

The functional thrombin receptor is associated with the plasmalemma and a large endosomal network in cultured human umbilical vein endothelial cells

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SUMMARY

The functional thrombin receptor, normally expressed by endothelial cells and platelets, is a member of the G protein-coupled, seven membrane-spanning-domain receptor family and is thought to be responsible for most, if not all, the cell stimulatory effects of thrombin. Upon binding, thrombin cleaves the receptor's N-terminal ectodomain, unmasking a new N terminus, which by itself activates the receptor. Using antibodies to different domains of the human thrombin receptor, we have localized the receptor in cultured human umbilical vein endothelial cells by indirect immunofluorescence and immunoelectron microscopy. We found the receptor expressed on the plasmalemma of cultured endothelial cells in individual units rather than in clusters, at lower concentration than, and at different sites from, thrombomodulin. We also found the

receptor associated with a distinct, intracellular, transferin receptor-containing, tubulovesicular network. The thrombin receptor-positive structure spread from the perinuclear region to the periphery of the cells, exhibiting a number of varicosities interconnected by branching tubular elements, strikingly similar to an image recently described for a continuous endosomal reticulum. Our results provide morphological evidence for the presence of the functional thrombin receptor at relative low density on the surface of cultured endothelial cells (compared to thrombomodulin) and in relatively large quantities inside the cells, associated with an endosomal compartment.

Key words: thrombin receptor, endothelium, endosome

INTRODUCTION

Thrombin (TH) is the key enzyme in the control of coagulation vs anticoagulation reactions in the blood plasma and at the surface of the vascular endothelium (Furie and Furie, 1988; Esmon, 1993). Its circulating form initiates clot formation by cleaving fibrinogen to fibrin; yet upon binding to thrombomodulin (TM), a 74 kDa class I glycoprotein of the endothelial cell plasmalemma, TH changes conformation and proteolytic specificity, triggering a series of anticoagulant reactions that begins with the proteolytic activation of Protein C and ultimately inhibits further TH generation (Esmon, 1989). Besides its crucial role in coagulation, TH is known to activate a variety of cell types, including platelets, endothelial cells, lymphocytes, monocytes, and vascular smooth muscle cells (Shuman, 1986).

Vu et al. (1991a,b) used functional expression cloning of human mRNA, derived from a megakaryocyte-like cell line, in *Xenopus laevis* oocytes to identify, clone and sequence a new ~70 kDa thrombin receptor (TR) normally expressed by endothelial cells and platelets. They showed that TR belongs to the family of G protein-coupled receptors characterized by seven membrane-spanning domains and that TR has an N-terminal ectodomain of 100 amino acid residues that comprises a

Protein C-like TH cleavage site followed by a hirudin-like TH binding domain (Vu et al., 1991a,b; Liu et al., 1991). Upon binding, TH specifically cleaves (between R41 and S42) part of the receptor's ectodomain, thereby unmasking a new N terminus that, acting as a tethered ligand, activates the receptor by an entirely new mechanism of receptor by ligand activation (Lefkowitz, 1991; Coughlin, 1993). More recent work has identified the minimal effective signal at the new N terminus required for receptor activation (Hui et al., 1992), shown that synthetic peptides containing that signal activate TR in the absence of TH (Scarborough et al., 1992) and demonstrated that specificity of TR for agonist peptide is defined by its extracellular surface (Gerszten et al., 1994). Recently, Bahou et al. (1993) have isolated, cloned and stably expressed the identical receptor from a human umbilical vein endothelial cell (HUVEC) library.

Using antibodies to different TR domains, we have localized this receptor on the surface of human umbilical vein endothelial cells and found it expressed at lower surface density and at different sites than TM. We also found that in many endothelial cells a large fraction of TR is associated, like TM, with an intracellular, tubulovesicular network distinct from the ER and the Golgi complex, but similar to a recently described type of continuous endosomal system (Hopkins et al., 1990).

MATERIALS AND METHODS

Cells and reagents

Cells and reagents were obtained from the following sources: human umbilical vein endothelial cells (HUVEC), endothelial growth (EGM) and basal medium (EBM) from Clonetic Corporation (San Diego, CA); bovine serum albumin (BSA), affinity-purified, alkaline phosphatase-conjugated goat anti-rabbit IgG and Sigma 104 phosphatase substrate from Sigma Chemical Company (St Louis, MO); affinity-purified, fluorescein-conjugated goat anti-mouse and goat anti-rabbit F(ab)₂ fragments from Cappel Organon Teknika Corporation (Westchester, PA); Vectashield mounting medium from Vector Laboratories (Buckingham, CA).

Endothelial cell culture

HUVEC were grown to confluency in T75 tissue culture flasks or in 3.5 cm plastic Petri dishes with or without glass coverslips, using EGM containing 2% fetal bovine serum as per supplier's instructions. The cells were checked regularly for the presence of von Willebrand Factor and Weibel-Palade bodies (Jaffe et al., 1973) and were washed in EBM prior to use.

Antibodies

Antibodies were generously provided by the following investigators: (i) rabbit IgG to the hirudin-like domain of the human TR (IgG 1809) (Hung et al., 1992), by Dr S. R. Coughlin (Department of Medicine, University of California, San Francisco, CA); (ii) rabbit IgG to a recombinant fusion protein comprising 160 amino-terminal amino acids of the human TR (anti-TR¹⁻¹⁶⁰); and (iii) rabbit IgG to a synthetic peptide that represents the amino-terminal residues 34-52 encompassing the predicted TH-cleavage site of the same receptor (anti-TR³⁴⁻⁵²), by Dr B. S. Coller and Dr F. W. Bahou (Bahou et al., 1993) (Department of Medicine, University of New York at Stony Brook, NY) (In all cases IgG fractions were obtained by Protein A purification; Fig. 1 gives the positions of the peptides used as antigens on the primary structure of TR); (iv) rabbit antiserum to human TM, by Dr P. W. Majerus (Maruyama and Majerus, 1985) (Washington University, School of Medicine, St Louis, MO); (v) mouse monoclonal antibodies to the cytoplasmic tail of the human transferrin receptor, by Dr I. Trowbridge (Hopkins and Trowbridge, 1983) (Salk Institute, San Diego, CA); (vi) rabbit antiserum to resident proteins of the endoplasmic reticulum (ER), by Dr D. Louvard (Louvard et al., 1982) (Pasteur Institute, Paris, France); (vii) rabbit antiserum to α -mannosidase II, by Dr M. G. Farquhar (this lab) (Velasco et al., 1993); and (viii) rabbit IgG to von Willebrand Factor from Dako Corporation (Carpenteria, CA).

