Biochemical Pharmacology, Vol. 53, pp. 487–491, 1997.

ISSN 0006-2952/97/$17.00 + 0.00
PII S0006-2952(96)00735-6

Nonproteolytic Activation of the Thrombin Receptor Promotes Human Umbilical Vein Endothelial Cell Growth but not Intracellular Ca^{2+}, Prostacyclin, or Permeability

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ABSTRACT. Both thrombin and the synthetic tetracapeptide thrombin receptor-activating peptide (TRAP), recently described as a peptide mimicking the new amino terminus created by cleavage of the thrombin receptor, stimulated the proliferation of human umbilical vein endothelial cells (HUVEC) in culture. Although to a lesser extent, F-14, a tetradecapeptide representing the residues 365–378 of human prothrombin, also promoted HUVEC growth, thereby demonstrating that thrombin can stimulate HUVEC growth via both a proteolytic and a nonenzymatic pathway. Thrombin-TRAP-, and F-14-induced HUVEC growth were inhibited by a thrombin receptor oligodeoxynucleotide antisense, showing that the growth-inducing effects of all 3 compounds were mediated through the same thrombin receptor. Thrombin and TRAP also stimulated intracellular Ca^{2+} increase, monolayer permeability increase, and prostacyclin release in HUVEC. None of these effects was observed with F-14, suggesting that thrombin-induced intracellular Ca^{2+} release, permeability increase, and prostacyclin release in HUVEC required catalytic cleavage of the receptor, whereas thrombin-induced growth might also be due to activation of the thrombin receptor through a nonproteolytic pathway. BIOCHEM PHARMACOL 53;4:487–491, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. thrombin receptor; endothelial cells; mitogenesis; intracellular free calcium; prostacyclin; permeability

Molecular cloning of the thrombin receptor from human platelets and endothelial cells and subsequent experiments with mutant receptors have revealed a unique mode of activation of this G-protein-coupled receptor [1, 2]. It is now clear that proteolytic cleavage of the extracellular N-terminal domain of the thrombin receptor generates a new amino-terminal domain that acts as a tethered ligand to activate the receptor. Indeed, peptides corresponding to this new N-terminal domain (TRAPs)§ have been shown to activate thrombin receptors [1, 3, 4]. However, under some experimental conditions, nonproteolytic activation of thrombin receptors has also been observed [3]. Thus, the synthetic tetradecapeptide F-14, derived from a region of the thrombin B-chain in close proximity to, but not in, the active site of this serine protease, has been shown to induce mitogenic effects on J774 macrophage-like cells [5] and HUVEC [3], suggesting that α-thrombin can activate cells without proteolytic cleavage of its receptor. The molecular interactions between the thrombin receptor and both the tethered (TRAP) and the synthetic peptide F-14 have not been defined, however. Previous studies from our laboratory have suggested that F-14 acts on endothelial cell thrombin receptors at a binding site different from the site through which TRAP activates HUVEC growth [3], but it is conceivable that both peptides interact with different binding sites on the same receptor. Experiments were, therefore, designed to determine if (1) the thrombin receptor involved in the growth-promoting effect of F-14 on HUVEC is the same as that activated by TRAP and (2) nonproteolytic activation of this receptor by F-14 is able to induce other thrombin-related effects on HUVEC. To assess if the mitogenic effects of TRAP and F-14 were mediated through the same receptor (i.e. the thrombin receptor described by Vu et al. [1]), we used an antisense oligodeoxynucleotide (ODN) that has been previously demonstrated to induce a selective downregulation of this receptor in human aortic smooth muscle cells and in the rabbit carotid artery [6]. Moreover, we determined the effect of F-14, in comparison with that of thrombin and TRAP, with regard to intracellular free Ca^{2+} levels, cell permeability changes, and prostacyclin (PGI_2) release by HUVEC.

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§ *Abbreviations:* $[Ca^{2+}]_i$, intracellular free calcium; ECGF, endothelial cell growth factor; HUVEC, human umbilical vein endothelial cells; ODN, oligodeoxynucleotides; PSS, physiological salt solution; TRAP, thrombin receptor activating peptide; PGI_2, prostacyclin.

