. Cell extracts were loaded onto 1-ml packed columns of AG1x8 anion exchange resin (100–200 mesh size; Bio-Rad) after columns have been washed with 2 ml of 2 M ammonium formate, 0.1 M formic acid, 2 ml of H_2O, and 4 ml of 20 mM NH_4OH, pH 9.0. After loading, the columns were washed with 3 ml of 40 mM NH_4OH and two times with 4 ml of 40 mM ammonium formate. The samples were eluted with 2 ml of 2 M ammonium formate and 0.1 M formic acid. The resulting inositol mono-, bis-, and trisphosphate were quantified by scintillation counting.

Platelet Preparation and Aggregation. Human venous blood was collected for healthy, drug-free volunteers into ⅛ volume of ACD containing prostaglandin I_2 (85 mM sodium citrate, 111 mM glucose, 71.4 mM citric acid, 1.6 μM PGI_2). Platelet-rich plasma was prepared by centrifugation at 160*g* for 20 min at room temperature. Platelet-rich plasma was centrifuged for 10 min at 730*g* and the platelet pellet resuspended in CGS (13 mM sodium citrate, 30 mM glucose, 120 mM NaCl). The platelets were collected by centrifugation at 730*g* for 10 min and resuspended at a concentration of 4×10^8 platelets/ml in HEPES-Tyrode's buffer (10 mM HEPES, 138 mM NaCl, 5.5 mM glucose, 2.9 mM KCl, 12 mM $NaHCO_3$, pH 7.4) containing 0.1% BSA, 1 mM $CaCl_2$, and 1 mM $MgCl_2$. This platelet suspension was kept for 30 min at 37°C before use in aggregation assays. Stimulation by different agonist peptides or inhibition of TRAP-dependent aggregation by antagonist compounds was determined in 96-well microtiter plates at room temperature. The total reaction volume of 0.2 ml/well included 6×10^7 platelets in HEPES-Tyrode's buffer and different concentrations of the agonist peptides (for stimulation of aggregation) or serial dilutions of antagonist compounds and 2 μM TRAP, which induces submaximal aggregation. The absorption of the samples was then determined at 490 nm using a microtiter plate reader (Softmax; Molecular Devices, Menlo Park, CA), resulting in the 0-min reading. The plates were then agitated for 5 min on a microtiter plate shaker and the 5-min reading obtained in the plate reader. Aggregation was calculated from the decrease of absorbance at 490 nm at 5 min compared with 0 min and expressed as percentage of the decrease in the TRAP control samples corrected for changes in the unaggregated control samples. Dose-response curves (EC_{50} values) and IC_{50} values were derived by nonlinear regression analysis form at least three independent experiments using the Prism software (GraphPad, San Diego, CA)

Membrane Preparations. Forty-eight hours after transfection, COS-7 cells were detached with PBS and 5 mM EDTA, washed with PBS, and the cell pellets were frozen at −20°C. Human platelet membranes were prepared from apheresis units obtained from blood banks. Platelets were washed with CGS (13 mM sodium citrate, 30 mM glucose, 120 mM NaCl) and pellets were frozen. COS-7 cells and

platelet pellets were thawed, homogenized in 20 mM Tris-HCl, pH 7.5, containing 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and sonicated for 10 s. Nuclear debris and intact cells were removed by centrifugation at 1000*g* for 10 min. The supernatant was centrifuged at 35,000*g* for 30 min and the resulting pellet was resuspended in 25 mM Tris-HCl, pH 7.5, 25 mM $MgCl_2$, 10% sucrose containing 0.1 mM phenylmethylsulfonyl fluoride, 50 μg/ml antipain, 1 μg/ml aprotinin, 40 μg/ml bestatin, 100 μg/ml chymostatin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin. Protein concentrations were determined according to the Bradford method. Membranes were aliquoted and frozen at −70°C.

[^{3}H]C721-40 Binding Assays. We developed a high-affinity TRAP to be used as a radioligand. [^{3}H] C721-40 [Ser-(pFPhe)-Har-Leu-Har-Lys-(^{3}H-Tyr)-NH_2] was prepared by Amersham by catalytic tritiation of an iodotyrosyl-containing precursor to a specific activity of 46 Ci/mmol.

[^{3}H]C721-40 binding to COS-7 and platelet membranes was determined using a rapid filtration assay. Binding assays were performed in 96-well plates (ultralow; Costar, Cambridge, MA). Total binding was determined by incubating the membranes (10 μg from COS-7 cells and 25 μg from platelets) with various concentrations (0.1–200 nM) of [^{3}H]C721-40 and assay buffer (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 0.1% BSA) in a final volume of 0.2 ml. For competition experiments, 50 μl of displacing compound was added. Nonspecific binding was determined in the presence of 20 μM unlabeled C721-40. After a 30-min incubation at 23°C, reactions were stopped by addition of ice-cold 20 mM HEPES, 138 mM NaCl, pH 7.5 and immediate filtration through Whatman GF/C filters (presoaked for 2–3 h in 10 mM HEPES containing 0.5% polyethylenimine and 0.1 M *N*-acetyl glucosamine) using a cell harvester (Skatron, Sterling, VA). The filters were washed four times, and membrane-bound radioactivity was determined in a scintillation counter. Specific binding was determined by subtraction on nonspecific binding from total binding. The radioligand saturation data were analyzed by nonlinear least square fitting using the EBDA and LIGAND programs (McPherson, 1983; Munson and Rodbard, 1989). The radioligand competition binding data were analyzed using the Prism software (GraphPad). Equilibrium inhibition constants were determined according to the equation $K_I = IC_{50}/(1 + [L]/K_D)$ where IC_{50} is the concentration of competing ligand required for 50% inhibition of

