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Internalization and Recycling of Activated Thrombin Receptors*

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James A. Hoxie, Menaka Ahuja, Elizabeth Belmonte, Shelly Pizarro, Robert Parton‡, and Lawrence F. Brass§

From the Departments of Medicine and Pathology, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the ‡European Molecular Biology Laboratory, D-6900 Heidelberg, Germany

Shortly after activation by either thrombin or the tethered ligand domain peptide SFLLRN, thrombin receptors undergo homologous desensitization, temporarily losing their ability to respond to both agonists. We have examined the role of receptor internalization and recycling in this process using receptor-directed antibodies as probes. The results show within 1 min of activation >85% of the approximately 200,000 thrombin receptors on megakaryoblastic human erythroleukemia (HEL) and CHRF-288 cells are sequestered into endosomes via coated pits, after which the majority are transferred to lysosomes. This process does not require proteolysis of the receptor and occurs with sufficient speed to play a major role in the regulation of thrombin receptor function. Although most of the internalized receptors are ultimately degraded, approximately 25% return to the cell surface. These recycled receptors are in a state in which they can respond to SFLLRN but not thrombin; nor do they self-activate despite the apparent continued presence of the tethered ligand. In contrast to other G protein-coupled receptors, which are internalized and then recycled in an activatable state, recovery of the thrombin response occurs only after the expression on the cell surface of adequate numbers of newly synthesized receptors.

Thrombin is a serine protease that is able to evoke biological responses from a variety of cells, including platelets. Recent evidence suggests that most, if not all, of these responses are mediated by a single class of cell surface receptors belonging to the family of G protein-coupled receptors (1, 2). Based upon cDNA sequencing, the human platelet thrombin receptor is thought to be formed by a single polypeptide chain with seven transmembrane domains and an extracellular N terminus. According to current models, receptor activation occurs when thrombin cleaves the N terminus between residues Arg^{41} and Ser^{42}, exposing a tethered ligand for the receptor (1, 2). In support of this model, peptides containing the initial 6 residues of the neo-N terminus, SFLLRN, have been shown to mimic the effects of thrombin on platelets (1, 3–9) and antibodies directed against the tethered ligand domain have been shown to inhibit platelet activation by thrombin (10, 11).

One of the characteristics of cellular responses to thrombin is that receptor activation produces a state of homologous desensitization in which the readdition of thrombin fails to evoke a second response (12–15). We have previously shown that desensitization affects responses to the receptor-derived peptide SFLLRN, as well as thrombin (16, 17). Using the megakaryoblastic HEL[1] cell line as a model, we found that activation by either thrombin or SFLLRN prevented a subsequent response to both agonists, even though responses to other agonists were preserved. Notably, recovery from the desensitized state occurred in two distinct phases. In the first phase, which was detectable within 30 min and continued over several hours, there was a partial (30–40%) recovery of responsiveness to SFLLRN but no recovery of the response to thrombin when the initial desensitization was caused by thrombin. When the initial desensitization was caused by SFLLRN, recovery of responsiveness to both agonists occurred at the same time. This first phase of recovery could be delayed by inhibitors of serine/threonine phosphatases but was unaffected by cycloheximide. In the second phase of recovery, which required as much as 20 h to complete, there was a full return of receptor responses to both SFLLRN and thrombin. This phase of recovery could be inhibited by cycloheximide, suggesting that it was at least partly due to the synthesis of new receptors (17).

These results suggested a model in which both receptor synthesis and dephosphorylation play a role in the recovery of thrombin receptor function on desensitized cells. However, it was unclear from those studies why cells desensitized by thrombin recover their ability to respond to SFLLRN in advance of their ability to respond to thrombin, and whether this might be due to the reactivation of previously inactivated receptors. It was also unclear whether receptor internalization might play a role in the regulation of receptor function, as it appears to do for β-adrenergic receptors (18, 19). To answer these questions, the present studies examine the effects of thrombin on the surface distribution, internalization, and recycling of thrombin receptors. The results show that activated thrombin receptors, like β-adrenergic receptors, can be rapidly internalized. Some of these internalized receptors are eventually recycled to the cell surface. However, in contrast to β-adrenergic receptors, recycled thrombin receptors lose their capacity to respond to thrombin, even while recovering their ability to be activated by SFLLRN.

EXPERIMENTAL PROCEDURES

Monoclonal Antibodies—The preparation of murine monoclonal antibodies against residues 42–55 (SFLLRNPNDKYEPF) of the

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§ To whom correspondence should be addressed: Hematology-Oncology Div., Silverstein 7, University of Pennsylvania, 3400 Spruce St., Philadelphia, PA 19104. Tel.: 215-573-3540; Fax: 215-573-2189.

[1] The abbreviations used are: HEL cells, human erythroleukemia cells; CHRF-288 cells, Children's Hospital Research Foundation cell line number 288; LH, lutropin hormone; LAMP, lysosome-associated membrane protein; FITC, fluorescein isothiocyanate; HIV, human immunodeficiency virus.

human thrombin receptor is described elsewhere (10). Based upon binding assays with peptide fragments, two of the antibodies, ATAP87 and ATAP138, are specific for epitope(s) within the sequence NPNDKYEPF. The third, ATAP2, recognizes an epitope within SFLLRNPN. These antibodies bind to the surface of intact platelets and inhibit thrombin-induced platelet activation (10). Antibody WEDE15 was prepared against a peptide corresponding to thrombin receptor residues 51–64 (KYEPFWEDEEKNES) and reacts in binding assays with the immunizing peptide but not peptide SFLLRNPNDKYEPF. All four of these antibodies are of the IgG_1 subtype. The anti-transferrin receptor antibody RPN511 was obtained from Amersham Corp. Antibody H4A3, directed against the human lysosomal protein LAMP-1, was a gift from Dr. J. T. August (Johns Hopkins University, Baltimore, MD) (20). Monoclonal antibodies BA6 and A2A9 directed against CD9 and the glycoprotein IIb-IIIa complex, respectively, are described elsewhere (21, 22). IgG_1 monoclonal antibodies that were used as isotype-matched negative controls included P3X63 (23), EH1, and DA6. The latter two antibodies are reactive with the HIV-1 *nef* protein and HIV-2 gp120 envelope molecules, respectively.[2]

Immunofluorescence Microscopy—For surface staining of intact cells, CHRF-288 or HEL cells were incubated at 37 °C in the presence or absence of an agonist, washed once, and then incubated with primary antibody for 30 min at 4 °C. Afterward, the cells were washed again, incubated for 30 min at 4 °C with a 1:20 dilution of FITC-conjugated goat anti-mouse IgG (Tago, Burlingame, CA), washed, and then fixed with 1% paraformaldehyde before being cytocentrifuged onto glass slides for microscopy. For intracellular staining, the cells were cytocentrifuged onto glass slides, fixed with methanol:acetone (50:50), and then stained with an optimal concentration of antibody. In the co-localization studies, the permeabilized cells were incubated with either the anti-transferrin receptor antibody or the anti-LAMP antibody, followed by rhodamine-conjugated goat anti-mouse IgG (Tago). Antibody EH1 was then added as a blocking reagent, followed by FITC-conjugated antibody ATAP2.