MATERIALS AND METHODS

Chemicals

Human α-thrombin (3000 NIH U/mg) was purchased from the Centre regional de transfusion sanguine (Strasbourg, France). The synthetic peptide TRAP (SFLLRNPNDKYEPF) was purchased from Bachem (Basel, Switzerland). All culture reagents were from Boehringer Mannheim (Mannheim, Germany). Eighteen-base phosphorothioate antisense oligodeoxynucleotides (ODN) complementary to codons 116 to 122 of the human thrombin receptor cDNA [1] (5′-GTT-CCT-GAG-AAG-AAA-TGA-3′) and the corresponding phosphorothioate sense ODN were from Appligene (Illkirch, France). F-14 (LLYPPWNKNFTEND) was from Neosystem (Strasbourg, France).

Culture and Proliferation Assays of HUVEC

HUVEC were cultured as described [3] in 96-well microplates in RPMI 1640 medium supplemented with 10% fetal calf serum, endothelial cell growth factor (ECGF) (30 μg/mL) (Sigma, St Quentin-Fallavier, France), and heparin (100 μg/mL) (Sigma). Cells were routinely used from the third to the sixth passage. For proliferation assays, cells were plated sparsely (10^4 cells/well) in 24-well cluster plates (Nunc, Polylabo, Strasbourg, France) in DMEM culture medium + 0.5% fetal calf serum. After 2 days, fresh medium was added to the dishes (DMEM + 0.5% fetal calf serum and the different concentrations of the ODNs to be tested). Thrombin (100 nM), TRAP (10 μM), F-14 (100 μM) or ECGF/heparin (30 + 100 μg/mL) were added 24 hr later and, after 3 days in culture, cells were detached from triplicate wells by trypsin treatment (0.05% trypsin – 0.02% EDTA) and counted in a Coulter counter (Coultronics, Courbevoie, France).

Binding of [^{125}I]-Thrombin to HUVEC

Thrombin was radiolabeled with Na^{125}I (Amersham, Les Ulis, France) using Iodo-Gen™ beads (Pierce Chemical Co., Interchim, Montluçon, France) for 7 min at 22°C. Unbound iodine was removed by passing the labeled compounds over a PD-10 column (Pharmacia LKB Biotechnology Inc., Saint Quentin en Yvelines, France) preequilibrated with PBS with 0.01% Tween 80. Thrombin was labeled to a specific activity of 2–4 μCi/μg and retained ca. 80% of its amidolytic activity. Experiments studying the specific binding of [^{125}I]-thrombin to HUVEC were performed on adherent cells, cultured in 24-well cluster plates. Prior to binding experiments, confluent cells ($2–4 \times 10^5$ cells/well) were preincubated for 24 hr with 10 μM ODNs. Culture medium was removed and cells were washed twice with 1 mL of ice-cold PBS. Incubations were carried out in a total 0.2 mL volume of PBS that contained [^{125}I]-thrombin (10 nM). Triplicate incubations were carried out at 0°C for 30 min and were terminated by the addition of 1 mL of ice-cold assay buffer. Cells were then rapidly washed twice with 1.5 mL ice-cold incubation buffer and the radioactivity measured after digestion of the cell monolayer with 0.5 mL of an aqueous 0.3% Triton X100 solution (w/v).

Intracellular Free Calcium [Ca^{2+}]$_i$ Measurements

HUVEC cultured in 75 cm^2 flasks were detached with a nonenzymatic cell dissociation solution (Sigma), scraped from the flasks, centrifuged, and resuspended in physiological salt solution (PSS, composition: NaCl 145 mM, KCl 5 mM, $MgCl_2$ 1 mM, $CaCl_2$ 1 mM, glucose 5.6 mM, Hepes/NaOH 5 mM pH 7.4.), containing fura-2/AM (1 μM) and incubated at 37°C for 30 min. The cell suspension was then diluted 5 times with PSS, and incubated for a further 60 min at 37°C. After 2 washes with PSS to remove extracellular fura-2, cells were resuspended in PSS and kept in the dark at room temperature. Experiments were carried out under constant stirring in a PTI spectrofluorometer using ca. 75,000 cells in 3 mL fluorescence cuvettes at 37°C. Calibration was carried out on Triton X-100 (0.1%)-solubilized cells and intracellular free calcium concentrations calculated as described by Grynkiewicz et al. [7].