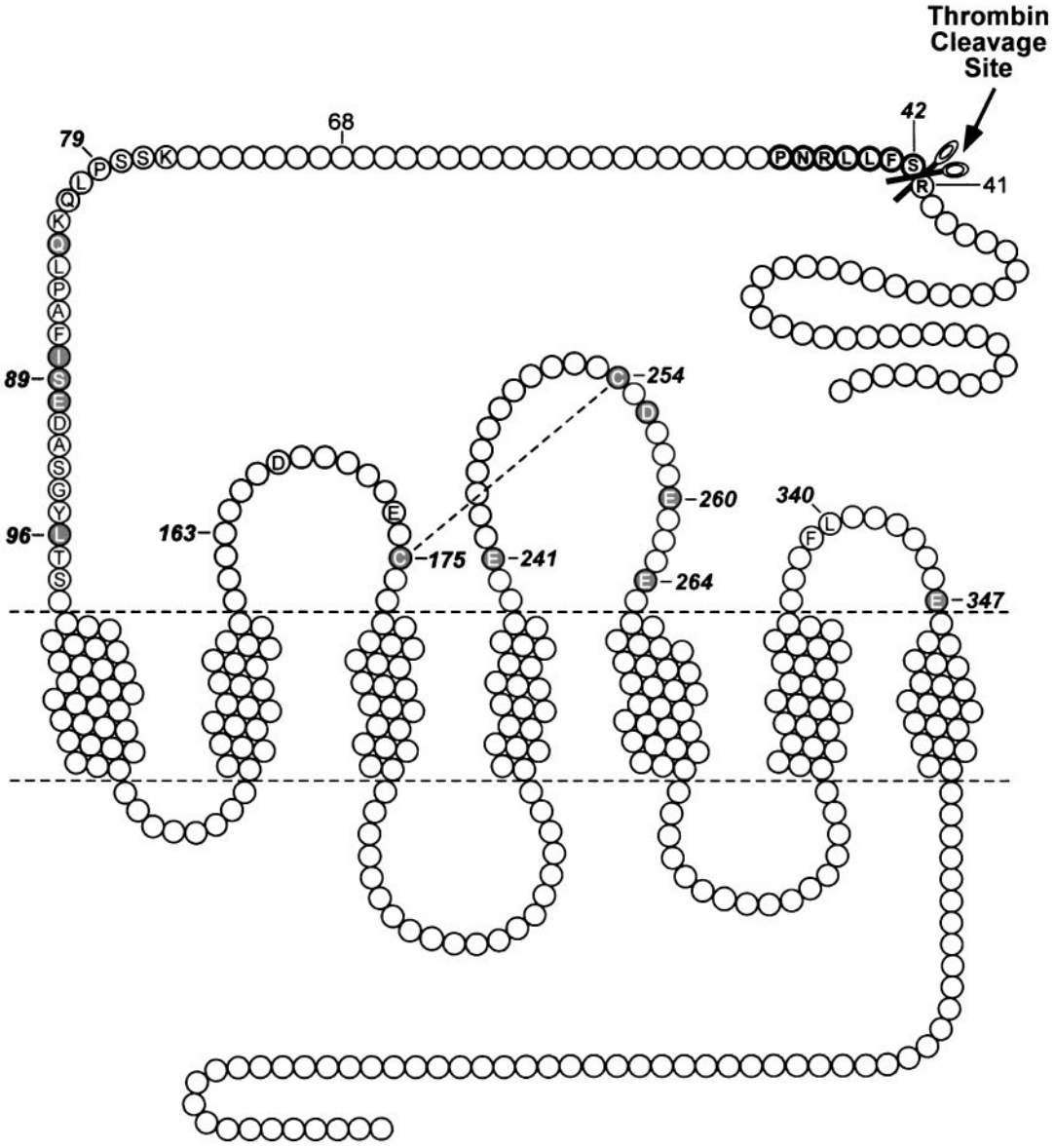

Fig. 1. Hypothetical human PAR-1 topology. Gray circles show the mutation sites in which replacement by alanine significantly altered the ability of TRAP or both thrombin and TRAP to activate the receptor. Bold circles represent the first seven amino acids residues of the tethered ligand (SFLLRNP). The arrow indicated residue R41 where thrombin cleaves PAR-1.

[^{3}H]C721-40 binding, [L] is the concentration of [^{3}H]C721-40 (10 nM), and K_D is the affinity constant of [^{3}H]C721-40 as determined by saturation binding.

Cell Surface Enzyme-Linked Immunosorbent Assay (ELISA). Cells were plated in 12-well dishes at 6×10^4 cells/well and transfected with 0.25 μg of DNA using the FuGene reagent. Forty-eight hours after transfection, cells were incubated with 1 nM thrombin for 10 min at 37°C. After receptor cleavage the cells were fixed in 4% paraformaldehyde for 10 min at 23°C and washed twice in PBS containing 3% BSA (Trejo et al., 1998). The cells were then incubated with a cleavage-sensitive PAR-1 monoclonal antibody, 61-1 (1 μg/ml), for 1 h at room temperature, followed by 30-min incubation at 23°C with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:6000 dilution). Both antibodies were diluted in PBS containing 1% BSA. Cells were washed twice in PBS and 3% BSA and incubated with the chromogenic substrate *O*-phenylenediamine dihydrochloride, 0.4 mg/ml. The reaction was stopped 10 min later by addition of 0.75 N HCl and the absorbance was read at 405 nm using a microplate reader (Molecular Devices).

Results

Analysis of PAR-1 Mutants Expressed in *X. laevis* Oocytes. A series of mutant PAR-1 receptors were constructed and expressed in *X. laevis* oocytes. Figure 1 shows the sites of the mutations in PAR-1. The mutations were localized to regions of the putative extracellular surface of the receptor previously identified as being important for receptor-ligand interaction. These mutations included both deletions as well as individual amino acid substitutions to further localize crucial contact points and are listed in Table 1. The functional responses of the mutant receptors were determined by assaying the level of calcium efflux due to stimulation by either thrombin or TRAP. The results (Table 1) show that a number of the mutations affect responses to agonist stimulation. In almost every case the mutation has a decidedly greater effect on the receptor's response to TRAP compared with that of thrombin. Mutations to specific residues in the amino terminus (I88A, S89A, and L96A), the second extracellular loop (D256A), and the third extracellular loop (E347A) were identified that had a profound effect on the functional responses to the agonist peptide. A number of mutants in the amino terminus had no effect in the responses to either thrombin or TRAP, including F87A, E90Q, D91N, S93A, Y95A, and T97A. A PAR-1 deletion mutant lacking amino acids 68 through 93 of the amino terminus has almost completely lost the ability to respond to TRAP yet still retains the ability to generate a maximal response to thrombin, albeit at a higher concentration than the wild-type receptor. Negatively charged residues were replaced by alanine in two of the single amino acid mutations (D256A and E347A) that affected the functional responses of the receptor. A previous study has indicated a potential role for an electrostatic interaction between R46 of the agonist peptide and E260 of the second extracellular loop (Nanevicz et al., 1995). Therefore, the D256 and E347 residues were replaced with asparagine and glutamine, respectively, to evaluate the importance of a negative charge at those positions. The D256N mutant responses to both agonists were affected much less than those of the D256A mutant. In contrast, the responses of the E347A and E347Q mutants were reduced to a similar extent. These results suggest that the negative charge of E347 may have a role in receptor activation, whereas the negative charge of the aspartic acid residue at position D256 appears to be less important. In summary, these analyses indicated that several residues in the extracellular surface of the receptor are important in mediating functional responses to agonists. In addition, these results suggest that responses to TRAP and the tethered ligand are not comparably affected by the mutations. Those mutants that were shown to have an effect on receptor responses to PAR-1 agonists were examined further in transiently transfected COS-7 cells to correlate their functional responses with their ability to bind a radiolabeled TRAP.