Immunofluorescence

Unless otherwise indicated, all procedures were carried out at room temperature (RT). HUVEC, grown on coverslips, were: (i) fixed for 1 hour in PBS + 2% formaldehyde freshly prepared from paraformaldehyde; (ii) washed (in PBS, 3 × 5 minutes); (iii) permeabilized (in PBS + 0.1% Triton X-100, 10 minutes); (iv) washed (in PBS, 3 × 5 minutes); (v) quenched (in PBS + 0.1% BSA = PBSA, 30 minutes); (vi) incubated for 1 hour in the first antibody (diluted 1:100 in PBSA); (vii) washed (in PBSA, 3 × 5 minutes); and (viii) incubated for 1 hour with the appropriate reporter antibody (diluted 1:100 in PBSA), followed by final washing (in PBS, 3 × 5 minutes) and mounting of the coverslips on glass slides using Vectashield mounting medium.

For double immunofluorescence, fixed, permeabilized and quenched cells were incubated with a mixture of first antibodies (anti-TR³⁴⁻⁵² and anti-transferrin receptor, both diluted 1:100 in PBSA) followed by a mixture of appropriate reporter antibodies (diluted 1:100 in PBSA).

Formaldehyde by itself induces variable degrees of cell permeabi-

lization at RT. Therefore, to investigate the distribution of TR on the cells' surface, unfixed HUVEC were chilled on ice and incubated at 4°C for 1 hour with anti-TR antibodies (IgG 1809 or anti-TR³⁴⁻⁵²) diluted 1:100 in EBM containing 0.1% BSA, followed by washing, fixation and processing as above.

Anti-TR¹⁻¹⁶⁰, which was raised against a denatured, recombinant fusion protein, gave a strong positive signal only when the cells were treated for 10 minutes at -20°C with 100% acetone prior to immunostaining.

Reacted cells were examined and photographed in a Zeiss Axiophot 20 microscope for transmitted light (phase-contrast) and incident light fluorescence using appropriate filters. Micrographs were taken at 1000× magnification using Kodak T-max 400 film.

Immunoelectron microscopy

Preembedding immunogold labeling

HUVEC monolayers, grown in plastic Petri dishes, were: (i) fixed for 6 hours at RT with a paraformaldehyde-lysine-periodate mixture (PLP) (McLean and Nakane, 1974); (ii) washed (PBS, 5 × 2 minutes); (iii) incubated for 12 hours at 4°C in rabbit anti-TR IgG (IgG 1809 or anti-TR³⁴⁻⁵² diluted 1:100 in PBSA); (iv) washed (PBSA, 4 × 15 minutes); and finally (v) incubated for 6 hours at 4°C in affinity-purified goat anti-rabbit IgG conjugated to 5 nm colloidal gold particles (diluted 1:50 in PBSA).

For simultaneous visualization of TR and TM on the surface of HUVEC, a double immunogold labeling protocol was applied, using as reporters 5 nm and 15 nm colloidal gold-Protein A complexes (Slot and Geuze, 1984). Briefly, PLP-fixed HUVEC were incubated for 12 hours with anti-TR (IgG 1809 or anti-TR³⁴⁻⁵² diluted 1:100 in PBSA) followed for 6h by 5 nm Protein A-gold (diluted 1:100 in PBSA). After washing (PBSA; 4 × 15 minutes), the cells were quenched for 20 minutes with free Protein A (250 μ g/ml) (to block residual unreacted IgG) and then incubated for 12 hours with anti-TM

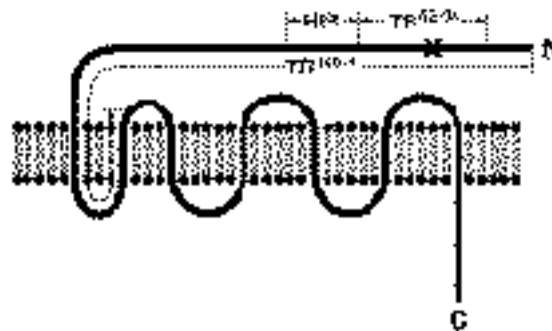


Fig. 1. Thrombin receptor model indicating parts of the sequences used as antigens. The functional TR has seven membrane-spanning domains and is a G protein-coupled receptor molecule with a unique TH cleavage site (X) within its N-terminal ectodomain. Upon binding TH cleaves the receptor, unmasking a new N terminus that acts as a tethered ligand and activates the receptor. Antibodies used in this study bind to epitopes within the sequences indicated. IgG 1809: rabbit IgG raised to a synthetic peptide representing the hirudin (HIR)-like TH binding domain of TR (Hung et al., 1992). Anti-TR³⁴⁻⁵²: rabbit IgG raised to a synthetic peptide representing amino acid residues 34-52 within the N terminus of TR encompassing the TH cleavage site X (Bahou et al., 1993). Anti-TR¹⁻¹⁶⁰: rabbit IgG raised to a recombinant fusion protein representing the N-terminal 160 amino acid residues of TR, which include the first two membrane-spanning domains (Bahou et al., 1993). As indicated all antibodies recognize epitopes of TR C-terminal to the TH cleavage site and therefore can not distinguish intact (uncleaved) from activated (cleaved) receptors.

followed for 6 hours by 15 nm Protein A-gold as above. In control experiments, the first antibodies (anti-TR and anti-TM) were either omitted or applied in a reversed sequence.

To test for antibody-induced TR internalization, fresh unfixed HUVEC were chilled on ice, incubated for 1 hour at 4°C in anti-TR (IgG 1809 or anti-TR³⁴⁻⁵² diluted 1:100 in PBSA) followed by washing (PBSA, 4 × 15 minutes) and incubation for 1 hour at 4°C in a reporter antibody, i.e. goat anti-rabbit IgG conjugated to 5 nm gold (diluted 1:50 in PBSA). After final washing (PBSA; 4 × 15 minutes) the cells were warmed up to 37°C for 10 minutes followed by processing for electron microscopy as described below.

After final incubation and washing steps, antigen-antibody-Protein A-gold complexes were stabilized by fixation in 1.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer, pH 7.4, containing 5% sucrose; postfixed in acetate-veronal-buffered 1% OsO₄, pH 7.4; stained en bloc with 0.5% uranyl acetate in the same buffer, dehydrated in a graded ethanol series and embedded in Epon.

Cryoultramicrotomy and immunogold labeling

Cells grown in a T75 flask were fixed in PLP for 6 hours at RT, scraped from the bottom of the flask, pelleted in a microfuge, cryoprotected by infiltration with 2.3 M sucrose in PBS containing 20% polyvinylpyrrolidone and then mounted on aluminum nails and frozen in liquid N₂ (Tokuyasu, 1989). Thin cryosections prepared from these pellets were collected on carbon/Formvar-coated nickel grids and incubated for 12 hours at 4°C in anti-TR³⁴⁻⁵² (diluted 1:100 in PBSA); followed for 2 hours by goat anti-rabbit IgG 5 nm gold conjugates (diluted 1:50 in PBSA). Grids were stained in 2% neutral uranyl acetate (10 minutes) in 0.2% methylcellulose, and 2% Carbowax (Tokuyasu, 1986).

