Biochem. J. (1989) 259, 35–40 (Printed in Great Britain)



# Stability of the thrombin–thrombomodulin complex on the surface of endothelial cells from human saphenous vein or from the cell line EA.hy 926

Alain BERETZ,* Jean-Marie FREYSSINET,* Josiane GAUCHY,* Didier A. SCHMITT,* Claude KLEIN-SOYER,* Cora-Jean S. EDGELL† and Jean-Pierre CAZENAVE*

*INSERM U.311, Service d'Hémostase et de Thrombose, Centre Régional de Transfusion Sanguine, 10 rue Spielmann, 67085 Strasbourg, France, and †Department of Pathology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27514, U.S.A.

Protein C activation by α-thrombin on the surface of endothelial cells depends on an essential membrane-glycoprotein cofactor, thrombomodulin. In the present study we have monitored the activity of thrombin–thrombomodulin complexes on human saphenous-vein endothelial cells (HSVEC) or on the endothelial cell line EA.hy 926. Cell monolayers were exposed for 5 min to 8.5 nM human α-thrombin and then washed to remove unbound thrombin. The cells were then incubated at 37 °C for 5–180 min. At the end of the respective incubation periods, purified human protein C (120 nM) was added in order to assay the activity of the thrombin–thrombomodulin complexes present on the cell surface. HSVEC pre-exposed to thrombin retained their full capacity to promote protein C activation up to 90 min after free thrombin was removed. This capacity then decreased slowly to reach 56% of control value after 180 min of incubation. Original activity was $3.8 \pm 0.9$ pmol of activated protein C formed/min per ml per $10^6$ cells (mean ± S.E.M., $n = 5$). The capacity of protein C activation of EA.hy 926 cells remained constant for 120 min after free thrombin was removed, then decreased to 76% of control after 180 min. Original activity was $2.0 \pm 0.4$ pmol of activated protein C formed/min per ml per $10^6$ cells (mean ± S.E.M., $n = 3$). Similar results were obtained with cells fixed with 3% paraformaldehyde. However, during the 5–180 min incubation period, non-fixed cells of both types were capable of significantly internalizing fluorescent acetylated low-density lipoprotein. In the experimental protocol used here, an eventual inhibition of thrombin internalization by protein C can be excluded, as protein C is only added at the end of the incubation period. We conclude that there is no evidence of rapid internalization of thrombin–thrombomodulin complexes on HSVEC or the EA.hy 926 cell line, as assessed by the ability of membrane-bound thrombin to activate protein C.

## INTRODUCTION

Protein C is the vitamin K-dependent precursor of a serine proteinase, activated protein C, which has potent anticoagulant capacity. Thrombin is the only known physiological activator of protein C, in the presence of an essential endothelial-cell cofactor, thrombomodulin (Esmon, 1987). Thrombomodulin is a glycoprotein with an $M_r$ of 75000, showing structural similarities to the low-density-lipoprotein (LDL) receptor (Jackman *et al.*, 1986, 1987; Suzuki *et al.*, 1987; Wen *et al.*, 1987). Endothelial cells would contain between 30000 and 50000 thrombomodulin molecules per cell, which represent about 50–60% of the total thrombin-binding sites (Maruyama & Majerus, 1985). The binding of thrombin to thrombomodulin has two anticoagulant functions: the neutralization of thrombin's procoagulant activities and the acceleration of the activation of protein C [for a review, see Freyssinet & Cazenave (1988)].

The fate of thrombin after it has bound to thrombomodulin has not been precisely elucidated. The bound thrombin is still susceptible to inhibition by plasma antithrombin III and subsequent removal from the circulation (Owen *et al.*, 1986). A covalent adduct of thrombin with endothelial cells is created by the interaction of the serine proteinase with a secreted inhibitor or the proteinase nexin; the enzyme–inhibitor complex is then taken up and degraded (Savion *et al.*, 1981). Thrombin inactivated with di-isopropyl phosphofluoridate ($iPr_2P$–F) ($iPr_2P$–thrombin) and labelled with $^{125}I$ binds to the endothelium, can be displaced by an excess of $iPr_2P$–thrombin, but remains bound to high-affinity binding sites for periods of up to several hours without being processed (Owen *et al.*, 1986). On the other hand, it was shown that, in the A549 cell line and partially in human umbilical-vein endothelial cells, thrombin–thrombomodulin complexes were internalized, followed by intracellular degradation of thrombin and reappearance of thrombomodulin on the cell surface after 30 min (Maruyama & Majerus, 1985). If thrombomodulin is emerging as the major binding site of thrombin on the endothelial surface, its role in the eventual intracellular processing of the enzyme remains to be fully elucidated. The activity of thrombomodulin-bound thrombin

Abbreviations used: $iPr_2P$-F, di-isopropyl phosphofluoridate; PBS, phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/0.9 mM-$CaCl_2$/0.5 mM-$MgCl_2$/1.5 mM-$KH_2PO_4$/6.5 mM-$Na_2HPO_4$, pH 7.4; 0.295 osM); S-2238, *H*-D-phenylalanyl-L-pipecolylarginine *p*-nitroanilide dihydrochloride; S-2366, L-pyroglutamyl-L-prolyl-L-arginine *p*-nitroanilide hydrochloride; LDL, low-density lipoprotein; DiI-Ac-LDL, acetylated LDL labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; APC, activated protein C; a.t.u., antithrombin unit; HSVEC, human saphenous-vein endothelial cell; Tyrode/albumin buffer and HAT are defined in the text; EGF, epidermal growth factor.

should be a good estimate of the amount of thrombin bound to the endothelial-cell receptor. The present study was designed to monitor with time the ability of human α-thrombin bound to human saphenous-vein endothelial cells (HSVECs) or the endothelial cell line EA.hy 926 to activate human protein C.