TABLE 1

Analysis of PAR-1 mutants expressed in *X. laevis* oocytes

Wild-type or mutant PAR-1 receptors were expressed in *X. laevis* oocytes and EC_{50} values for ^{45}Ca efflux in response to thrombin and SFLLRNP were evaluated. Responses were determined as a percentage of the control value, which was the maximum response of PAR-1 to either thrombin or SFLLRNP. EC_{50} values shown are representative of those obtained in at least two separate experiments performed in triplicate.

Construct	EC_{50}	
	Thrombin	SFLLRNP-NH_2
	nM	μM
Wild-type	0.05 ± 0.04	0.5 ± 0.3
Deletions		
ΔNH3 (68–93)	1 ± 0.8	>10
ΔEC1 (163–174)	>10	NT
ΔEC2 (240–267)	>10	NT
ΔEC3 (339–344)	0.1 ± 0.06	>50
Point mutants		
F87A	0.05 ± 0.04	0.5 ± 0.3
I88A	0.05 ± 0.03	30 ± 15
S89A	0.05 ± 0.04	30 ± 18
D91N	0.05 ± 0.03	0.5 ± 0.2
S93A	0.05 ± 0.02	0.5 ± 0.3
Y95A	0.05 ± 0.04	0.5 ± 0.3
L96A	0.05 ± 0.04	20 ± 10
EC1DE (D167A/E173A)	0.05 ± 0.03	0.5 ± 0.3
EC2DE (E241A, D256A, E260A, E264A)	>10	>40
E241A	0.05 ± 0.04	0.5 ± 0.3
D256A	0.1 ± 0.07	>40
D256N	0.05 ± 0.04	5 ± 3
E260A	0.05 ± 0.03	0.5 ± 0.4
E264A	0.05 ± 0.04	0.5 ± 0.3
E347A	0.1 ± 0.08	>50
E347Q	0.5 ± 0.3	>40
C175S	10 ± 2	>40
C175S, C254S	10 ± 1	>40

NT, not tested: the mutants that were not responsive to thrombin were not tested for TRAP responses.

Functional Analysis of PAR-1 Mutants Expressed in COS Cells: PI Hydrolysis. COS-7 cells have been reported to have very few endogenous PAR-1 receptors (Ishihara et al., 1997). Therefore these cells were transiently transfected with the mutant constructs. Functional responses of the PAR-1 mutants were evaluated by determining agonist-stimulated phosphoinositide hydrolysis. The untransfected cells responded to either thrombin (Fig. 2A) or TRAP (Fig. 2B) only at relatively high concentration of the agonists, confirming that native expression of PAR-1 in COS cells is very low. Cells transfected with the wild-type receptor responded to thrombin and TRAP with EC_{50} values of 30 ± 20 pM (Fig. 2A) and 120 ± 50 nM (Fig. 2B), respectively. The responses to TRAP and thrombin obtained with the mutants are summarized in Table 2. In general, these results were very similar to the results obtained from the calcium efflux experiments in the *X. laevis* oocytes. Again the responses to TRAP were diminished much more than the responses to thrombin. The EC_{50} values of the thrombin response curves ranged from

wild-type responses to greater than 30 nM. The extracellular amino terminal region point mutants' responses to thrombin were unaffected, whereas most of the receptors with mutations in the second or third extracellular loops did show moderate loss of responsiveness to thrombin. In contrast, the shift in the EC_{50} value for responses to TRAP was at least 100-fold higher than for the wild-type receptor in all of the mutants except for D256N and E260A. The D256N mutant had only a modest effect on responses to both thrombin and TRAP. C721-40 also elicited a PI hydrolysis response in COS-7 cells expressing PAR-1 with an EC_{50} value of 100 nM.