The final preparations were examined and micrographed using a Philips CM 10 electron microscope.

Morphometry

To determine the labeling density for TR and TM, the number of Protein A-gold particles bound to the apical (luminal) plasmalemma was counted, and normalized per 1 μm membrane profile. The total sample used for counting amounted to 841 μm of endothelial plasmalemma, collected from 126 micrographs. Conversion to density per 1 μm² surface was calculated by assuming a thin-section thickness of 60 nm average.

Enzyme-linked immunosorbent assay (ELISA) on intact HUVEC

To quantitate bound anti-TR antibodies on intact vs permeabilized cells, HUVEC were split into 96-well plates at a seeding density of 5000 cells/well.

For cell surface binding, confluent HUVEC were chilled on ice and incubated for 1 hour at 4°C with anti-TR³⁴⁻⁵² or anti-TR¹⁻¹⁶⁰ diluted in EGM (ranging from 1:100 to 1:20 000) followed by washing (3 × 10 minutes in cold EGM) and fixation (2% formaldehyde, freshly prepared from paraformaldehyde, in PBS for 1 hour) and then processed through steps (iv) and (v) followed by steps (viii) to (x) as indicated below. For antigen present inside HUVEC, the cells were: (i) fixed as above; (ii) washed (PBS, 3 × 5 minutes); (iii) permeabilized for 10 minutes in PBS containing 0.1% Triton X-100; (iv) washed (PBS, 3 × 5 minutes); (v) quenched (PBSA, 30 minutes); (vi) incubated with the first antibodies as above; (vii) washed (PBSA, 3 × 5 minutes); and then (viii) incubated for 1 hour in affinity-purified alkaline phosphatase-conjugated goat anti-rabbit reporter antibody (diluted 1:20 000 in PBSA); followed by (ix) final washing (PBS; 3 × 5 minutes); and (x) incubation for 3 hours at 37°C in 100 μl/well of alkaline phosphatase substrate solution (1 mg/ml *p*-nitrophenylphosphate in 0.1 M diethanolamine buffer, pH 9.8). The relative intensity of the color reaction was quantified, using a model 750 microplate reader (Cambridge Technology, Inc., Watertown, MA).

Control experiments

As controls for immunofluorescence, immunogold, and ELISA experiments the first antibodies were either omitted or replaced by an irrelevant rabbit IgG.

RESULTS

Localization of TR by indirect immunofluorescence

To obtain information on the localization and distribution of TR limited to the plasmalemma and unaltered by fixation, fresh unfixed HUVEC were incubated at 4°C with anti-TR antibodies followed by a reporter antibody and then processed for microscopy as given in Materials and Methods. This procedure revealed a diffuse, low intensity staining (Fig. 2A), indicating that TR is evenly distributed on the cell surface without detectable clustering, as was shown to be the case for TM under identical conditions (Fig. 2B). When cells were fixed and permeabilized before immunostaining, anti-TR³⁴⁻⁵² revealed the presence of a strongly reactive, distinct, intracellular, tubulovesicular network that spread from the perinuclear region to the periphery of the cells (Fig. 3A) and was present in ~90% of the cells. A similar surface and intracellular staining pattern was observed with the antibody designated as IgG 1809, raised against a synthetic peptide from the hirudin-like domain of TR. At high magnification, varicosities interconnected by branching tubules and cisternae were found along the network, giving an image strikingly similar to that described by Hopkins and coworkers for a continuous type of endosomal reticulum (Hopkins et al., 1990). A generally similar, strong staining pattern was obtained with anti-TR¹⁻¹⁶⁰ only when the cells were treated with acetone prior to antibody incubation (Fig. 4). This could be explained by the fact that anti-TR¹⁻¹⁶⁰ was raised against a denatured, recombinant fusion protein, electroeluted from SDS-gels (Bahou et al., 1993). It also could mean that the antibody recognizes epitopes within the first two membrane-spanning domains of the receptor (see Fig. 1) that become accessible upon acetone treatment, resulting in a significantly enhanced fluorescence signal. As already described for anti-TR³⁴⁻⁵², anti-TR¹⁻¹⁶⁰ gave only relatively sparse labeling of the plasmalemma.

To identify the intracellular, TR-positive structure, we have carried out immunofluorescence tests with antibodies to marker proteins of different subcellular compartments. Polyclonal antibodies to ER proteins revealed a reticular staining pattern (Louvard et al., 1982), finer and more widely spread throughout the cytoplasm of the cells than the TR-positive network (Fig. 5A). Anti-α-mannosidase II, an antibody to a generally accepted Golgi membrane antigen (Velasco et al., 1993), gave a typical Golgi signal in the form of a cluster of densely packed vacuoles or cisternae, capping one pole of the nucleus (Fig. 5B). In contrast, double immunofluorescence experiments using rabbit anti-TR antibodies and mouse monoclonal antibodies to the transferrin receptor (a well documented endosomal marker) (Hopkins and Trowbridge, 1983; Hopkins et al., 1990) revealed extensive colocalization of both molecules in the distinct, intracellular, tubulovesicular network described above (Fig. 3A,B).

Localization of TR by immunogold labeling

When fixed HUVEC monolayers were incubated with anti-TR followed by a colloidal gold-conjugated reporter antibody, a small number of gold particles was found associated with the apical (luminal) (Fig. 6A) as well as with the basal (abluminal) (not shown) plasmalemma. Gold particles were found mostly isolated or, rarely, in small groups of two or three. No significant association of gold particles with the introversions of coated pits or plasmalemmal vesicles was observed.

When unfixed HUVEC were incubated with anti-TR gold at 4°C followed by warming up to 37°C gold particles were found in non-clathrin-coated (not shown) but, rarely, also in clathrin-coated, pits and vesicles and in multivesicular body type endosomes. (Fig. 6B,C,D). These findings indicate that TR can be internalized from the cell surface to endosomes even in the absence of its physiological ligand, TH.

On thin frozen sections of PLP-fixed cell pellets, processed

for immunogold labeling with anti-TR, only a few gold particles were found associated with the outer aspect of the plasma membrane. The majority of gold labeling was found associated with non-coated vesicles and endosomes within the cytoplasm (Fig. 7A,B), most likely representing the fine-structural equivalent of the tubulovesicular system visualized by immunofluorescence. There was no labeling of the ER or the Golgi complex.