HUVEC Monolayer Permeability

For monolayer permeability determinations, HUVEC were seeded at high density (5×10^5 cells/cm^2) on fibronectin-coated polycarbonate (0.4 μm pore diameter) cell culture inserts for 24-well plates (Costar, Cambridge, MA). Permeability measurements were carried out 24 hr later, essentially as described by Patterson et al. [8]. Briefly, cell culture inserts were floated on 40 mL of constantly stirred abluminal PSS solution using polystyrene rings. The luminal medium was replaced with PSS containing 0.67 mg/mL of Evans Blue dye adsorbed to 40 mg/mL of fatty acid-free bovine serum albumin. Every 10 min, 200 μL of abluminal solution was removed and optical density at 620 nm determined on a microplate reader. Data were expressed as clearance values, and statistical analysis and permeability constant calculations were performed as described by Cooper et al. [9].

Prostacyclin Release

HUVEC (maximum passage 4) were grown to confluence in fibronectin (5 μg/cm^2)-coated 24-well plates. Experiments were carried out as described by Royston et al. [10], using prostacyclin release values during a 2-hr preincubation period to correct for basal release. Prostacyclin was measured as its stable degradation product 6-keto-prostaglandin $F_{1\alpha}$ using an enzyme immunoassay kit from Amersham (Les Ulis, France). Experiments were carried out in triplicate.

Statistical Analysis

All data are expressed as mean ± SE. EC_{50}values and the corresponding SE were determined by fitting the logistic equation to the data from individual experiments as described by DeLéan et al. [11].

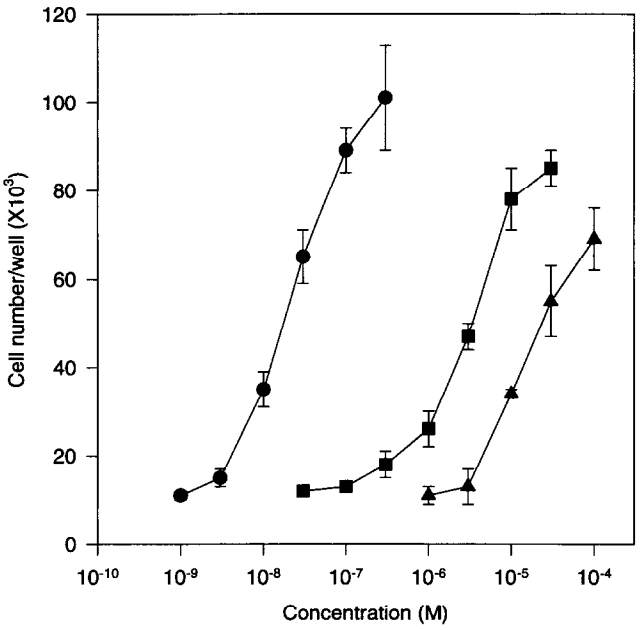

FIG. 1. HUVEC growth in the presence of thrombin, TRAP, or F-14. HUVEC were seeded (10^4 cells/well) in culture medium containing 0.5% fetal calf serum and the indicated concentrations of α-thrombin (●), TRAP (■), and F-14 (▲). After 3 days in culture, cells were trypsinized and counted. Data are reported as mean cell density ± SD (n = 9).

RESULTS AND DISCUSSION

Effect of an Antisense ODN on the Binding of [^{125} I]-Thrombin to HUVEC

Consistent with data obtained by us on other types of vascular cells and on HUVECs [3], [^{125} I]-thrombin bound to a single class of high-affinity binding sites on HUVECs. The dissociation binding constant (K_D) and the maximal binding capacity (B_{max}) values were 6.3 ± 0.9 nM and 8.9 ± 0.9 × 10^6 sites/cell (n = 3), respectively. This specific binding was inhibited by a 24 hr preincubation of HUVEC with 10 μM of the antisense ODN (95 ± 7% inhibition, n = 9). On the contrary, sense ODN-treated cells demonstrated an unchanged level of [^{125}I]-thrombin binding compared to untreated HUVEC (4.3 ± 2.1% inhibition, n = 9). These observations, therefore, show that 24 hr incubation with the antisense ODN strongly downregulates thrombin receptor expression and confirm the results obtained with the same ODNs on human aortic smooth muscle cells [6].