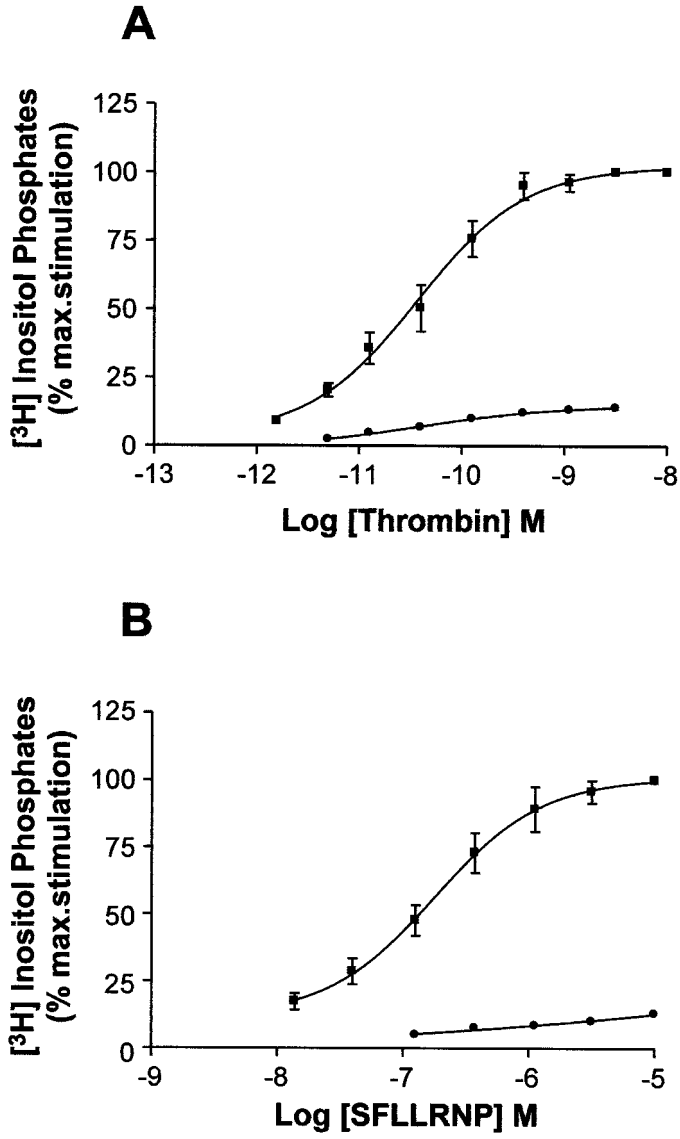

Fig. 2. Phosphoinositide production in COS-7 cells untransfected or transiently expressing wild-type human PAR-1. Untransfected or PAR-1 transfected COS-7 cells were incubated for 45 min at 37°C (see *Experimental Procedures* for details). A, dose response to thrombin. B, dose response to SFLLRNP. The circles (●) represent the untransfected COS-7 cells and the squares (■) represent the cells transfected with the wild-type PAR-1. Data are presented as a percentage of accumulation of tritiated inositol phosphates over basal levels and represented the mean of at least three individual experiments performed in triplicates. Maximal responses for both thrombin and SFLLRNP ranged from 15- to 20-fold increase in [^{3}H]inositol phosphate accumulation over basal levels. EC_{50} values were determined using the GraphPad Prism software. EC_{50} values for thrombin = 30 ± 20 pM and for SFLLRNP = 120 ± 50 nM.

A similar shift in functional responses ($EC_{50} > 30$ μM) was also observed when the C721-40 peptide was used as an agonist in the ΔEC3, L96A, and D256A mutants (data not shown). The deletion of amino acids 68 to 93 resulted in a complete loss of response to TRAP, whereas the EC_{50} value for thrombin was only 10-fold higher than the wild-type. This result is in contrast to an earlier report indicating that deletion of a stretch of amino acids from the same region (83–89) resulted in loss of response to both agonists (Bahou et al., 1994). To investigate this conflict, we constructed the identical deletion mutant reported by Bahou et al. (1994) and evaluated its response to thrombin and TRAP. The results of the functional analyses of this mutant (83–89) indicate that this deletion does cause a much greater loss of responsiveness to thrombin than the 68 to 93 deletion (Table 2).

ELISA Assay of PAR-1 Expression. To evaluate the expression levels of the different mutants, an ELISA assay was performed. PAR-1 mutants were detected immunologically at the plasma membrane surface by an antibody to the extracellular amino terminus. The thrombin receptor antibody 61-1 is cleavage-sensitive (Norton et al., 1993), so the cells were preincubated with thrombin before the antibody addition. This assay is specific for receptor proteins in which the amino-terminal sequence is accessible to the extracellular medium. Almost all of the mutants evaluated by the ELISA assay exhibited levels of expression similar to those observed with the wild-type receptor (Table 3). The only exception was the C175S mutant that expressed only 53% the level of receptors compared with the wild-type PAR-1 expression level. These results indicated that most of the mutants were expressed and transported to the cell surface at levels such that the effects of the mutations on functional responses were not simply due to a reduction in expression.

Binding of a Radiolabeled TRAP to PAR-1. To determine the effects of the extracellular mutations on ligand

TABLE 2

Phosphoinositide production in COS-7 cells transiently transfected with wild-type or mutant PAR-1

Transfected COS cells were incubated for 45 min at 37°C (see *Experimental Procedures* for details). The accumulation of tritiated inositol phosphates was determined in response to thrombin and TRAP. EC_{50} values were determined using the GraphPad Prism software. Data are presented as means ± S.D. of at least three independent experiments, each performed in triplicate.

Construct	EC_{50}	
	Thrombin	SFLLRNP-NH_2
	nM	*μM*
Wild-type	0.03 ± 0.02	0.12 ± 0.05
Deletions		
ΔNH3 (68–93)	0.30 ± 0.16	>180
ΔNH3 (83–89)	>10	>100
ΔEC1 (163–174)	>10	>100
ΔEC3 (339–344)	0.06 ± 0.03	>60
Point mutants		
I88A	0.04 ± 0.02	>100
S89A	0.01 ± 0.005	30 ± 20
L96A	0.03 ± 0.02	40 ± 10
EC2DE (E241A, D256A, E260A, E264A)	>30	>100
D256A	0.30 ± 0.07	>60
D256N	0.24 ± 0.09	7.0 ± 3.0
E260A	0.15 ± 0.04	0.48 ± 0.13
E347A	0.30 ± 0.11	>60
E347Q	0.26	>100
C175S	>10	>100
C175S, C254S	>30	>100

binding, we established a high-affinity ligand-binding assay using a radiolabeled [^{3}H]TRAP. We have previously validated this novel binding assay (D. Oksenberg, unpublished data) in membranes from human platelets. Our results in platelet membranes for the affinity constant (K_d = 25 nM) and the number of sites (B_{max} = 4 pmol/mg of protein) are similar to those previously reported (Ahn et al., 1997). The binding properties and biological activities of the tethered ligand-derived peptides listed in Table 4 were determined to examine their relationship. Table 4 shows a comparison between the binding K_i values and the EC_{50} values in platelet aggregation for different agonist peptides. An alanine, glycine (Table 4), and proline (data not shown) scanning of tethered ligand peptide sequence indicates the importance of the different residues in the tethered ligand (Phe^2, Leu^4, Arg^5) that are responsible for both binding and biological activity. Figure 3 shows a plot of binding K_i values in platelet membranes versus biological activity (EC_{50} or IC_{50} for platelet aggregation) with a correlation coefficient of 0.94. The results demonstrate that there is a good correlation between the peptides binding and biological activity (Fig. 3), as was found with heterocyclic (Hoekstra et al., 1998) and aminoindole (Andrade-Gordon et al., 1999) peptide-mimetic antagonists based on the SFLLR motif. In contrast, biologically inactive peptides such as SALLR or SGLLR did not compete for ligand binding (Table 4).