Colocalization and quantitation of TR and TM by double immunogold labeling

To compare the localization, distribution and surface density of TR and TM on the surface of HUVEC we have used simultaneous labeling with Protein A conjugated to gold particles of different dimensions as reporters. Anti-TR gold particles were found at distant sites, different from those of particles marking TM clusters (Fig. 8). The surface density of the two

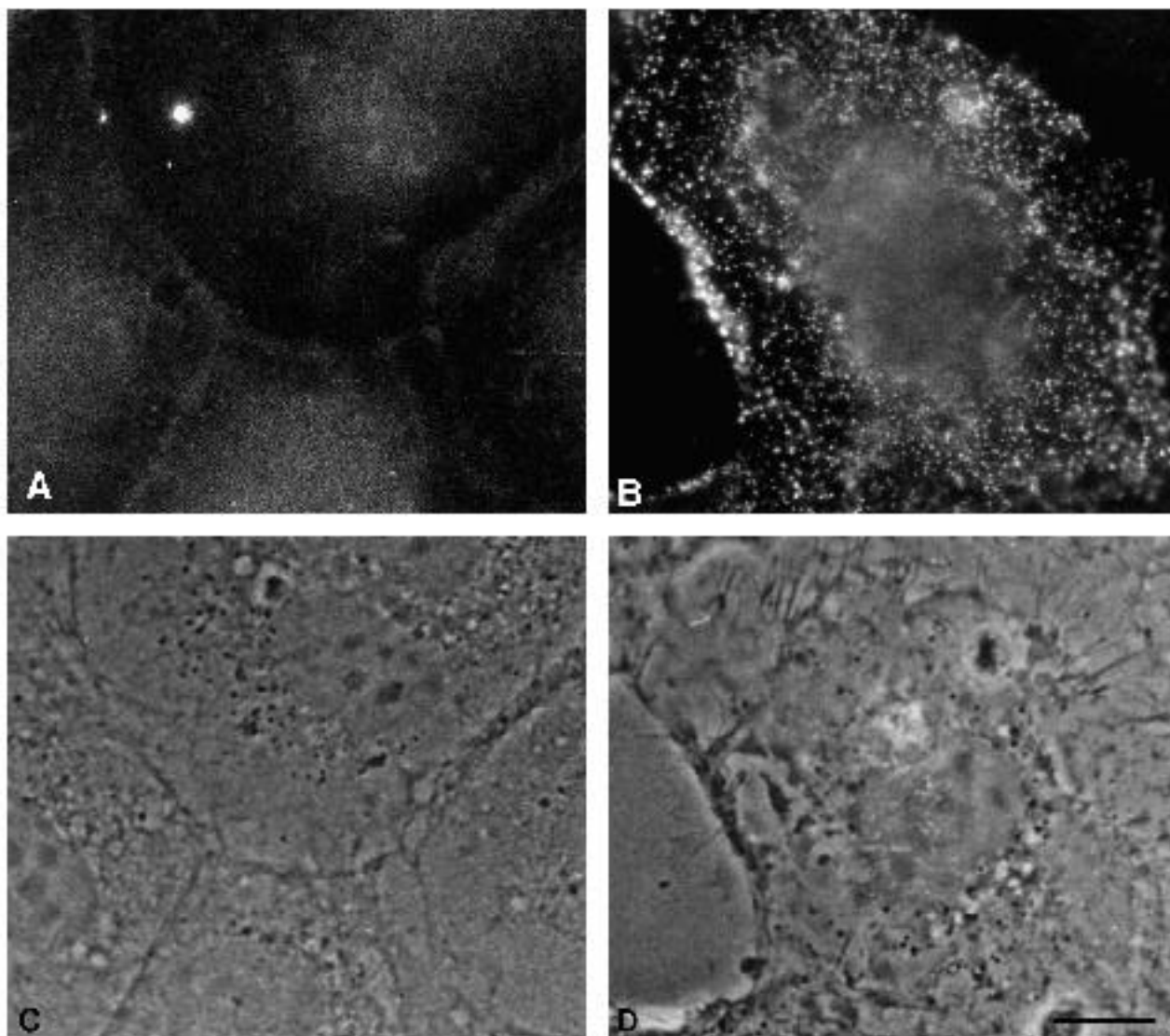


Fig. 2. Localization of TR (A) and TM (B) on the surface of HUVEC by indirect immunofluorescence. Fresh, unfixed cells were incubated with IgG 1809 or anti-TR³⁴⁻⁵² (A), or anti-TM (B), followed by a rhodamine-conjugated reporter antibody. TR is detected as a weak, diffuse signal (A) compared to TM, which is found in a distinct, strong, punctate pattern (B). Note that in A the signal is stronger at the center of the cells than at the periphery, most likely caused by the shape of unpermeabilized cells. (C and D) Corresponding phase-contrast images. Bar, 10 μ m.

reporters was assessed by counting Protein-A gold particles, measuring the length of the relevant plasmalemmal profiles and normalizing particle counts to $10 \mu\text{m}^2$ of plasmalemma calculated from a defined sample as given in Materials and

Methods. An average of 14 reporter gold particles for anti-TM were found per $10 \mu\text{m}^2$ plasmalemma compared to ~ 2 reporter gold particles for anti-TR for the same cell surface area. These numbers, which are most probably underestimates of the real surface density of receptor molecules, suggest that a relatively high concentration of TM is expressed on the surface of HUVEC compared to a relatively low concentration of TR.

Quantitation of TR on intact HUVEC by ELISA

To quantitate the relative TR concentration on the surface versus the inside of HUVEC, we have carried out ELISAs with anti-TR antibodies on intact versus permeabilized cells, as given in Materials and Methods. The results obtained show that only a relatively small number of TR molecules are expressed on the cell surface compared to a more than 10-fold larger number present within the cells (Fig. 9). This large pool of intracellular TR is most likely associated with the endosomal compartment described above.

Control experiments

Control experiments for light- and electron-microscopic immunocytochemistry as well as ELISA, carried out as described in Materials and Methods, were negative.