Electron Microscopy—CHRF-288 cells were incubated with 50 nM thrombin at 37 °C. At various times (0–5 min) aliquots were removed and added to a large volume of ice-cold medium. All further manipulations were performed at 4 °C. The cells were washed by centrifugation and resuspended in cold medium containing a 1:100 dilution of antibody ATAP2 ascites. After 30 min the cells were washed several times and then incubated with goat anti-mouse IgG adsorbed to 5-nm gold (Aurion, The Netherlands). After additional washing the cells were sedimented, fixed in 1% glutaraldehyde in 100 mM cacodylate buffer, pH 7.35, and embedded in Epon. Sections were stained with uranyl acetate and lead citrate and viewed in a Zeiss EM10 microscope (24).

Flow Cytometry—After incubation with the desired agonist for varying time intervals, CHRF-288 or HEL cells (2×10^6 cells/ml) were fixed in suspension in 1% paraformaldehyde, kept on ice for 30 min and then washed and resuspended in staining buffer (phosphate-buffered saline, 0.02% sodium azide, 0.2% bovine serum albumin, pH 7.4). Primary antibodies were added in the form of either undiluted hybridoma culture supernatant or an optimal dilution of ascites (1:400 to 1:1,000). After 30 min the cells were washed again, incubated with a 1:40 dilution of FITC-labeled goat anti-mouse IgG (Tago) for an additional 30 min, and then fixed in 4% paraformaldehyde for analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Binding Studies—The binding of radioiodinated ATAP138 to CHRF-288 cells and HEL cells was quantitated essentially as described previously (10). In brief, the antibody was incubated with the cells (1×10^6/ml) for 20 min at 37 °C, after which aliquots of the cell suspension were sedimented through silicone oil to separate bound from free antibody. [^{14}C]Inulin was used to correct for unbound antibody trapped within the cell pellet, although this correction proved to be negligible.

Other Methods and Materials—Human α-thrombin (≈3,000 units/mg) was provided by Dr. J. Fenton (New York State Department of Health, Albany, NY). Peptides were prepared as described (4). CHRF-288 cells (25), which were originally derived from a patient with acute megakaryoblastic leukemia, were a gift from Drs. M. Lieberman and D. Witte (University of Cincinnati College of Medicine, Cincinnati, OH) and were maintained in RPMI 1640 medium with 20% fetal calf serum. HEL cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium with 10% fetal calf serum (16). Changes in the cytosolic free Ca^{2+} concentration were measured using Fura-2 as previously described (17).

RESULTS

Thrombin receptor desensitization and internalization were studied with HEL cells (26) and CHRF-288 cells (25), two human cell lines with megakaryoblastic properties. The HEL cell thrombin receptor has a nucleotide sequence that is identical to that reported for Dami cells (1) except for the substitution of Leu for Val^{238} and Cys for Ser^{364} in the fourth and seventh predicted transmembrane domains.[3] The number of thrombin receptors on the surface of CHRF-288 and HEL cells was determined using ATAP138, a monoclonal antibody directed against the tethered ligand domain of the thrombin receptor (the specificities of the various monoclonal antibodies used in these studies are described in greater detail under "Experimental Procedures"). On average, ATAP138 bound to 209,000 ± 6,000 sites on CHRF-288 cells (Fig. 1*A*) and 160,000 ± 40,000 sites on HEL cells (data not shown). Since the same antibody binds to approximately 1,800 sites/cell on platelets (10) and since HEL and CHRF-288 cells have roughly 10 times the diameter and 100 times the surface area of platelets, the density of thrombin receptors on the two cell lines appears to be similar to platelets.

Data showing the response of HEL cells to thrombin and the desensitization of the HEL cell thrombin receptor by thrombin and SFLLRN have been presented elsewhere (16, 17). Comparable results with CHRF-288 cells are shown in Fig. 1. As was seen with HEL cells, both thrombin and the SFLLRN peptide caused a transient increase in the cytosolic free Ca^{2+} concentration in CHRF-288 cells (Fig. 1*B*). This response was no longer present when the cells were incubated with thrombin for 10 min, washed, and then rechallenged immediately with either thrombin or SFLLRN (Fig. 1*C*). Three hours later, the cells were still unresponsive to thrombin but had partially regained their ability to respond to SFLLRN (Fig. 1*D*).

Loss of Receptors from the Cell Surface following Activation—The distribution of thrombin receptors on the surface of CHRF-288 cells was examined by fluorescence microscopy using antibody ATAP2, which is also directed against the tethered ligand domain of the receptor N terminus. On resting cells ATAP2 binding sites were present on all cells and uniformly distributed across the cell surface (Fig. 2*A*). However, after exposure to thrombin for 10 min little or no antibody binding could be detected (Fig. 2*B*). An identical loss of ATAP2 binding sites occurred when the cells were incubated with SFLLRN, rather than thrombin (not shown), but the binding of monoclonal antibodies directed against the plasma membrane protein CD9 (Fig. 2, *C* and *D*) and the glycoprotein IIb-IIIa complex (not shown) was unaffected by either thrombin or SFLLRN.

Flow cytometry was used to quantitate the effects of thrombin on antibody binding to the surface of CHRF-288 cells. Exposure to thrombin for 10 min decreased antibody binding by an average of 87% using any of three antibodies directed against the receptor-tethered ligand domain: ATAP2, ATAP87, and ATAP138 (Fig. 3*A*). Similar results were also obtained with a fourth antibody, WEDE15, which is directed against a domain of the receptor N terminus that includes the putative thrombin binding site (not shown). By comparison, there was no decrease in the binding of the anti-CD9 antibody, BA6 (Fig. 3*A*), or in antibodies directed against glycoproteins IIb and IIIa (not shown). The loss of ATAP2 binding was not

[2] J. Hoxie, unpublished observations.

[3] A. Tarver, M. Poncz, and L. Brass, unpublished observation.