Effect of α-Thrombin, TRAP, and F-14 on the Proliferation of HUVEC in Culture

Native α-thrombin stimulated the growth of HUVEC in vitro in a dose-dependent manner (Fig. 1). The concentration of α-thrombin required to obtain maximal cell proliferation (A_{max}) was between 0.3 and 1 μM with a half-maximal response (ED_{50}) at approximately 30 nM. TRAP exhibited a significant dose-dependent mitogenic effect for HUVECs when tested at concentrations ranging from 30 nM to 30 μM (Fig. 1). The ED_{50} of TRAP was ca. 100-fold above that observed for native α-thrombin. Because this peptide can only be created by cleavage of the thrombin receptor [1], our results confirm that enzymatic activity of α-thrombin is necessary for its mitogenic effect on HUVEC. Consistent with data already shown [3], a thrombin-derived tetradecapeptide fragment named F-14, representing residues 365–378 of the human prothrombin sequence (insertion loop of the thrombin B chain) stimulated HUVEC growth over a concentration range of 1 μM to 100 μM, with optimal stimulation occurring between 30 and 100 μM (Fig. 1). These data suggest that thrombin can stimulate HUVEC growth via 2 different ways, one being dependent on its catalytic activity, the other mediated by a specific sequence located within the insertion loop of its B chain, separate and distinct from its active center and located at the surface of the thrombin molecule [3].

To establish if this nonenzymatic activity of thrombin might be due to a specific interaction with the known thrombin receptor, HUVEC were incubated with an antisense ODN previously shown to downregulate the expression of the thrombin receptor in human vascular smooth muscle cells [6] and in HUVEC, as shown above. As shown in Table 1, the mitogenic effect of thrombin and TRAP was totally abolished following a 24-hr preincubation of HUVEC with the antisense ODN, whereas thrombin and TRAP-induced HUVEC growth were not affected by the corresponding sense ODN. The mitogenic effect of endothelial cell growth-promoting factor, ECGF/heparin, was not altered by the antisense ODN, showing that it specifically affected the mitogenic signal of thrombin via its cell surface receptor. Under the same experimental conditions, the mitogenic effect of F-14 was strongly reduced following preincubation of HUVEC with the antisense ODN, thereby showing that F-14-induced HUVEC proliferation occurred through the thrombin receptor described by Vu et al. [1]. This observation is of considerable importance because it demonstrates for the first time that this receptor can be activated through a nonproteolytic pathway, distinct and additional to the TRAP-mediated activation [3].

TABLE 1. Effect of ODNs on the mitogenic effect of thrombin, TRAP, and F-14

	Cell number/well (×10^3)	
Mitogens	**Sense ODN**	**Antisense ODN**
Saline	11 ± 1	10 ± 2
ECGF/heparin	110 ± 13	107 ± 14
Thrombin	89 ± 5	12 ± 5
TRAP	78 ± 2	13 ± 2
F-14	69 ± 7	10 ± 1

Growth-arrested HUVEC (10^4 cells/well) were incubated for 24 hr in the presence of the phosphorothioate ODNs. Cells were then rinsed and grown in the presence of saline, ECGF/heparin (30 + 100 μg/mL), thrombin (100 nM), TRAP (10 μM), or F-14 (100 μM). After 3 days in culture, cells were trypsinized and counted. Each point represents the mean cell number ± SD (n = 9).

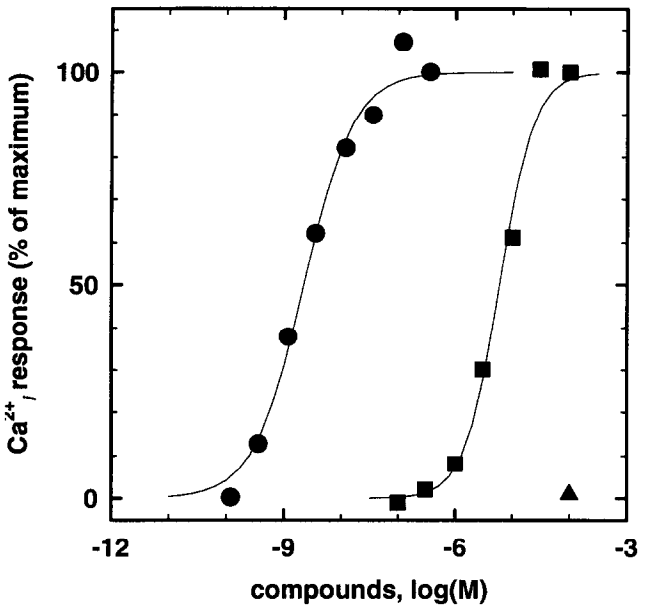

FIG. 2. Effect of thrombin, TRAP, and F-14 on intracellular free Ca^{2+} levels in HUVEC. Increasing doses of thrombin (●), TRAP (■), and F-14 (▲) were added to confluent HUVEC. Intracellular free Ca^{2+} was measured as described in Materials and Methods. Data are expressed as percent of the maximal increase of $[Ca^{2+}]_i$ (340 ± 120 and 850 ± 330 nM for thrombin and TRAP, respectively) and are the mean of 3 to 4 experiments. SE were less than 10% of the mean.