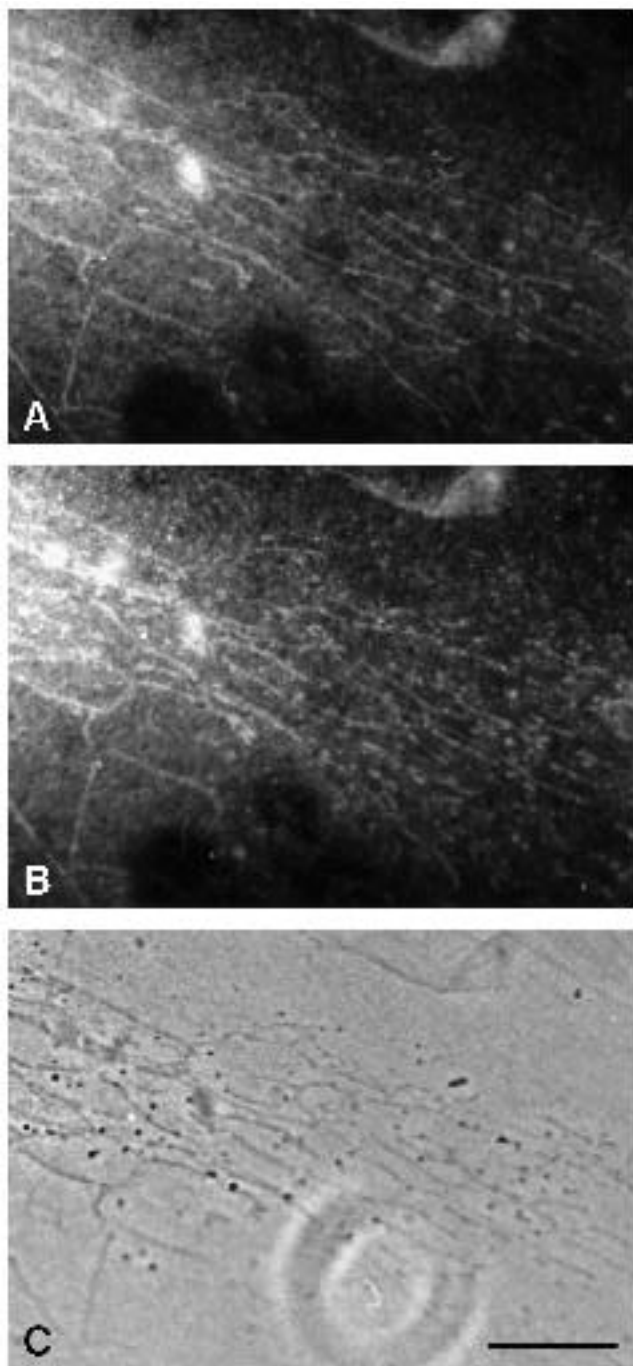


Fig. 3. Colocalization of TR (A) and transferrin receptor (B) in HUVEC by double immunofluorescence. Fixed and permeabilized HUVEC were incubated with anti-TR³⁴⁻⁵² and anti-transferrin receptor antibodies followed by a mixture of appropriate reporter antibodies (conjugated to different fluorophores). Micrographs A (TR) and B (transferrin receptor) demonstrate extensive colocalization of both molecules within a distinct, intracellular tubular network, which is also seen in the corresponding phase-contrast image C. Bar, $10 \mu\text{m}$.

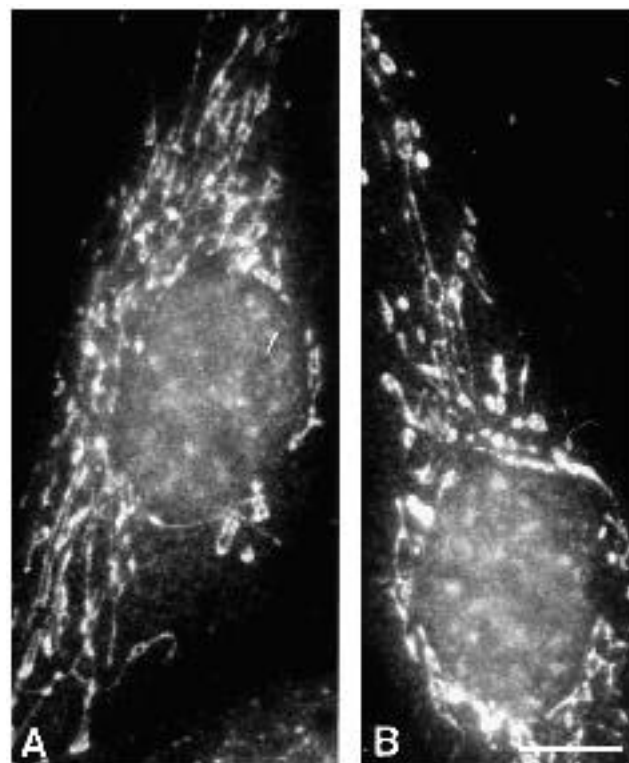


Fig. 4. Localization of TR in fixed and permeabilized HUVEC. Fixed cells were treated with acetone as described in Materials and Methods, prior to incubation with anti-TR¹⁻¹⁶⁰ followed by a rhodamine-conjugated second antibody. TR is found at apparently high concentration within the cells, associated with a continuous, tubulovesicular network which spreads from the perinuclear region to the periphery of the cells. Micrographs A and B demonstrate morphological variants of the described network in different cells. Bar, $10 \mu\text{m}$.

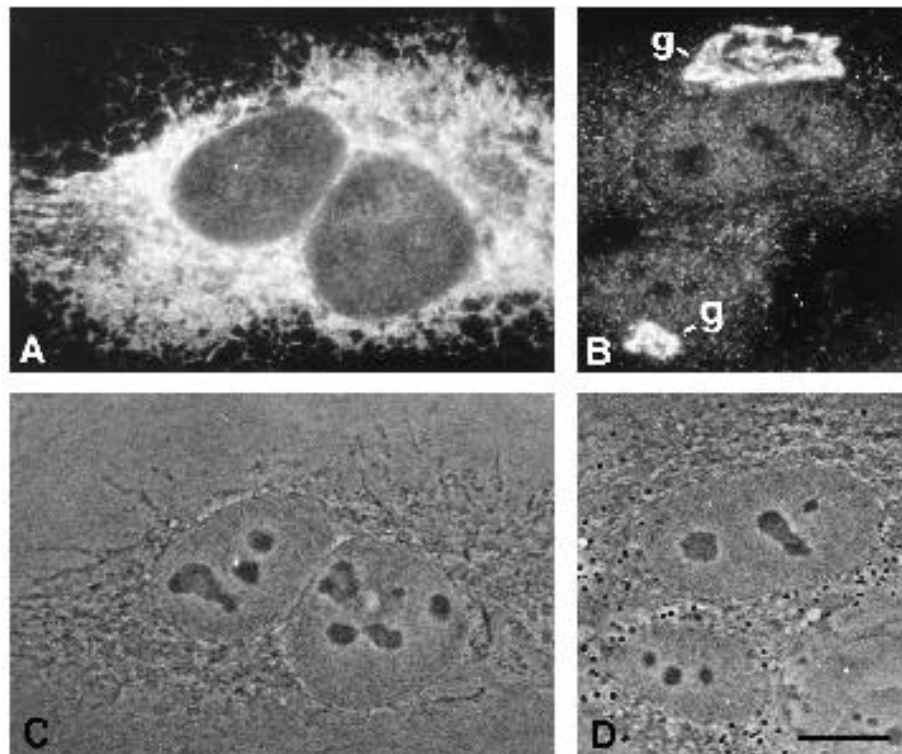


Fig. 5. Visualization of the endoplasmic reticulum (A) and the Golgi complex (B) by indirect immunofluorescence in HUVEC. Fixed, permeabilized cells were incubated with antibodies to resident proteins of the endoplasmic reticulum (A) and to α -mannosidase II, a marker protein of the Golgi complex (g, in B). The antibodies exhibit strong and specific staining of the respective cellular compartments, which are both clearly different from the prominent, TR-containing, tubulo-vesicular network demonstrated in Figs 2 and 3. (C and D) Corresponding phase-contrast images. Bar, 10 μ m.