Competition experiments using different agonist peptides and a PAR-1 receptor antagonist BMS 200261 (Bernatowicz et al., 1996) were also performed in both platelets and COS-7-expressing PAR-1. Table 5 shows representative data indicating that the results obtained in membranes from the COS-PAR-1 transfected cells are very similar to those obtained in platelets, thus validating the use of the transfected COS cells in this study. Both the native (SFLLRNP) and modified [S(pFPhe)SHarLHarK, SFChaChaRK] TRAPs displace the [^{3}H]SpFPheHarLHarKY ([^{3}H]C721-40) binding with similar K_i values in platelets and COS-PAR-1 membranes. BMS 200261, a previously described PAR-1 antagonist, also inhibits [^{3}H]C721-40 with similar affinity in both cell types. A scrambled peptide [(pFPhe)SHarLHarK], a PAR-2 (SLIGKVD)-, or a PAR-4 (GYPGKV)-specific agonist were unable to displace [^{3}H]C721-40 binding. Saturation binding isotherms were performed with membranes from COS-7 cells transfected with PAR-1 (Fig. 4). Membranes from untransfected cells had a nearly undetectable level of specific binding (Fig. 4). The percentage of specific binding for the PAR-1

TABLE 3

ELISA detection of wild-type and PAR-1 mutants on the surface of COS-7 cells

COS-7 cells transiently expressing the wild-type or mutant receptors were incubated with thrombin for 10 min at 37°C. Expression levels were detected in fixed cells with a PAR-1 antibody as described. The expression levels are presented as a percentage of wild-type PAR-1 (100%). Data presented are mean of three independent experiments.

Construct	Expression
	%
Wild-type	100
Deletions	
ΔNH3 (68–93)	78 ± 5
ΔEC3 (339–344)	91 ± 17
Point mutants	
I88A	90 ± 14
S89A	93 ± 16
L96A	100 ± 6
EC2DE (E241A, D256A, E260A, E264A)	83 ± 10
D256A	101 ± 7
D256N	NT
E260A	NT
E347A	92 ± 12
E347Q	102 ± 10
C175S	57 ± 6
C175S, C254S	70 ± 9

NT, not tested.

TABLE 4

Effect of different SFLLR-derived peptides on platelet aggregation and binding

Aggregation of washed platelets induced by various SFLLR-derived peptides was measured. Different concentrations (10^{-9}–10^{-4} M) of the peptides were added and the EC_{50} values were determined. Competition binding studies for the different peptides were performed using [^{3}H]721-40 as the radioligand and membranes prepared from human platelets. The IC_{50} values were determined by adding different concentrations of the peptides (10^{-9}–10^{-4} M). K_i values were determined using the equation $K_i = IC_{50}/(1 + [L]/K_D)$ where IC_{50} is the concentration of competing ligand required for 50% inhibition of [^{3}H]C721-40 binding, [L] is the concentration of [^{3}H]C721-40 (10 nM) and K_D is the high-affinity constant of [^{3}H]C721-40 as determined by saturation binding (K_D for platelets = 25 ± 10 nM). Both EC_{50} and IC_{50} values were analyzed by nonlinear regression fit using the GraphPad Prism software.

	Binding IC_{50}	Aggregation (WP) EC_{50}
	μM	
SFLLRNP-NH_2 (C152-48)	1.35 ± 0.15	1 ± 0.3
SFChaChaRK-NH_2	0.17 ± 0.04	0.3 ± 0.2
S(pFPhe)HarLRK-NH_2	0.13 ± 0.02	0.4 ± 0.25
S(pFPhe)HarLHarK-NH_2 (C721-40)	0.08 ± 0.01	0.3 ± 0.2
(pFPhe)SHarLHarK-NH_2	>100	>100
SFLLR-NH_2	1.5 ± 0.5	0.8 ± 0.1
AFLLR-NH	1.1 ± 0.15	0.8 ± 0.2
S**A**LLR-NH_2	>100	>100
SF**A**LR-NH_2	1.9 ± 0.7	3.2 ± 0.3
SFL**A**R-NH_2	10 ± 2	4.8 ± 0.8
SFLL**A**-NH_2	>100	>100
GFLLR-NH_2	1.3 ± 0.5	1.8 ± 0.7
S**G**LLR-NH_2	>100	>100
SF**G**LR-NH_2	4.7 ± 1.2	12 ± 3
SFL**G**R-NH_2	86 ± 12	>100
SFLL**G**-NH_2	>100	>100

WP, washed platelets.

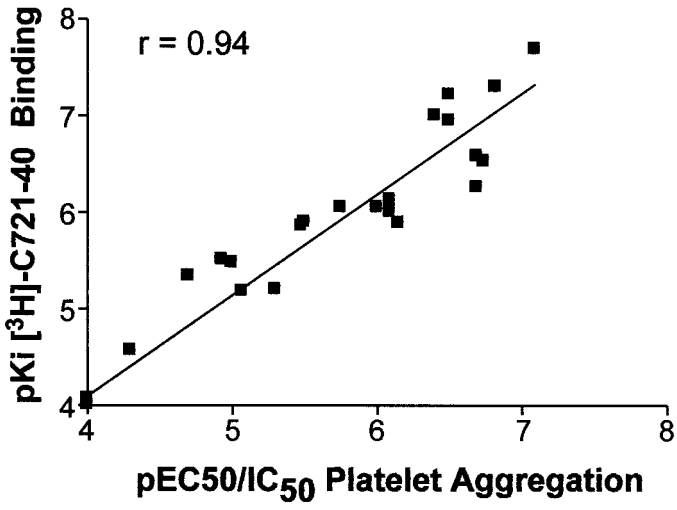

Fig. 3. Correlation between K_i values obtained in binding assays versus EC_{50}/IC_{50} values in platelet aggregation for different SFLLR-derived peptides. The EC_{50} (platelet aggregation) and K_i ([^{3}H]C721-40 binding) values plotted correspond to those described in Table 4. In addition IC_{50} values in platelet aggregation and K_i values in ligand binding (data not shown) for selected PAR-1 antagonists (Andrade-Gordon et al., 1999) were also plotted. Competition and aggregation data were obtained as described in Table 4.