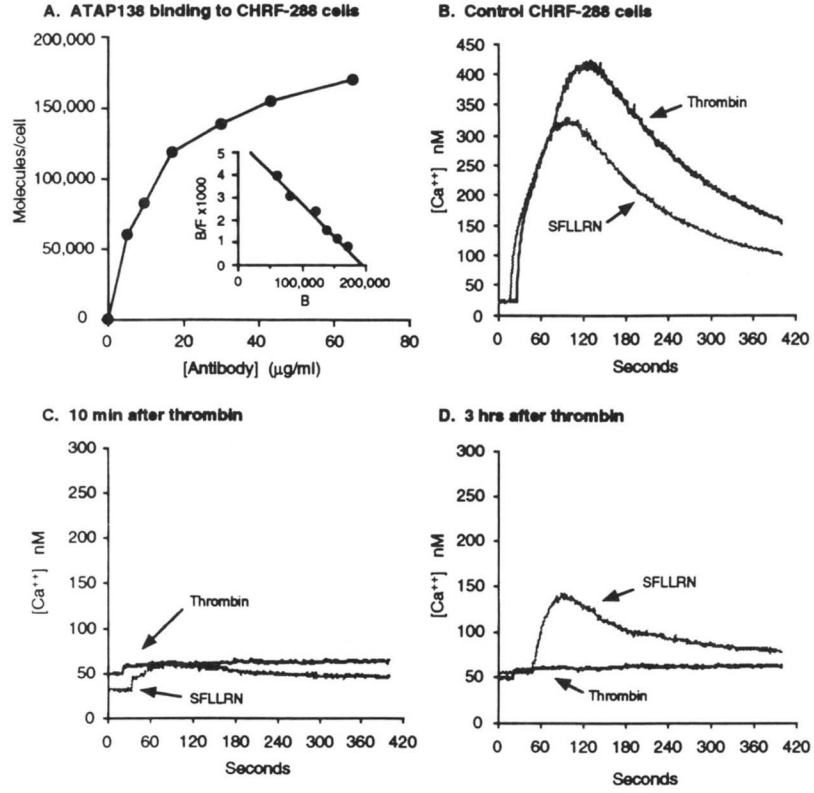

FIG. 1. **CHRF-288 cells.** *A*, binding of radioiodinated anti-thrombin receptor antibody, ATAP138, to CHRF-288 cells. In three such studies the mean number of antibody binding sites was 209,000 ± 6,000/cell with a K_D of 71 ± 2 nM (mean ± S.E.). *B*, the cytosolic free Ca^{2+} concentration was measured in Fura-2-loaded CHRF-288 after stimulation with thrombin (50 nM) or the tethered ligand peptide, SFLLRN (25 μM). In *C* and *D*, cells were incubated with thrombin for 10 min, resuspended in fresh medium, and then stimulated with thrombin or SFLLRN either 10 min or 3 h later.

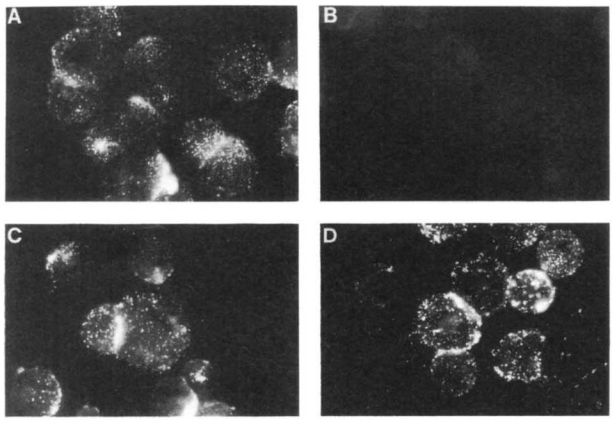

FIG. 2. **Fluorescence microscopy of intact CHRF-288 cells.** After a 10-min incubation in the presence or absence of 20 nM thrombin, CHRF-288 cells were resuspended in fresh medium and incubated with either the thrombin receptor antibody, ATAP2, or the CD9 antibody, BA6, followed by FITC-labeled anti-mouse secondary antibody. *A*, resting cells with ATAP2; *B*, thrombin-stimulated cells with ATAP2; *C*, resting cells with BA6; *D*, thrombin-stimulated cells with BA6.

prevented by resuspending the thrombin-treated cells in fresh medium or by adding a 5-fold excess of the thrombin-binding protein hirudin, indicating that it was not due to occlusion of the antibody binding site by thrombin. The kinetics of receptor loss were exceedingly rapid, with a 50% decrease occurring within 30 s of thrombin addition and >85% occurring within 1 min (Fig. 3*B*).

Internalization of Activated Thrombin Receptors—To determine whether receptor internalization is responsible for the loss of activated thrombin receptors from the cell surface, CHRF-288 cells were incubated with thrombin or SFLLRN and then fixed and permeabilized before being stained with receptor antibody ATAP2. In unstimulated cells antibody binding occurred at scattered sites within the cells as well as on the cell surface (Fig. 4*A* and the *first panel* of Fig. 5). The intracellular sites included small punctate structures as well as larger structures with a vesicular appearance. Exposure of the cells to thrombin for 10 min prior to fixation caused a dramatic increase in both types of intracellular staining, particularly in the large vesicular structures (Fig. 4, *C* and *D*). Since the same changes occurred in cells activated with SFLLRN (Fig. 4*B*), they appear to not require proteolysis of the receptor. On the other hand, there was no intracellular staining, either before or after thrombin, with the CD9 antibody, BA6 (Fig. 4, *E* and *F*) and no binding under either set of conditions of an isotype-matched control antibody, DA6, directed against the HIV-2 glycoprotein, gp120 (not shown). Therefore, these results suggest that activated thrombin receptors are selectively internalized from the cell surface and that this process is promoted by activation of the receptor but does not require proteolysis of the receptor N terminus.[4]

The changing distribution of the internalized receptors over time is shown in Fig. 5. For these studies, CHRF-288 cells were fixed and permeabilized at intervals as long as 120 min after the addition of thrombin. After 1 min, a time by which antibody binding to the cell surface had decreased by 85%, ATAP2 bound predominantly to numerous punctate cytoplasmic structures that were distributed in a perinuclear pattern. At this early time point few of the vesicular structures noted in Fig. 4 were visible, but by 10 min such structures were prominent. At 30 and 60 min, the vesicular structures were distributed asymmetrically, typically clustering to one

[4] The conclusion that the newly detectable intracellular receptors were initially on the cell surface is also supported by our preliminary observations with a monoclonal antibody whose epitope spans the proposed site of cleavage by thrombin. As would be predicted, this antibody shows an intracellular distribution of receptors identical to that in Fig. 1 (*B–D*), when CHRF-288 cells are activated non-proteolytically with SFLLRN but does not recognize the intracellular receptors in cells activated by thrombin (L. Brass, S. Pizarro, M. Ahuja, E. Belmonte, J. Stadel, and J. Hoxie, unpublished observations).