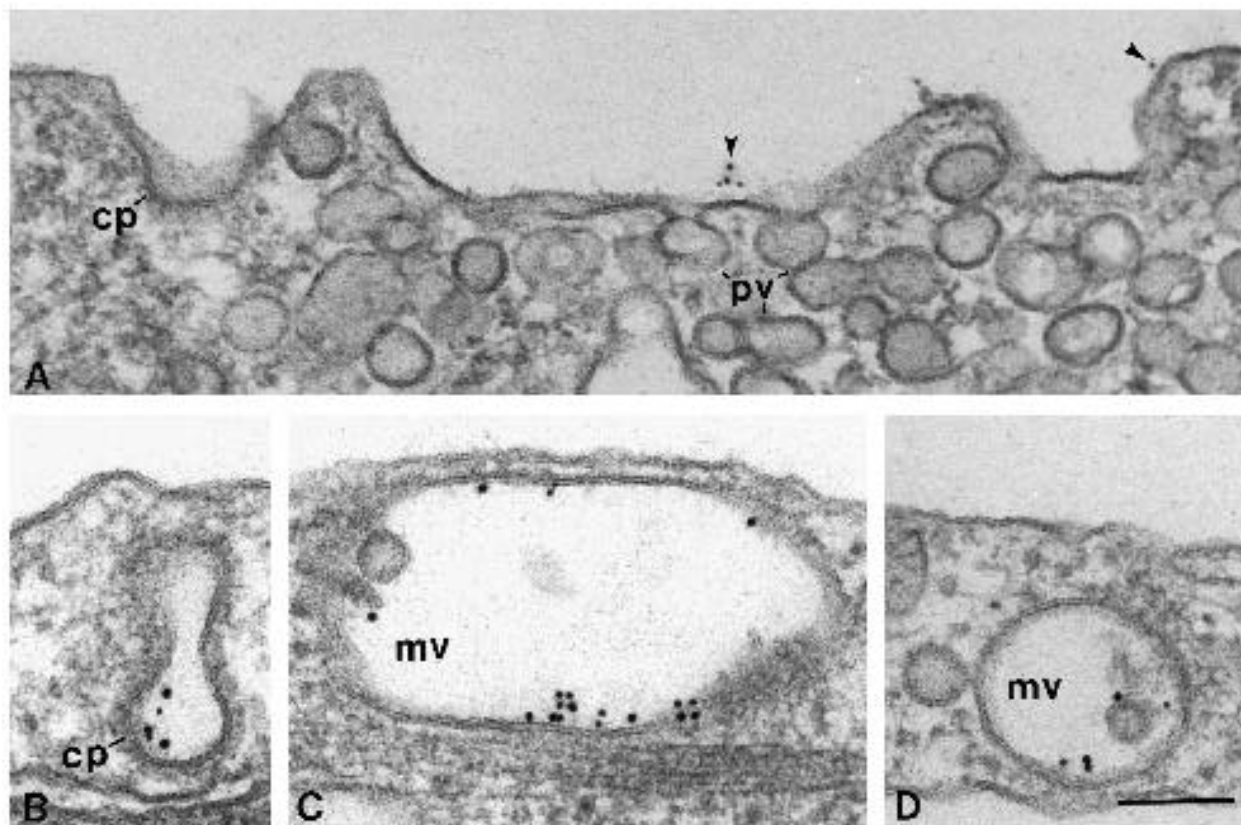


Fig. 6. Localization of TR by preembedding immunogold labeling in HUVEC. Fixed cells were incubated with IgG 1809 or anti-TR³⁴⁻⁵² followed by 5 nm colloidal gold/goat anti-rabbit IgG. Only a small number of gold particles were found on the plasmalemma (arrowheads in A) and no association with plasmalemmal vesicles (pv) (or their introits) and coated pits (cp) was observed. When fresh, unfixed cells were incubated at 4°C for 1 hour followed by warming up to 37°C for 15 minutes, gold particles were found internalized via coated pits (cp) and vesicles (B); and by plasmalemmal vesicles (not shown) to endosomes of the multivesicular body (mv) type (C and D). Bar, 0.1 μ m.