transfected cells was approximately 85% of total binding. The K_D of [^{3}H]C721-40 for the wild-type receptor was 14 ± 7 nM. Most of the mutant receptors bound the ligand with an affinity within 2- to 3-fold of that of the wild-type receptor (Table 6). The only exceptions were those mutants that had exhibited very poor responses to both thrombin and TRAP. These mutants (ΔEC1, EC2DE, and C175S) exhibited a very low specific binding, sometimes indistinguishable from untransfected cells. We also determined the K_i value for the native TRAP SFLLRNP in both WT and mutant receptors (Table 6). Similar to the data obtained with the C721-40 there were no major differences in K_i values between the WT and mutant receptors. The number of binding sites on the cells expressing the wild-type receptor was 74 ± 10 pmol/mg of protein (Fig. 4). The B_{max} value for the mutant receptors are reduced 3- to 4-fold compared with the wild-type receptor expression (Table 6). This is in contrast to the results of the ELISA assay that indicated similar levels of expression for wild-type and mutant receptors. This reduction in sites could translate into a reduced responsiveness to agonists and thereby explain the decreased potency of the agonist peptide in the functional assays. To test this possibility, the wild-type expression construct was cotransfected with the empty vector at a ratio of 1:10 to reduce the level of expression of PAR-1 without altering the transfection conditions. The level of receptor expression and the functional responses of the transfected cells were then evaluated. The affinity constant (K_d = 9 nM) was similar to the one reported for the WT receptor (14 nM) and the B_{max} value was 20 pmol/mg membrane protein (Fig. 5B) versus 74 pmol/mg of protein for the WT receptor. This level of expression (20 pmol/mg of protein) was comparable with that of the majority of the receptor mutants. Functional analysis of the cells transfected with 10 times less DNA showed (Fig. 5A) that the EC_{50} values for both thrombin (66 pM) and TRAP (330 nM) were only modestly reduced compared with those for thrombin (30 pM) and TRAP (120 nM) in the cells transfected with normal amounts of DNA. These results show that the striking differences in the functional responses of the mutants to thrombin and TRAP are not a consequence of the lower level of ligand binding sites seen with the PAR-1 mutants.

Another possibility for the reduced number of binding sites in cells expressing the mutant receptors is a loss of high-affinity sites as a consequence of uncoupling G proteins from the receptors. We therefore evaluated C721-40 binding to

TABLE 5

[^{3}H]C721-40 to platelet and COS-PAR-1 membranes

Competition binding studies were used to determine the K_i values of different agonist peptides using [^{3}H]C721-40 as the radioligand and membranes prepared from human platelets or from COS-7 cells expressing PAR-1. IC_{50} values were determined using the GraphPad Prism software. K_i values were determined using the equation $K_i = IC_{50}/(1 + [L]/K_D)$ where IC_{50} is the concentration of competing ligand required for 50% inhibition of [^{3}H]C721-40 binding, [L] is the concentration of [^{3}H]C721-40 (10 nM), and K_D is the high-affinity constant of [^{3}H]C721-40 as determined by saturation binding (K_D for platelets = 25 ± 10 nM and for COS PAR-1 = 14 ± 7 nM). Data represent the mean of at least three independent experiments performed in triplicate.

	K_i	
	Platelets	COS PAR-1
	μM	
S(pFPhe)HarLHarK-NH_2	0.04 ± 0.01	0.04 ± 0.01
SFChaChaRK-NH_2	0.08 ± 0.04	0.035 ± 0.02
SFLLRNP-NH_2	0.7 ± 0.15	0.6 ± 0.13
P(Fphe)SHarLHarK-NH_2	>100	>100
SLIGKVD-NH_2	>100	>100
GYPGKV-NH_2	>100	>100
BMS 200261	0.035 ± 0.01	0.02 ± 0.01

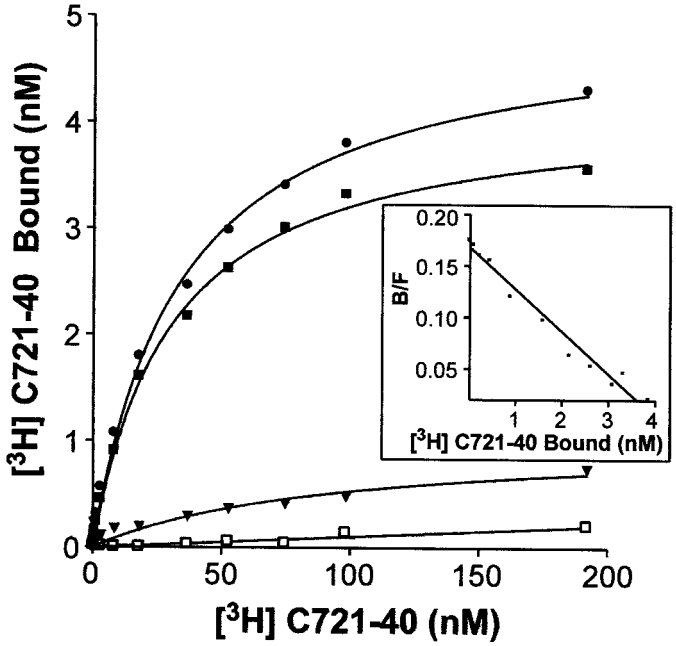

Fig. 4. Saturation binding analysis of [^{3}H]C721-40 binding to PAR-1. Membranes (10 μg) from COS-7 cells transfected with human wild-type PAR-1 were incubated with various concentrations of [^{3}H]C721-40 (0.1–200 nM) and of unlabeled C721-40 (0.1–200 μM). Each point represents total binding (●), specific binding (■), or nonspecific binding (▼) and is the average of triplicate determinations from one representative experiment. Specific binding to membranes from vector-transfected COS-7 cells (□) is also shown. Inset, Scatchard analysis of the data. The data were analyzed using LIGAND. The K_D = 21 nM and the B_{max} = 81 pmol/mg of protein. Similar results were obtained in four additional experiments.

TABLE 6

Ligand binding profile of wild-type and mutant PAR-1 receptors

Saturation binding experiments were performed using [^{3}H]C721-40 and membranes prepared from COS cells transfected with wild-type or mutant PAR-1 receptors. Equilibrium constant (K_D) and receptor densities (B_{max}) were determined using the LIGAND program. Data represent the mean of at least three independent experiments performed in triplicate. Equilibrium constants (K_i) for SFLLRNP were determined in WT and PAR-1 mutants using [^{3}H]C721-40 as radioligand.