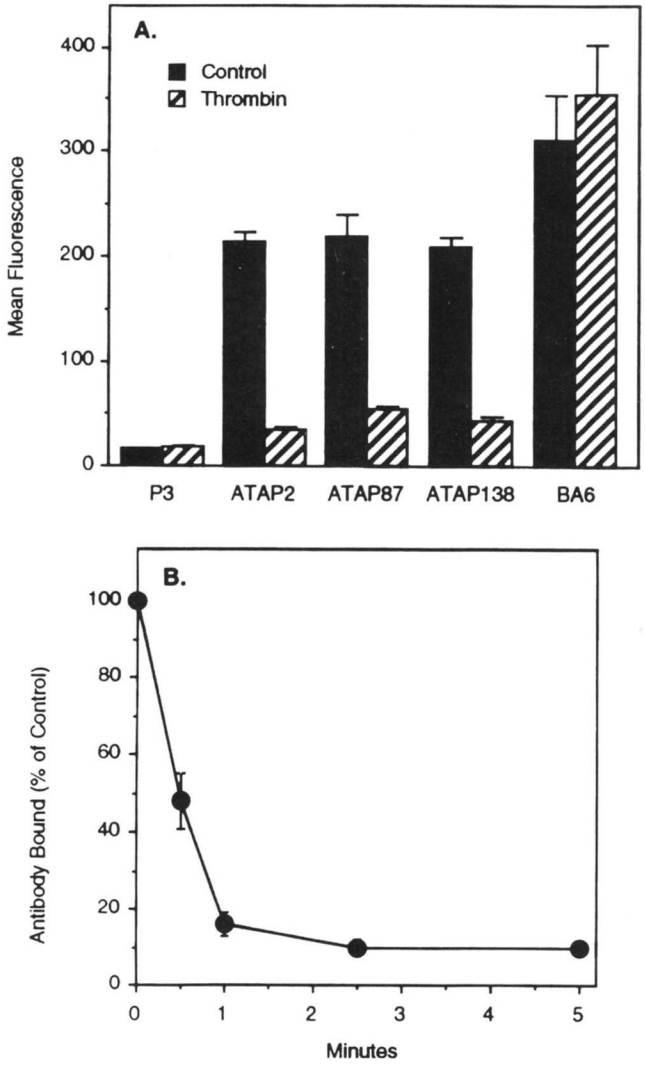

FIG. 3. **Flow cytometric analysis of antibody binding to CHRF-288 cells.** *A*, CHRF-288 cells were incubated for 10 min in the absence (*solid bars*) or presence (*hatched bars*) of 50 nM thrombin, followed by one of the monoclonal antibodies shown and FITC-labeled anti-mouse secondary antibody. The cells were then analyzed by flow cytometry to quantitate cell surface fluorescence. ATAP2, ATAP87, and ATAP138 are anti-thrombin-receptor antibodies. BA6 is an anti-CD9 antibody. P3 is an isotype-matched control murine monoclonal antibody. The results shown are from 4–8 determinations expressed as mean ± S.E. *B*, CHRF-288 cells were fixed and stained with antibody ATAP2 or ATAP138 after incubation with 50 nM thrombin for the times shown. The results are the mean ± S.E. of 4 studies.

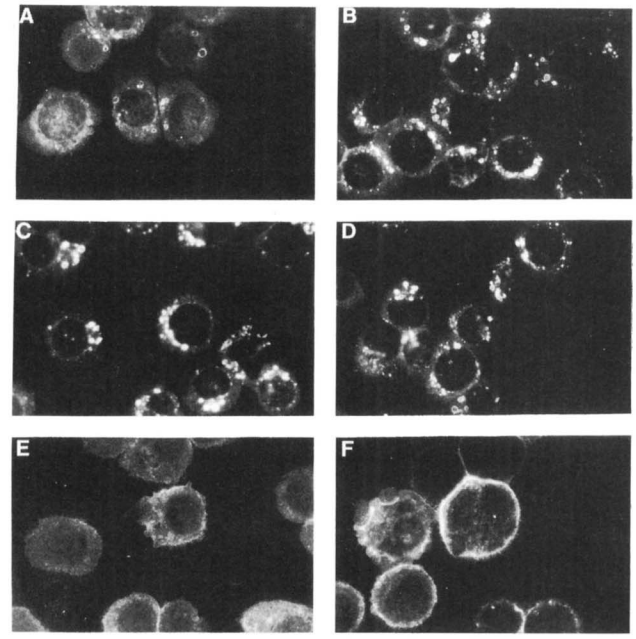

FIG. 4. **Fluorescence microscopy of permeabilized CHRF-288 cells.** CHRF-288 cells were incubated for 10 min at 37 °C with 50 nM thrombin or 50 μM SFLLRN, then washed, fixed, and permeabilized with methanol/acetone and stained with primary antibody. *A*, control cells with thrombin receptor antibody ATAP2; *B*, SFLLRN-treated cells with antibody ATAP2; *C* and *D*, thrombin-treated cells with antibody ATAP2; *E*, control cells with anti-CD9 antibody BA6; *F*, thrombin-treated cells with antibody BA6.

side of the nucleus. By 120 min fluorescence of structures in the cytoplasm had generally decreased, but there was a discernible increase in cell surface fluorescence, a result subsequently confirmed by flow cytometry of recovering cells (see below).

Localization of the Internalized Receptors—To identify the cellular compartments involved in thrombin receptor internalization, co-localization studies were performed with two other monoclonal antibodies: RPN511, which binds to the human transferrin receptor and was used as a marker for endosomes (27, 28); and H4A3, which recognizes the lysosome-associated membrane protein, LAMP-1, and was used to identify lysosomes (20). Fig. 6 shows a paired exposure of CHRF-288 cells that were fixed and permeabilized after a 1-min incubation with thrombin and stained with ATAP2 (*top*) and RPN511 (*bottom*). The punctate perinuclear structures typically seen with ATAP2 at this early time point were also recognized by the transferrin receptor antibody. These particular structures were not, however, detectable with the anti-LAMP-1 antibody, H4A3 (not shown). Fig. 7 shows a paired exposure when the CHRF-288 cells were stained with ATAP2 (*top*) and H4A3 (*bottom*) 30 min after the addition of thrombin. At this time point the compartments recognized by the two antibodies overlapped, with the vesicular structures recognized by the thrombin receptor antibody clustering around the region of anti-LAMP-1 staining and, in some, but not all, cases binding both antibodies. These results indicate that activated thrombin receptors are rapidly incorporated into endosomes and then more slowly transported into lysosomes.

Entry of Receptors through Coated Pits in the Plasma Membrane—Immunoelectron microscopy was used to examine the initial steps of thrombin receptor internalization. In the studies shown in Fig. 8, CHRF-288 cells were incubated with thrombin for 1 min at 37 °C, then labeled at 4 °C with antibody ATAP2 followed by gold-conjugated secondary antibody. One minute after the addition of thrombin, clusters of receptors were seen in and around pits within the plasma membrane that resemble typical clathrin-coated pits. There was no clustering of receptors in non-coated pits, even at longer times. The involvement of coated pits in thrombin receptor internalization is similar to that recently reported for lutropin hormone (LH) receptors (29) but contrasts with the results of studies on β-adrenergic receptors, where internalization appears to involve non-coated pits (30), suggesting that distinct mechanisms for internalization may be utilized by different G protein-coupled receptors.

Recycling of Activated Thrombin Receptors—As was noted earlier, CHRF-288 and HEL cells that have been desensitized by thrombin recover 30–40% of their initial responsiveness to SFLLRN over a period of 2–3 h (Fig. 1 and Ref. 17), but during the same period there is no recovery of the response to thrombin. This early phase of recovery of the SFLLRN

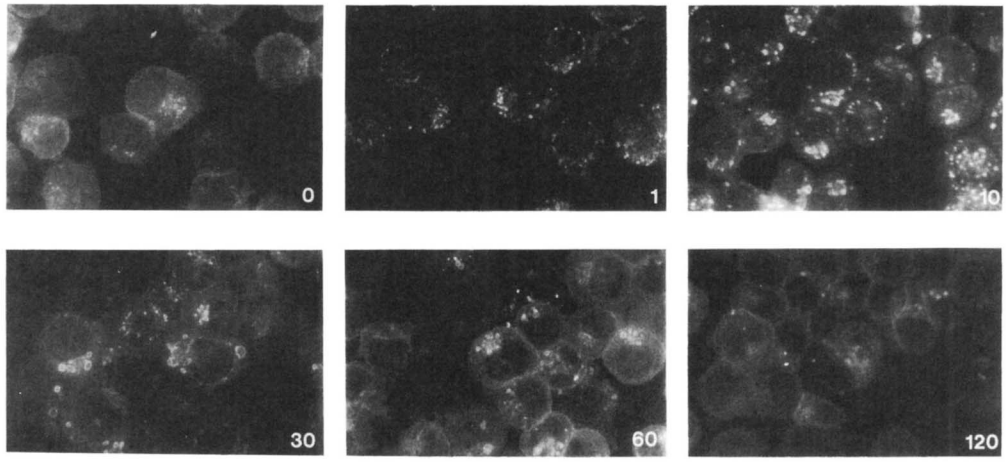

FIG. 5. **Time course of thrombin receptor internalization.** CHRF-288 cells were incubated at 37 °C for up to 120 min. Thrombin (20 nM or approximately 2 units/ml) was added at time zero. Hirudin (10 units/ml) was added after 10 min to remove any thrombin still bound to the cell surface. At each time shown, aliquots of the cell suspension were washed, fixed with methanol/acetone, and then stained with thrombin receptor antibody, ATAP2.