Effect of Thrombin, TRAP, and F-14 on $[Ca^{2+}]_i$, Permeability, and Prostacyclin Release

Thrombin induced a rapid transient increase of $[Ca^{2+}]_i$, this effect was dose-dependent, the half-maximal effect being observed at 2.0 ± 0.3 nM (n = 3) (Fig. 2). The effect of thrombin (1 U/mL, 12 nM) was totally blocked by hirudin (2-fold molar excess: 24 nM, not shown), suggesting that this activity of thrombin was due to the cleavage of the cellular thrombin receptor as proposed previously [12]. Transient $[Ca^{2+}]_i$ increase was also obtained with TRAP, whose half-maximal effect was seen at concentrations 1000-fold higher than those necessary for thrombin to induce the same effect (EC_{50} = 6.0 ± 0.8 μM, n = 4) (Fig. 2). These results are similar to those already reported in HUVEC [12, 13]. At concentrations as high as 100 μM, F-14 did not affect $[Ca^{2+}]_i$. The absence of any stimulatory effect of F-14 on $[Ca^{2+}]_i$ was somewhat unexpected, considering that increase in $[Ca^{2+}]_i$ is classically associated with the mitogenic effect of growth factors [14]. However, $[Ca^{2+}]_i$ increase is a transient effect, one essentially complete several min after stimulation, whereas growth promotion requires several hr [15]. We, therefore, determined if F-14 was able to stimulate other events needing longer incubation times in the presence of thrombin, such as increase in cell monolayer permeability or PGI_2 production [12]. To determine the effect of thrombin, TRAP, and F-14 on HUVEC monolayer permeability, HUVEC were grown on permeable polycarbonate membranes. As shown in Fig. 3, basal permeability of HUVEC monolayers was very low under these conditions (corresponding to a permeability constant of 1.6 10^{-6} cm s^{-1}), and only slightly increased after prolonged incubation times (Fig. 3). As described by others [12], addition of thrombin dramatically increased the permeability of a HUVEC monolayer after a short lagtime, and permeability remained at a high level for the remainder of the experiment. These effects of thrombin were dose-dependent and were observed at concentrations similar to those that increased $[Ca^{2+}]_i$ in HUVEC (EC_{50} = 4.0 ± 0.5 nM, n = 3). Similar responses were obtained with TRAP, which required much higher concentrations than thrombin for the same response (EC_{50} = 110 ± 25 μM, n = 2). F-14, even at a high concentration (1 mM), had no effect on HUVEC permeability.

As shown in Fig. 4, thrombin and TRAP also increased the release of PGI_2 (measured as 6-keto-prostaglandin $F_{1\alpha}$) from HUVEC, with the half-maximal effects being observed at 2.0 ± 0.2 nM (n = 4) and 110 ± 19 μM (n = 2) for thrombin and TRAP, respectively. F-14 up to 1 mM had no effect on PGI_2 production by HUVEC.

Therefore, it appears that F-14 is able to induce growth responses in HUVEC by stimulating the same thrombin receptor as the one described by Vu et al. [1], but through