Constructs	[^{3}H]C721-40		SFLLRNP
	K_D	B_{max}	K_i
	nM	*pmol/mg protein*	*μM*
Wild-type	14 ± 7	74 ± 10	0.6 ± 0.13
Deletions			
ΔNH3 (68–93)	10 ± 3	13 ± 3	1.7
ΔNH3 (83–89)	13 ± 2	10 ± 1	3.2
ΔEC1 (163–174)	ND	ND	ND
ΔEC3 (339–344)	17 ± 11	14 ± 1	0.8
Point mutants			
I88A	31 ± 11	22 ± 7	1.5
S89A	24 ± 8	21 ± 10	1.5
L96A	41 ± 19	18 ± 4	0.8
EC2DE (E241A, D256A, E260A, E264A)	ND	ND	ND
D256A	ND	ND	ND
D256N	45 ± 9	14 ± 7	1.1
E260A	40 ± 27	33 ± 16	0.55
E347A	26 ± 10	16 ± 5	1.35
E347Q	30 ± 12	16 ± 6	2
C175S	ND	ND	ND
C175S, C254S	ND	ND	ND

ND, not detected. No K_D, B_{max}, or K_i values could be calculated for these mutants because they exhibited very low specific binding (less than 30% of total binding).

membranes from COS cells expressing wild-type PAR-1 in absence or presence of guanosine-5′-*O*-(3-thio)triphosphate (GTPγS; 100 μM). The results showed a K_d value of 25 ± 8 nM and 42 ± 9 nM and a B_{max} value of 79 ± 12 and 83 ± 8 pmol/mg of protein in absence or presence of GTPγS. This indicates that coupling to G proteins is not necessary for high-affinity binding of C721-40 to PAR-1.

Evaluation of C721-40 as an Antagonist. The C721-40 peptide does not elicit a functional response from most of the mutant receptors yet this peptide has a similar affinity for both WT and mutant receptors. This raises the possibility that C721-40 could behave as an antagonist for these mutants. To evaluate this possibility, two mutant receptors (ΔEC3 and L96A) were preincubated for 5 min with two different saturating concentrations of C721-40 (3 and 10 μM). Thrombin was then added at different concentrations. Both control and C721-40-treated cells display similar responses to thrombin (data not shown), indicating that C721-40 was unable to inhibit responses of these mutants to thrombin.

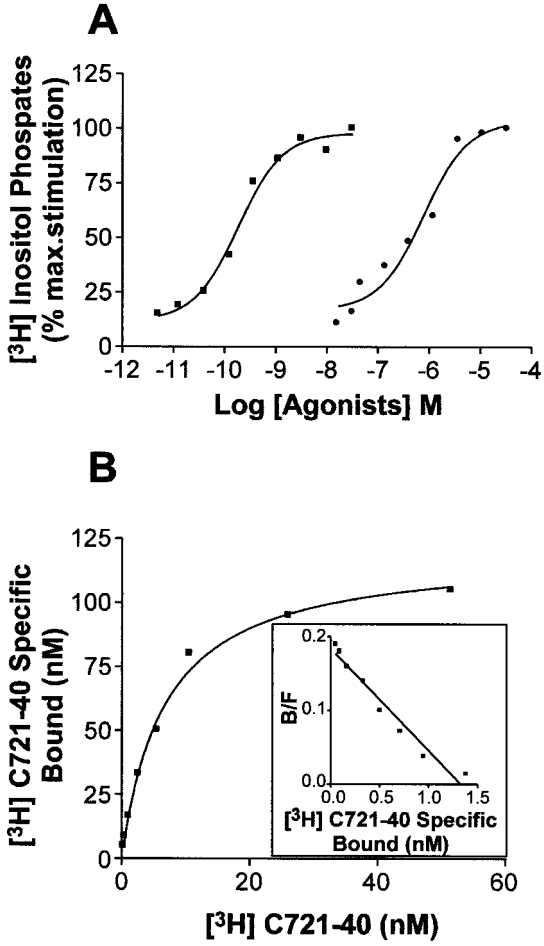

Fig. 5. Analysis of COS cells transiently transfected with PAR-1 and vector cDNA in 1:10 ratio. A, cells were incubated at 37°C for 45 min with either thrombin or TRAP. The accumulation of tritiated inositol phosphates was determined in response to thrombin (■) and TRAP (●). Data are presented as a percentage of accumulation of tritiated inositol phosphates over basal levels. Each point is the average of triplicate determinations from one representative experiment. Maximal responses for both thrombin and SFLLRNP ranged from 10- to 15-fold increase in [^{3}H]inositol phosphate accumulation over basal levels. EC_{50} values were determined using the GraphPad Prism software. EC_{50} for thrombin = 66 pM and for SFLLRNP = 330 nM. Similar results were obtained in an additional experiment. B, saturation binding analysis. Membranes (10 μg) from COS-7 cells transfected with the wild-type PAR-1 and vector cDNA (1:10 ratio) were incubated with various concentrations of [^{3}H]C721-40 (0.1–200 nM) and of unlabeled C721-40 (0.1–100 μM). Inset, Scatchard analysis of the data. The data were analyzed using LIGAND. Each point represents specific binding and is the average of triplicate determinations from one representative experiment. The K_D = 9 nM and B_{max} = 20 pmol/mg of protein. Similar results were obtained in two additional experiments.

Discussion

The objective of this study was to determine the impact of mutations to the thrombin receptor on its ability to interact with two agonists; the tethered ligand exposed by cleavage of the receptor by thrombin and the synthetic peptide mimetic of the tethered ligand. Two main conclusions can be drawn from the results of this study: 1) There are significant functional differences in the interaction of the agonist peptide and the tethered ligand with the receptor. 2) The mutations significantly reduce functional responses to the peptide with little effect on its ability to bind to the receptor. To our knowledge, this is the first time that it has been demonstrated that mutations on the extracellular surface of a G protein-coupled receptor can uncouple receptor binding from functional activity.

The differential impact of the mutations on functional responses to the two agonists could be explained by differences in the mechanism of interaction or potency. The tethered ligand may interact with different or additional sites on the receptor and it has also been suggested that thrombin may have functions in activating the receptor in addition to exposure of the tethered ligand (Molino et al., 1995). Alternatively, being tethered to the receptor could result in a substantial difference in potency verses the free peptide. This is in agreement with previous reports, indicating that thrombin and TRAP can elicit distinct responses and that the soluble peptide is a weak agonist in comparison to the tethered version of the peptide (Vouret-Craviari et al., 1992; Lau et al., 1994; Lasne et al., 1995). We did find that high levels (>60 μM) of the free peptide could still induce a significant response (3–5-fold) with most mutants, suggesting that the difference between the two agonists may be due to the high local concentration of the tethered ligand rather than a mechanistic difference in receptor activation.