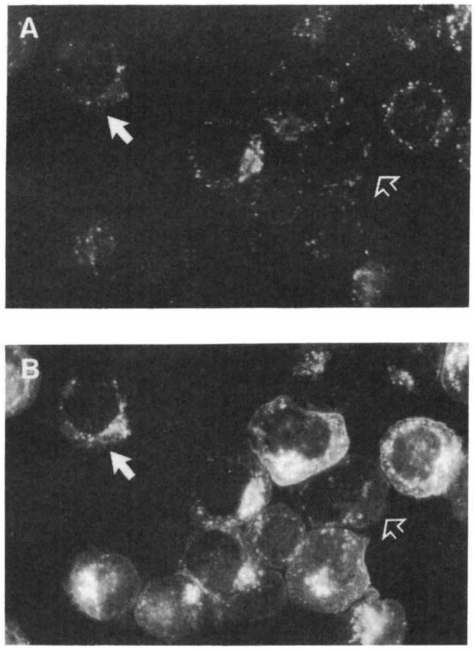

FIG. 6. **Co-localization of antibodies directed against thrombin receptors and transferrin receptors after 1 min with thrombin.** CHRF-288 cells were incubated with thrombin (20 nM) for 1 min at 37 °C and then stained with thrombin receptor antibody ATAP2 and the anti-transferrin receptor antibody RPN511, as described under "Experimental Procedures." *A*, fluorescence from the FITC-conjugated ATAP2; *B*, fluorescence from the transferrin receptor antibody detected with rhodamine-conjugated secondary antibody. The *filled* and *open arrows* point to two pairs of cells in which the location of the two antibodies can be compared.

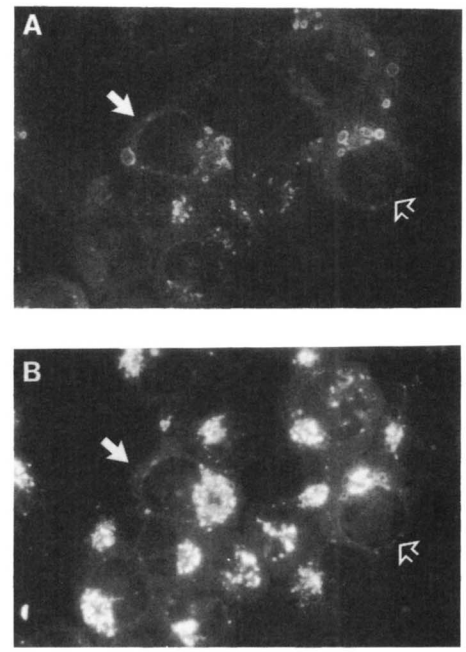

FIG. 7. **Co-localization of antibodies directed against thrombin receptors and LAMP-1 after 30 min with thrombin.** CHRF-288 cells were incubated with 20 nM thrombin for 30 min at 37 °C and then stained with thrombin receptor antibody ATAP2 and the monoclonal antibody H4A3, an antibody specific for the lysosomal membrane protein LAMP-1. *A*, fluorescence from the FITC-conjugated ATAP2; *B*, fluorescence from the anti-LAMP-1 antibody detected with rhodamine-conjugated secondary antibody. In the cell indicated by the *filled arrows*, the large ring visible to the left of the nucleus with the thrombin receptor antibody is not stained by the LAMP-1 antibody. In the cell indicated by the *open arrows*, several vesicles at the top of the cell are stained by both antibodies.

response is unaffected by cycloheximide, suggesting that it does not require receptor synthesis (17). Since the present studies show that activated thrombin receptors are rapidly internalized, we next tested whether the recovery of responsiveness to SFLLRN coincides with the reemergence of receptors on the cell surface. Fig. 9 shows the results that were obtained. In these studies CHRF-288 cells were incubated briefly with thrombin, after which the recovery of SFLLRN-induced changes in cytosolic Ca^{2+} was compared with the recovery of receptors on the cell surface measured by flow cytometry. On these cells, the initial exposure to thrombin caused a 90% decrease in both antibody binding and the magnitude of the SFLLRN-induced increase in intracellular Ca^{2+}. Over the next 3 h the peptide-induced Ca^{2+} signal recovered to approximately 40% of its simultaneous value in control cells and antibody binding recovered to approximately 30% of its initial value. The rates of recovery of the two events were similar, although at the earliest time points the recovery of antibody binding sites preceded the recovery of the Ca^{2+} signal. Identical results were obtained with anti-thrombin receptor antibody WEDE15 (not shown). Since there was no

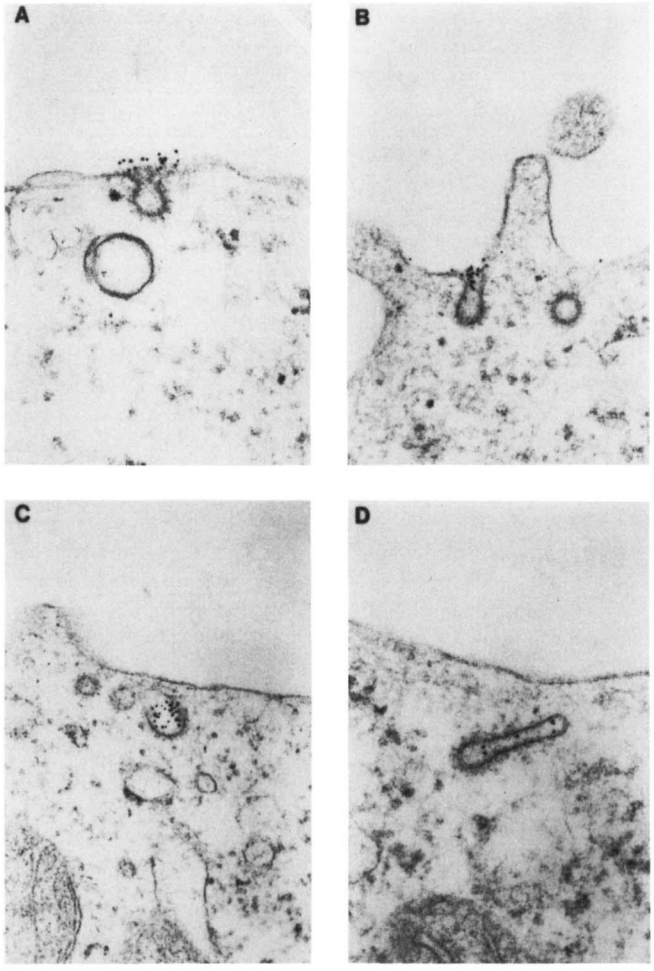

FIG. 8. **Electron microscopy of thrombin-stimulated CHRF-288 cells.** CHRF-288 cells were incubated with 50 nM thrombin for 1 min at 37 °C. Surface receptors were detected by incubating the cells with antibody ATAP2 followed by secondary antibodies adsorbed to 5-nm gold at 4 °C. Four representative images of the plasma membrane are shown. In *panels A–C*, gold particles are seen clustering in or near coated pits. *Panel D* shows a surface invagination connected by a tubule out of the plane of the section with a coated pit attached.