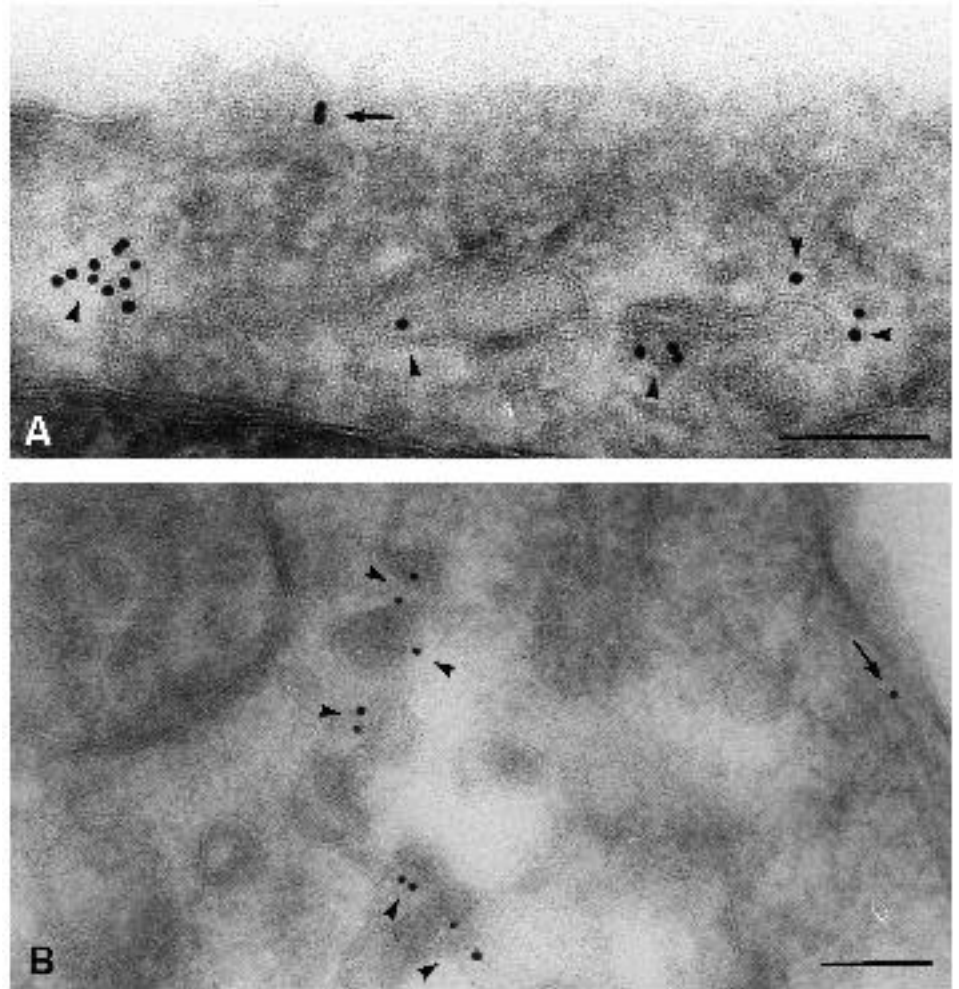


Fig. 7. Localization of TR by cryo-immunogold labeling of HUVEC. PLP-fixed cell pellets were processed for cryoultramicrotomy and immunogold labeling as described in Materials and Methods. Most gold particles were found inside the cells, associated with small, endosome-like vesicles (arrowheads) correlating with the distinct tubular structure seen by immunofluorescence in Figs 2 and 3. The cluster of gold particles seen against a light background in A is within vesicles, whose limiting membranes can be seen after longer photographic exposure. Few gold particles were associated with the plasmalemma (arrows). Bars, 0.1 μm.

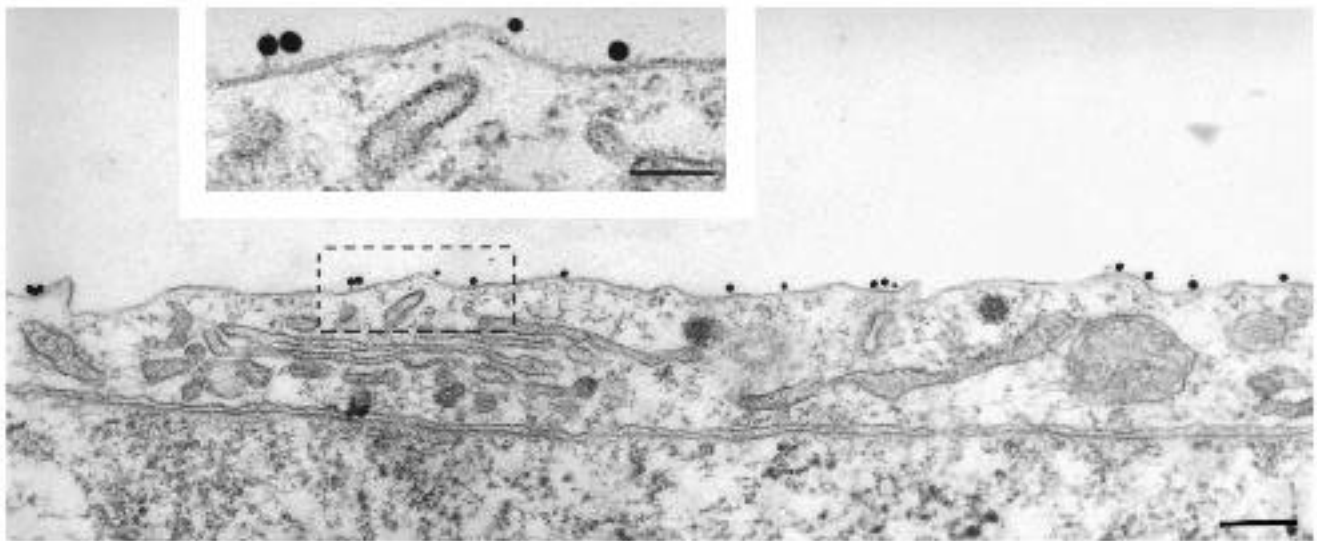


Fig. 8. Colocalization of TR and TM on the surface of HUVEC by double immunogold labeling. Cells were fixed and processed for double immunogold labeling as described in Materials and Methods. The 5-nm anti-TR gold particles were found in relatively small numbers, of about 2 particles per 10 μm² plasmalemma, compared to an average of 14 15-nm anti-TM-gold particles per 10 μm² cell surface area, at different sites from TM-gold. The enlarged inset clearly demonstrates cell surface association of different-sized gold particles which are separated from the plasmalemma by a small distance, representing the combined size of an antibody molecule and Protein A. One can also recognize the separate distribution of anti-TR (small, isolated particle in the middle of the inset) from the large anti-TM particles. Bars: 0.1 μm; 0.05 μm (inset).

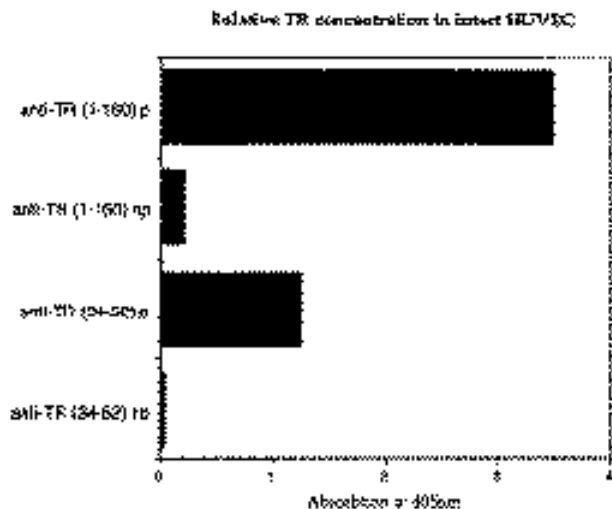


Fig. 9. Quantitation of relative TR concentration in intact HUVEC by ELISA. ELISAs on intact cells were performed as described in Materials and Methods using anti-TR³⁴⁻⁵² (raised against the corresponding synthetic peptide) and anti-TR¹⁻¹⁶⁰ (raised against a recombinant fusion protein). The figure illustrates results, that are representative of three consecutive ELISA experiments. The horizontal columns represent mean values of anti-TR antibody binding to intact HUVEC at a range of dilutions where the response was linear (antibody concentrations of 1:100 to 1:800 for both antibodies used). Both antibodies detect only a small amount of TR on the surface of fresh, non-permeabilized cells (np). When cells were permeabilized (p) before application of the antibodies, a consistently more than 10-fold higher intracellular concentration of TR was found. Since, for ELISA experiments cells were not treated with acetone prior to antibody incubation, results obtained with anti-TR¹⁻¹⁶⁰ relied on the relatively weak binding of that antibody to non-denatured cells. We assume, that the relative high concentration of TR inside the cells reflects the strong signal from the intracellular network, obtained by immunocytochemistry at the light- and electron-microscope levels.