The level of receptor expression was determined by an ELISA and a radioligand binding assay. The ELISA assay indicated that most of the mutants expressed at levels similar to that of the wild-type receptor. In contrast, the number of receptors detected with the binding assay was typically only 30% of the numbers of wild-type receptors. The conflicting results of the two assays may be explained by the different requirements of each assay. The antibody used in the ELISA will detect any receptor whose amino terminus is exposed on the extracellular surface of the cell, whereas the binding of the radioligand requires that the receptor is expressed and properly folded on the surface of the cell. In addition to the proper folding of the receptor, a number of the GPCRs have been shown to also require the coupling of G proteins to the receptor to provide a high-affinity site for their ligand. The small shift obtained in binding experiments in the presence of GTPγS suggests that the ability of PAR-1 to associate with G proteins is not critical for high-affinity ligand binding. To examine the possibility that this reduced

expression could account for the loss of functional responses; we expressed the WT receptor at the reduced levels observed with the mutants. Functional responses to TRAP were unaffected, confirming that the reduced levels of expression of the mutant receptors do not account for their loss of functional responses.

The results of the radioligand binding assays showed that most of the mutations had only a negligible effect on the ligand affinity for the mutant receptors. Even deletion of a considerable fraction of the extracellular amino terminal domain containing residues believed to be important for ligand interaction with the receptor resulted in no loss of binding affinity for the radioligand. Only a few of the analyzed mutations had a striking effect on ligand binding. These included the mutants affecting the theoretical disulfide bond linking the first and second extracellular loop (C175S, C175S/C254S), and EC2DE/A in which all four of the acidic residues in the EC2 were substituted by alanine. Disulfide bonds linking extracellular domains of GPCRs have been shown to be important for maintaining the conformation of the receptor and to allow ligand access to the binding pocket (Dohlman et al., 1990; Perlman et al., 1995). Thus, it was not surprising that a loss of binding was observed with these mutants. The results of the ELISA assay of these mutants also indicate the importance of these residues for receptor function. Previously, the E260 residue has been implicated in ligand-receptor interactions (Nanevicz et al., 1995). Only a modest effect on binding and functional responses was observed with the E260A mutant in this study. The importance of the negative charge of D256 was examined by comparing the effects of the D256A mutant to those of a D256N mutant. There is a dramatic shift in TRAP biological responses and C721-40 binding in the D256A mutant compared with the WT. These data confirm previously published information about the potential importance of this site for TRAP functional responses. Our data accentuate the importance of this site for binding interactions. In contrast, the D256N mutant shows only a moderate shift in TRAP EC_{50} and C721-40 binding. Together, these results suggest that although the negative charge of D256 is not important in receptor-ligand interactions, specific interactions with this residue are necessary for binding and functional responses. Recent data published by Al-Ani et al. (1999) have also shown a similar importance for the EC2 loop of PAR-2 in determining agonist function. When key residues (PEE) in the PAR2-EC2 were mutated to PRR, the potency of the agonist peptide SLIGRL was decreased much more (100-fold) than the potency of trypsin (only 7-fold). This would suggest a general phenomenon of PAR receptors where mutations in critical regions important for biological responses affect differently the activity of the free peptide versus the tethered ligand exposed by cleavage.

The results showing that most of the mutations had little or no effect on binding was unexpected because our observations and those reported in previous studies of the functional responses of the thrombin receptor had indicated that some of the mutated amino acid residues were responsible for receptor activation (Bahou et al., 1993; Nanevicz et al., 1995, 1996). Our results indicate that the mutations dissociate binding of the soluble peptide from its ability to activate the receptor. Previous mutagenesis studies with other G protein-coupled receptors have shown uncoupling of ligand binding and receptor activation. However, this is usually the result of disruption of the receptor-G protein coupling by mutations or deletions in the second or third intracellular loop (Cotecchia et al., 1992; Blin et al., 1995). Retention of functional responses to thrombin indicates that receptor-G protein coupling is not disrupted by the mutations. Preservation of the affinity of the ligand for the receptor with the loss of its ability to initiate signal transduction suggested the possibility that TRAP could act as an antagonist of thrombin activation of the mutant receptors. We tested this possibility in two mutants, ΔEC3 and L96A. The results obtained did not support this hypothesis. Preincubation of the cells with the high concentrations of TRAP used clearly had no ability to antagonize responses to thrombin in these experiments. This appears to be the first demonstration of mutations of the extracellular surface of a G protein-coupled receptor that results in a loss of functional responsiveness without compromising ligand binding.

In summary, this study has shown that there are profound differences in the activation of the thrombin receptor by the tethered ligand versus the free peptide. Mutations capable of virtually eliminating response to the synthetic peptide have little effect on the activation of the receptor by thrombin. Whether the source of these differences is due to the intramolecular mechanism of the tethered ligand activation or to mechanistic differences of receptor activation by the two agonists remains to be determined. Our findings also indicate that these mutations are able to dissociate receptor-specific agonist binding from functional activity. To our knowledge this has not been previously reported for other GPCRs, suggesting that it may be unique to receptors activated by a tethered ligand.

The potential value of thrombin receptor antagonists as therapeutic agents to specifically inhibit the cellular actions of thrombin has been recognized (Scarborough et al., 1994; Brass, 1997). The few putative PAR-1 antagonists reported so far (Seiler et al., 1995; Hoekstra et al., 1998) have varied in their ability to inhibit thrombin activation although they could effectively block stimulation by TRAP. An originally described PAR-1 antagonist BMS 200261 (Bernatowicz et al., 1996), which is able to inhibit both thrombin and TRAP in human umbilical vein endothelial cells (O'Brien et al., 2000), has also been reported as a partial agonist for both PAR-1 and PAR-2 (Kawabata et al., 1999). Only recently, the RW&J 56110 (Andrade-Gordon et al., 1999) compound has been described as a pure PAR-1 antagonist with the ability to inhibit both thrombin and TRAP functional responses in different cells. Our results suggest that the intrinsic efficacy of the tethered ligand vary from that of the soluble peptide. Therefore, the interactions between the tethered and soluble peptide with the receptor are very different, as shown in our study with PAR-1 mutants. Understanding the intrinsic differences in activation of the PAR-1 by thrombin and TRAP will facilitate the development of these and as yet undiscovered thrombin receptor antagonists.

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