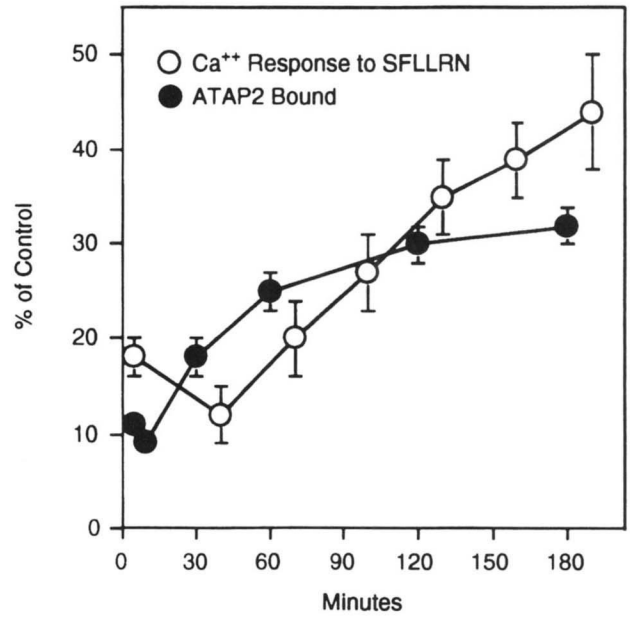

FIG. 9. **Recovery of thrombin receptors and SFLLRN responses in CHRF-288 cells desensitized with thrombin.** CHRF-288 cells were incubated with 50 nM thrombin for 10 min at 37 °C. At each time indicated one of two assays were performed: either 10 μM SFLLRN was added and the subsequent increase in cytosolic free Ca^{2+} was measured using Fura-2 fluorescence (*open symbols*) or the binding of ATAP2 and ATAP138 to the cell surface were measured by flow cytometry (*closed symbols*). The results shown are the mean ± S.E. of 10 Ca^{2+} determinations and 4 antibody measurements and are expressed as a percentage of the results obtained with the non-thrombin-treated cells at the same time.

recovery of the thrombin response during this period, these results suggest that the more rapid recovery of SFLLRN responsiveness is due to the reemergence of previously internalized receptors that are unable to interact with thrombin a second time.

DISCUSSION

Prior to the publication of the cloned sequence of the thrombin receptor, little was known about the mechanisms by which it is activated and inactivated. It was clear, however, that at least some of the cellular effects of thrombin involve G proteins and that, like other receptors which interact with G proteins, thrombin receptors are subject to homologous desensitization. These effects could be demonstrated with platelets but were best observed with cells in which exposure to thrombin is not a terminal event. For example, we found that HEL cell thrombin receptors undergo homologous desensitization and that this process does not require receptor proteolysis since it could also be observed with SFLLRN-containing peptides (17). In those studies recovery from the desensitized state evoked by thrombin occurred in two distinct phases. The first was detectable within 30 min and lasted several hours. During this period the cells responded to SFLLRN but not thrombin. This phase of recovery was unaffected by cycloheximide but could be delayed by inhibitors of serine/threonine phosphatases such as calyculin and okadaic acid, suggesting that receptor expression may be modulated by phosphorylation, either of the receptor itself or an associated protein (17). The second phase of recovery required up to 20 h to complete and could be inhibited with cycloheximide. This phase restored the full thrombin response and presumably reflects the resynthesis and surface expression of new receptors.

In the present studies we have extended these earlier observations by examining the role of receptor internalization and recycling in the loss and subsequent recovery of thrombin responses. Both of the cell lines that were studied express large numbers of functional thrombin receptors at a density similar to platelets, and in both cell lines thrombin responses were subject to homologous desensitization. Since thrombin binding studies have been hampered by the ability of thrombin to bind with high affinity to cell surface proteins other than its receptor, monoclonal antibodies rather than labeled ligands were used to detect and track the receptor. These antibodies bind to both the intact and the thrombin-cleaved forms of the thrombin receptor, making it possible to locate the receptor before and after activation.

The results show that activated thrombin receptors are internalized at a rate that is as fast or faster than the time required to demonstrate desensitization of receptor function. Within 1 min of activation, thrombin receptors were clustered in and around coated pits in the plasma membrane, after which they passed through at least three morphologically distinguishable cytoplasmic structures. The first, which had a punctate appearance by fluorescence microscopy, also contained transferrin receptors and presumably represents early endosomes. The second, which had a vesicular appearance,

was variably stained with the anti-LAMP-1 antibody and probably represents late endosomes or prelysosomes. These were detectable within 10 min of the addition of thrombin. The final structures, which were visible as early as 30 min, were intensely stained by the anti-LAMP-1 antibody and are presumably mature lysosomes. At approximately the same time that thrombin receptors could be detected in lysosomes, there was also a detectable recovery of antibody binding sites on the cell surface. Over a 2-h period the number of receptors on the surface of CHRF-288 cells returned to approximately 30% of its original value. This recovery paralleled the recovery of receptor function when the cells were stimulated with SFLLRN and coincided with the first phase of receptor resensitization during which the cells were unable to respond to thrombin.

A Model for Receptor Internalization and Recycling—Collectively, these observations suggest a working model for receptor internalization and recycling that accounts for the present observations and suggests directions that can be taken in future studies (Fig. 10). According to this model, thrombin receptors are internalized shortly after they are activated. The majority of the internalized receptors are sorted to lysosomes and presumably destroyed. However, approximately 25% are recycled back to the cell surface. It is the recycling of previously activated (and presumably cleaved) receptors that appears to account for the cycloheximide-resistant early phase of recovery during which the cells respond to SFLLRN but not thrombin. This discrepancy between the recovery of the SFLLRN and thrombin responses in thrombin-desensitized cells is a consequence of the novel mechanism by which thrombin activates its receptors. On the other hand, as would be predicted by the model, when the receptors are initially activated (and desensitized) by SFLLRN, the recovery of the thrombin and SFLLRN responses occurs equally rapidly (17), presumably because in this case the recycled receptors are intact and therefore capable of responding to either agonist. Regardless of whether the cells are initially desensitized by thrombin or SFLLRN, the recycled receptors are insufficient to restore full responses. Complete recovery occurs only after the cells synthesize new receptors and express them on their surfaces. Since some staining of lysosomal structures by the receptor-directed antibodies was seen even in "resting" CHRF-288 cells, there may be a much slower rate of constitutive internalization and perhaps recycling of receptors even in the absence of thrombin stimulation.