DISCUSSION

We have studied the distribution of TR on cultured human endothelial cells (HUVEC) and compared it with that of TM on the same specimens. To this intent we have used a number of immunocytochemical procedures and, in the case of TR, a number of different antibodies that recognize epitopes in the N-terminal part of the molecule between residues 1 and 160.

The salient findings are: as indicated by the results of the immunogold procedures, TR is distributed as single molecules or small clusters of molecules on the endothelial plasmalemma, whereas TM is present in relatively larger, separate clusters (see also Horvat and Palade, 1993). If we assume comparable binding affinities of antibodies to their antigens, the surface density of TR on the endothelial plasmalemma appears to be seven-fold less than that of TM. A large fraction of the total cellular TR antigen is found inside the cells; it is not present within exocytic compartments (ER or Golgi complex) but appears to be associated with a large polymorphic, continuous, transferrin receptor-containing endosomal network similar to that recently described by Hopkins et al. (1990). By ELISA on intact vs permeabilized cells, this intracellular endosomal pool

is at least 10 times larger than the surface pool. The general pattern of intracellular distribution of TR is similar to that of TM (Horvat and Palade, 1993).

Previous studies, using various immunocytochemical procedures and in situ hybridization protocols, have localized TR in human megakaryoblastic cells (Hoxie et al., 1993), in transfected rat fibroblasts (Ishii et al., 1993), in atheromatous plaques in which it appears to be highly concentrated in smooth muscle cells (Nelken et al., 1992) and in the mouse embryo during development, where its expression became restricted with time of maturation to certain neurons, the endothelium and the liver (Soifer et al., 1994). According to more recent evidence TR is also present in peripheral blood lymphocytes (Howells et al., 1993) and in human T lymphoblastoid cells (Tordai et al., 1993). As far as we know, our study is the first to be reported on TR localization and distribution at high resolution in endothelial cells.

As already mentioned in the Introduction, TH is the controlling enzyme in coagulation/anticoagulation reactions. In addition, it promotes platelet aggregation (Davey and Luscher, 1967) and 'activates' a variety of cell types, including endothelial cells (Shuman, 1986). For instance, TH stimulates the production of prostacyclin (Weckler et al., 1978), platelet activating factor (Prescott et al., 1984), plasminogen activator-inhibitor (Gelehrter and Sznyer-Laszyk, 1986) and platelet-derived growth factor (Daniel et al., 1986) by cultured endothelial cells. It also stimulates neutrophil adhesion to endothelia (Zimmerman et al., 1986), release of von Willebrand Factor and concurrent relocation of GMP 140 (P selectin) to the endothelial cell surface (Hattori et al., 1989). TH as well as the activating peptide were reported to stimulate E-selectin expression in HUVEC (Shankar et al., 1994) and to induce early events in the activation of T cells (Mari et al., 1994). It has been speculated that the common function of all these diverse activities is wound hemostasis followed by wound repair, which requires inflammatory reactions for debris clearing and cell proliferation for healing (Esmon, 1993).

Available evidence indicates that TM is the TH receptor involved in hemostasis and control of coagulation by anticoagulation reactions. This leaves TR as the main candidate for mediator of the other TH effects, such as the activation processes mentioned above and the mitogenic response to TH in a number of cell types including vascular smooth muscle cells (McNamara et al., 1993).

Our salient finding is the presence of a large TR pool located primarily in a continuous endosomal network in cultured endothelial cells (HUVEC). By contrast, there is no detectable colocalization of TR with molecules of the ER or the Golgi complex, indicating that the local TR concentration is below the level of detection by immunofluorescence. The large size of the intracellular TR pool raises the question of its possible functional significance. It could house a mobilizable intracellular reserve of TR to be brought to the cell surface by excess TH or other factors, or it could represent receptors removed from the cell surface to bring to an end their signal transduction activities.

At present, there are conflicting interpretations on the sub-cellular localization and distribution of TR, which may reflect functional differences among different cell types. In human platelets, for instance, TH-cleaved TR apparently remains at the cell surface with the new N terminus inserted in a 'protec-

tive environment', inaccessible to relevant antibodies (Norton et al., 1993). In contrast, receptor activated by TH cleavage or agonist peptide is rapidly internalized in cells of megakaryoblastic lines; most of it is degraded in lysosomes (Hoxie et al., 1993), but a fraction recycles to the cell surface, together with stored, uncleaved receptors (Brass et al., 1994). Finally, in transfected rat 1 fibroblasts, cleaved TR is internalized and degraded in lysosomes while uncleaved TR, stored apparently in the Golgi complex, can be recruited to the cell surface (Hein et al., 1994).

Work by Ishii et al. (1994) has established that upon TH binding TR is phosphorylated by an associated G protein-coupled receptor kinase and that experimental cotransfection of *Xenopus laevis* oocytes with TR and β -adrenergic receptor kinase 2 (but not other kinases) leads to phosphorylation of TR and desensitization or shut-off of its signaling activities.

These results suggest that internalization and recycling may play an important role in TR de- and resensitization in endothelial cells to permit fast recovery of TH responsiveness compared to the long time required for cell surface expression of newly synthesized TR. We assume that the high concentration of intracellular, endosomal TR in cultured endothelial cells, in the absence of TH, most likely represents stored, uncleaved receptors. Cleaved TR may be added to this pool upon the cells' exposure to TH. Since the antibodies used in our study can not distinguish cleaved (activated) from uncleaved (not yet activated) receptors, we were not able to check these assumptions. When appropriate reagents become available, future work could characterize the state of TR in various subcellular compartments, thereby advancing our understanding of the physiological significance of the large TR concentration in the endosomal network identified in our studies.

The authors gratefully acknowledge the excellent technical assistance of Michael McCaffery with the ultrastructural studies. This work was supported by National Heart, Lung and Blood Institute grant HL-17080 to G.E.P. and by a Fellowship of the Max Kade Foundation to R.H. Some of the data was presented in abstract form at the 33rd Annual Meeting of the American Society for Cell Biology, December 1993, in New Orleans.

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(Received 24 August 1994 - Accepted 10 November 1994)