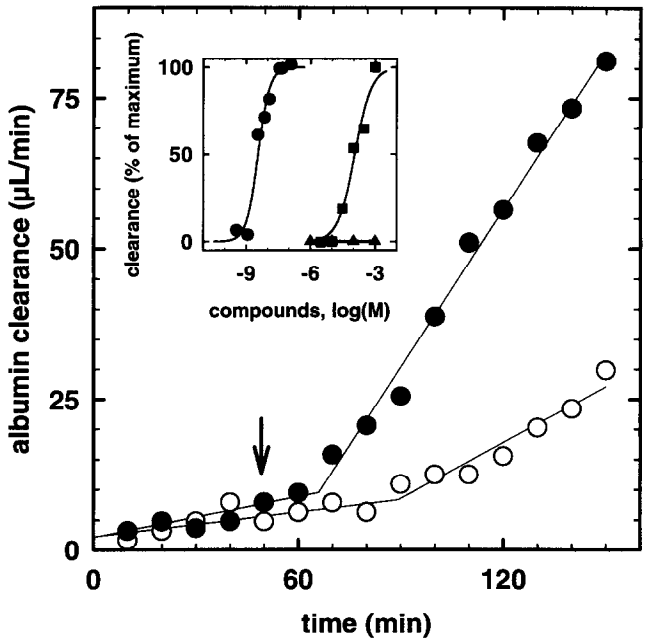

FIG. 3. Effect of thrombin, TRAP, and F-14 on the permeability of HUVEC monolayers. The permeability of HUVEC monolayers determined as the clearance of bovine serum albumin is plotted as a function of time in controls (○) and after the addition of thrombin 12 nM (●) (arrow). *Inset*: Dose-response relationships for the effect of thrombin (●), TRAP (■), or F-14 (▲) on HUVEC monolayer permeability. Data are the mean of 2 to 3 experiments and are expressed as a percentage of the maximal clearance, which was 0.50 ± 0.08 and 0.67 ± 0.03 μL/min for thrombin and TRAP14, respectively. SE represented ca. 15% of the mean values.

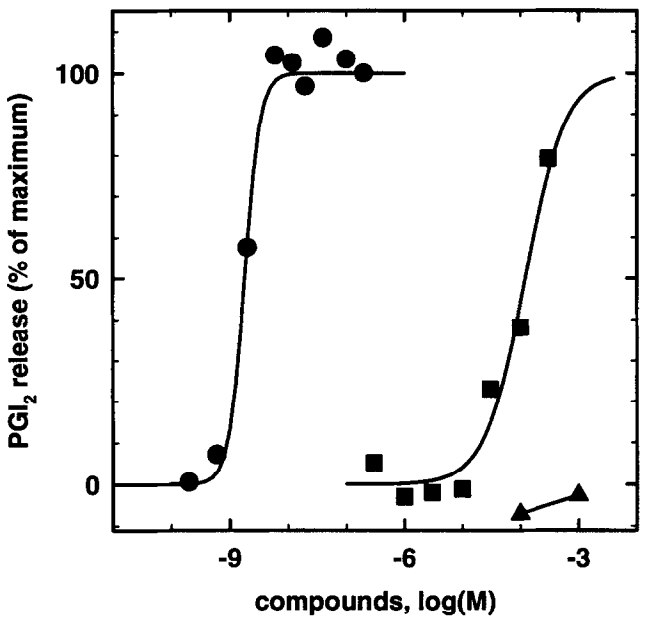

FIG. 4. Effect of thrombin, TRAP, and F-14 on PGI2 release from HUVEC. 6-keto-prostaglandin $F_{1\alpha}$ accumulation in the medium during a 30 min incubation period with thrombin (●), TRAP (■), or F-14 (▲) was measured by enzyme immunoassay as described in Materials and Methods. Data were corrected for basal release (5.3 ± 0.9 pg/well/30 min). The maximal effect corresponded to 60 ± 30 pg/well/30 min. Data are the mean of 2 to 4 experiments and are expressed as a percentage relative to the maximal effect of thrombin. SE represented ca. 15% of the mean values.

a nonproteolytic mechanism. This observation is unexpected because, up to now, all the cellular effects of thrombin have been demonstrated to occur via the catalytic activation of this receptor. However, F-14, unlike thrombin, does not induce a variety of other responses classically associated with activation of the thrombin receptor, such as intracellular Ca^{2+} release, cell monolayer permeability increase, or PGI_2 release [12]. This suggests that nonproteolytic thrombin receptor activation through the F-14 binding site can induce HUVEC growth, but that other responses necessitate cleavage of the thrombin receptor. The reason for such a difference remains unknown, but several recent reports suggest the role of several discrete regions within the thrombin receptor N-terminal extension as being necessary for nonproteolytic receptor activation [16, 17]. Although the molecular mechanisms of this effect remain poorly characterized, these regions may represent the binding site for peptide ligands that activate the thrombin receptor through a nonproteolytic mechanism. Alternatively, this peptide sequence may affect receptor phosphorylations, signaling mechanisms via critical conformational adjustments, as suggested by Bahou et al. [16]. Despite these unresolved issues, our data provide additional evidence for the complexity of thrombin receptor activation by defining a previously uncharacterized regulatory mechanism ultimately specifying receptor activation processes.

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