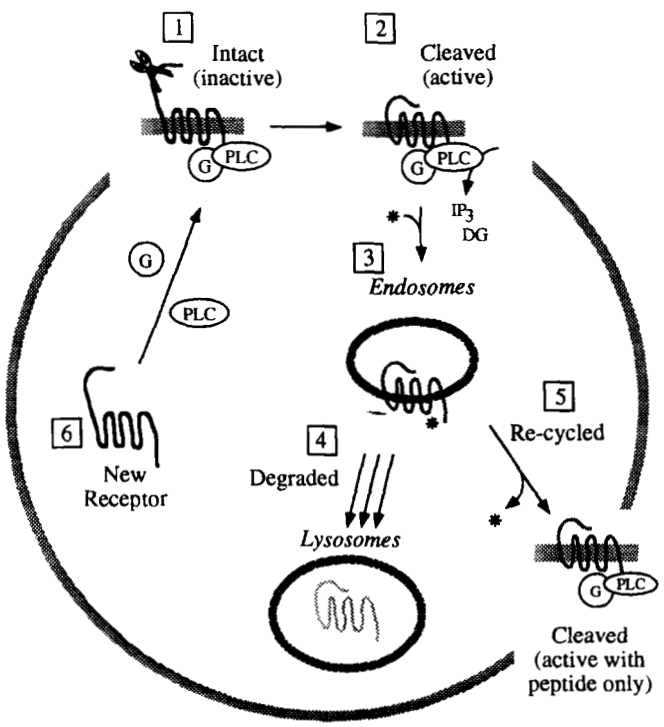

FIG. 10. **A working model for thrombin receptor internalization and recycling.** Intact thrombin receptors on the surface of responsive cells (*1*) are activated by proteolysis of the N terminus initiating G protein-mediated events such as the activation of phospholipase C_β (*2*). This is followed by receptor internalization which requires activation, but not proteolysis, of the receptor (*3*). Receptor phosphorylation (*) may occur at the same time. Once internalized, approximately 75% of the thrombin receptors on CHRF-288 cells are transferred to lysosomes and degraded (*4*), while approximately 25% are recycled to the cell surface, possibly after dephosphorylation (*5*). The recycled, thrombin-activated receptors are unable to respond to thrombin a second time but retain their ability to be activated by exogenous peptides containing the critical SFLLRN sequence. Responsiveness to thrombin is restored by the synthesis of new receptors (*6*).

Although not previously demonstrated for thrombin receptors, agonist-induced internalization has been studied with several other members of the G protein-coupled family of receptors, including β-adrenergic (18), muscarinic cholinergic (31), and LH receptors (29). For β-adrenergic receptors, loss of receptor function is generally placed in the context of three overlapping events: desensitization (loss of receptor-mediated responses), sequestration (loss of ligand binding sites from the cell surface), and down-regulation (loss of binding sites for membrane-permeable ligands). Although much of the data on β-adrenergic receptor internalization has been obtained by comparing the binding of membrane-permeable and membrane-impermeable agonists, von Zastrow and Kobilka (19) have recently used receptor-directed antibodies to track the movement of β-adrenergic receptors stably transfected into human embryonic kidney 293 cells. Their results show that β-adrenergic receptors internalize within 5–10 min of agonist addition and return to the cell surface within 30 min of agonist removal. In between, the receptors passed through an intracellular compartment that, as observed in the present studies, also contained transferrin receptors.

Although these results with β-adrenergic receptors are in some ways similar to those obtained with thrombin receptors, further comparisons point out several striking differences even among the relatively small number of G protein-coupled receptors that have been examined. These include differences in the rate and extent of internalization, as well as in the ultimate fate of the internalized receptors, all of which may reflect differences in the role of internalization and recycling as regulators of receptor function. For example, although the numbers vary, typically only 25–50% of activated β-adrenergic receptors (32, 33), muscarinic receptors (31), and LH receptors (29) are internalized. In contrast, we found that essentially all of the activated thrombin receptors on CHRF-288 and HEL cells are internalized. Second, of the β-adrenergic receptors that are internalized, nearly all are recycled unless the agonist remains present for longer than 60 min, in which case an increasing fraction of the receptors are degraded (33). By comparison, we found that the majority of internalized thrombin receptors are degraded, as are internalized LH receptors (29). Third, although recycled β-adrenergic receptors are responsive to their natural agonists, recycled thrombin receptors are unresponsive to thrombin, even though their ability to be reactivated SFLLRN shows that they are otherwise intact. Finally, differences may exist in the route by which G protein-coupled receptors are internalized; thrombin receptors (the present studies) and LH receptors (29) are

internalized through coated pits, whereas β-adrenergic receptors have been reported to be internalized through non-coated pits (30). Whether this particular difference is entirely receptor-specific, as opposed to cell-specific, remains to be determined, but as is discussed below, it has implications for the mechanism of internalization.

Additional Issues—The proposed model for thrombin receptor internalization and recycling shown in Fig. 10 raises a number of issues for future investigation. First, what are the signals that trigger receptor internalization? Presumably not proteolysis of the N terminus since receptors activated by SFLLRN are internalized as readily as those activated by thrombin. Receptor phosphorylation may be one such trigger, but at the moment the evidence that activated thrombin receptors become phosphorylated is still indirect. A related issue is whether thrombin receptor desensitization is due solely to receptor internalization or, as has been shown for β-adrenergic receptors, desensitization can occur even when internalization is blocked by mutating the receptor or inhibiting endocytosis (32–35). Interestingly, in the study by Yu *et al.* (33), blockade of internalization prevented *resensitization* of the β-adrenergic receptors, implying that processing of the internalized receptors, possibly by phosphatases, might be required for recovery of function. Our data with thrombin receptors are consistent this interpretation.

Second, what role does the structure of the thrombin receptor play in the mechanism of internalization? Based upon analogies with other proteins, the clustering of activated thrombin receptors in clathrin-coated pits could be directed by specific sites within the cytoplasmic domains of the receptor. For a number of other receptors these internalization motifs are characterized by an aromatic amino acid, usually a tyrosine, presented in the context of a tight turn (27). The existence of signal sequences within the cytosolic domains of G protein-coupled receptors has not been clearly established, but tyrosine residues have been implicated in the internalization of β_2-adrenergic receptors (36) and several such residues are present in the tail of the thrombin receptor. Internalization may also require the activation-dependent association of one or more accessory molecules with the thrombin receptor, such as the adaptor proteins that help to form the clathrin cage when mannose 6-phosphate/insulin-like growth factor II receptors and asialoglycoprotein receptors are internalized (27, 37, 38).

A third issue is the biological role of the recycled thrombin receptors, particularly since they do not respond to thrombin. Might peptides generated at the site of vascular injury reactivate these receptors leading to long term effects on the cell? Finally, why do recycled receptors not self-activate? Their ability to respond to SFLLRN suggests that they are coupled to G proteins. Why does the tethered ligand not reactivate the receptor? One possibility is that the N terminus of the receptor has undergone additional proteolytic processing. Proteolysis by plasma peptidases has been proposed (39), but the slow rate of that process relative to the short time that activated thrombin receptors remain on the cell surface makes intracellular processing more likely. Studies with peptides mimicking the tethered ligand domain suggest that the removal of the N-terminal serine residue would be sufficient to render it inactive, but proof that this occurs awaits the physical isolation and sequencing of the recycled receptors. Since antibody ATAP2 recognizes an epitope within the first 8 residues of the neo-N terminus, it is unlikely that a substantial portion of this region can have been removed. An alternative possibility is that thrombin has to be present to promote the interaction between the tethered ligand and remainder of the receptor immediately after receptor cleavage.

In conclusion, the studies presented in this manuscript show that activated thrombin receptors are removed from the surface of HEL and CHRF-288 cells with remarkable rapidity and largely, but not entirely, destroyed. This process of internalization and recycling is at least partly responsible for the loss and recovery of thrombin receptor function observed when thrombin receptors are activated. Whether internalization is solely responsible for desensitization and whether internalization also occurs on other thrombin-responsive cells is a subject of considerable interest, as is the role played by these processes in the biology of other vascular cells.

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REFERENCES

1. Vu, T.-K. H., Hung, D. T., Wheaton, V. I., and Coughlin, S. R. (1991) *Cell* **64,** 1057–1068
2. Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pagès, G., Pavirani, A., Lecocq, J.-P., Pouysségur, J., and Van Obberghen-Schilling, E. (1991) *FEBS Lett.* **288,** 123–128
3. Huang, R., Sorisky, A., Church, W. R., Simons, E. R., and Rittenhouse, S. E. (1991) *J. Biol. Chem.* **266,** 18435–18438
4. Vassallo, R. R., Jr., Kieber-Emmons, T., Cichowski, K., and Brass, L. F. (1992) *J. Biol. Chem.* **267,** 6081–6085
5. Hui, K. Y., Jakubowski, J. A., Wyss, V. L., and Angleton, E. L. (1992) *Biochem. Biophys. Res. Commun.* **184,** 790–796
6. Brass, L. F., and Woolkalis, M. J. (1992) *Biochem. J.* **281,** 73–80
7. Chao, B. H., Kalkunte, S., Maraganore, J. M., and Stone, S. R. (1992) *Biochemistry* **31,** 6175–6178
8. Seiler, S. M., Michel, I. M., and Fenton, J. W., II (1992) *Biochem. Biophys. Res. Commun.* **182,** 1296–1302
9. Sabo, T., Gurwitz, D., Motola, L., Brodt, P., Barak, R., and Elhanaty, E. (1992) *Biochem. Biophys. Res. Commun.* **188,** 604–610
10. Brass, L. F., Vassallo, R. R., Jr., Belmonte, E., Ahuja, M., Cichowski, K., and Hoxie, J. A. (1992) *J. Biol. Chem.* **267,** 13795–13798
11. Hung, D. T., Vu, T.-K. H., Wheaton, V. I., Ishii, K., and Coughlin, S. R. (1992) *J. Clin. Invest.* **89,** 1350–1353
12. Huang, E. M., and Detwiler, T. C. (1987) *Biochem. J.* **242,** 11–18
13. Paris, S., Magnaldo, I., and Pouysségur, J. (1988) *J. Biol. Chem.* **263,** 11250–11256
14. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G., and Watanabe, K. (1987) *J. Biol. Chem.* **262,** 8557–8565
15. Jones, L. G., McDonough, P. M., and Brown, J. H. (1989) *Mol. Pharmacol.* **36,** 142–149
16. Brass, L. F., Manning, D. R., Williams, A., Woolkalis, M. J., and Poncz, M. (1991) *J. Biol. Chem.* **266,** 958–965
17. Brass, L. F. (1992) *J. Biol. Chem.* **267,** 6044–6050
18. Perkins, J. P., Hausdorff, W. P., and Lefkowitz, R. J. (1991) in *The Beta-Adrenergic Receptors* (Perkins, J. P., ed) pp. 73–124, Humana Press, Clifton, NJ
19. von Zastrow, M., and Kobilka, B. K. (1992) *J. Biol. Chem.* **267,** 3530–3538
20. Mane, S. M., Marzella, L., Bainton, D. F., Holt, V. K., Cha, Y., Hildreth, J. E. K., and August, J. T. (1989) *Arch. Biochem. Biophys.* **268,** 360–378
21. Huang, M.-M., Indik, Z., Brass, L. F., Hoxie, J. A., Schreiber, A. D., and Brugge, J. S. (1992) *J. Biol. Chem.* **267,** 5467–5473
22. Bennett, J. S., Hoxie, J. A., Leitman, S. S., Vilaire, G., and Cines, D. B. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2417–2421
23. Kohler, H., and Milstein, M. (1975) *Nature* **256,** 495–498
24. Parton, R. G., Prydz, K., Bomsel, M., Simons, K., and Griffiths, G. (1989) *J. Cell Biol.* **109,** 3259–3272
25. Fugman, D. A., Witte, D. P., Jones, C. L. A., Aronow, B. J., and Lieberman, M. A. (1990) *Blood* **75,** 1252–1261
26. Tabilio, A., Rosa, J., Testa, U., Kieffer, N., Nurden, A. T., Del Canzio, M. C., Breton-Gorius, J., and Vainchenker, W. (1984) *EMBO J.* **3,** 453–459
27. Trowbridge, I. S. (1991) *Curr. Opin. Cell Biol.* **3,** 634–641
28. Smythe, E., and Warren, G. (1991) *Eur. J. Biochem.* **202,** 689–699
29. Ghinea, N., Hai, M. T. V., Groyer-Picard, M.-T., Houllier, A., Schoevaert, D., and Milgrom, E. (1992) *J. Cell Biol.* **118,** 1347–1358
30. Raposo, G., Dunia, I., Delavier-Klutchko, C., Kaveri, S., Strosberg, A. D., and Benedetti, E. L. (1989) *Eur. J. Cell Biol.* **50,** 340–352
31. Carola, E., Gamalero, S. R., Genazzani, E., and Costa, E. (1990) *J. Pharmacol. Exp. Ther.* **253,** 257–265
32. Kassis, S., Ólasmaa, M., Sullivan, M., and Fishman, P. H. (1986) *J. Biol. Chem.* **261,** 12233–12237
33. Yu, S. S., Lefkowitz, R. J., and Hausdorff, W. P. (1993) *J. Biol. Chem.* **268,** 337–341
34. Waldo, G. L., Northup, J. K., Perkins, J. P., and Harden, T. K. (1983) *J. Biol. Chem.* **258,** 13900–13908
35. Feldman, R. D., McArdle, W., and Lai, C. (1986) *Mol. Pharmacol.* **30,** 459–462
36. Valiquette, M., Bonin, H., Hnatowich, M., Caron, M. G., Lefkowitz, R. J., and Bouvier, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 5089–5093
37. Glickman, J. N., Conibear, E., and Pearse, B. M. F. (1989) *EMBO J.* **8,** 1041–1047
38. Beltzer, J. P., and Spiess, M. (1991) *EMBO J.* **10,** 3735–3742
39. Coller, B. S., Ward, P., Ceruso, M., Scudder, L. E., Springer, K., Kutok, J., and Prestwich, G. D. (1992) *Biochemistry* **31,** 11713–11720