## MATERIALS AND METHODS

### Materials

Materials were obtained from the following sources: cell-culture media {M199, RPMI 1640, Hepes, L-glutamine, penicillin, streptomycin, and trypsin–EDTA solution [trypsin (0.5 g/l)/EDTA (0.2 g/l)/NaCl (0.85 g/l)]} were from Gibco, Paisley, Renfrewshire, Scotland, U.K.; fatty-acid-free human serum albumin from Sigma, St. Louis, MO, U.S.A.; hirudin from Stago, Asnières, France; acetylated LDL labelled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL) from Biomedical Technologies, Cambridge, MA, U.S.A.; S-2366 (L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide hydrochloride) and S-2238 (*H*-D-phenylalanyl-L-pipecolylarginine *p*-nitroanilide dihydrochloride) from Kabi Vitrum, Stockholm, Sweden; $HAuCl_4$ (tetra-aurochloric acid) from Aldrich, Strasbourg, France. All other chemicals were obtained from Merck, Darmstadt, Germany, and were of analytical grade.

### Purification of human plasma proteins

Human α-thrombin [3000 National Institutes of Health (NIH) units/mg of protein] was obtained by activating purified prothrombin with Taipan-snake (*Oxyranus scutellatus scutellatus*) venom (Owen & Jackson, 1973; Freyssinet *et al.*, 1986). Protein C was purified to homogeneity (as judged by SDS/polyacrylamide-gel electrophoresis) from human plasma cryosupernatant as previously described (Freyssinet *et al.*, 1985).

### Cell culture

Human endothelial cells were obtained from fragments of saphenous veins obtained during coronary-bypass surgery. The veins were kept in Hank's balanced salt solution with 1% human serum albumin (Centre Régional de Transfusion Sanguine, Strasbourg, France). The vessels were opened with scissors, rinsed gently with PBS and the endothelial-cell layer gently scraped off with a scalpel blade. The blade was shaken in culture medium. The cells were cultivated by previously described method (Klein-Soyer *et al.*, 1986) that is a modification of those of Jaffe (1980) and Willems *et al.* (1982). The culture medium was M199/RPMI 1640 (1:1) containing 10 mM-Hepes, 2 mM-L-glutamine, antibiotics (100 units of penicillin and 100 μg of streptomycin/ml) and 30% pooled human serum. The suspension of scraped cells was homogenized with a silicone-treated Pasteur pipette and seeded in Petri dishes (35 mm diam.; Corning, New York, NY, U.S.A.) pretreated with a human plasma fraction enriched in fibronectin (56%) by cryoprecipitation. The cells were passaged using trypsin–EDTA at a split ratio of 1:4. Cells were used in these experiments at the second or third passage. Protein C cofactor activity was shown to be constant up to the eighth passage (Cazenave *et al.*, 1985). The human cells line EA.hy 926 was derived by fusing human umbilical-vein endothelial cells with the permanent cell line A549 (Edgell *et al.*, 1983). It was grown on dishes precoated with a human plasma fraction enriched in fibronectin. The medium was RPMI/M199 (1:1), containing 10 mM-Hepes, 2 mM-L-glutamine, antibiotics (100 units of penicillin or 100 μg of streptomycin/ml), HAT (100 μM-hypoxanthine/0.4 μM aminopterin/16 μM-thymidine) and 10% human pooled serum. The cells were passaged using trypsin–EDTA at a split ratio of 1:10.

### Protein C cofactor activity

Cells grown to confluence in 35 mm-diameter Petri dishes were washed three times with 1 ml of warm (37 °C) PBS and twice with Tyrode/albumin buffer (NaCl, 137 mM; KCl, 2.7 mM; $NaHCO_3$, 12 mM; $NaH_2PO_4$, 0.4 mM; $MgCl_2$, 1 mM; $CaCl_2$, 2 mM; Hepes, 5 mM; glucose, 1 g/l; human fatty-acid-free serum albumin, 0.35%, w/v; pH 7.35; osmolarity 0.295 osM). When cell fixation was required, 1 ml of 3% paraformaldehyde in PBS was added after washing the monolayer with PBS and left for 20 min at room temperature. The fixed cells were washed three times with PBS and were then processed similarly to the non-fixed cells.

A portion (1 ml) of warm (37 °C) Tyrode/albumin was added and 30 μl of human α-thrombin (30 NIH units/ml) or Tris/NaCl (50 mM-Tris/0.1 M-NaCl, pH 7.5) buffers were used as controls. The dishes were left for 5 min in the incubator [37 °C; $CO_2$/air (1:19); saturation humidity]. The cells were then washed twice with cold (4 °C) Tyrode/albumin, and reincubated at 37 °C in 1 ml of Tyrode/albumin for 5–180 min. After 5, 10, 20, 40, 60, 90, 120 and 180 min, two dishes were removed from the incubator, 10 μl of protein C (12 μM) was added, and the dishes were incubated at 37 °C for 15 min. As recommended by Salem *et al.* (1984), less-than-saturating concentrations of protein C were used in order to conserve material, and incubation times were adjusted so that less than 20% of the protein C was activated. The reaction was terminated by addition of 5 a.t.u. of hirudin in 5 μl, and the concentration of APC formed was determined by reference to a standard curve constructed with known amounts of APC (Freyssinet *et al.*, 1986). It was verified that the presence of hirudin had no effect on the amidolytic activity of activated protein C.

### Internalization of fluorescent acetylated LDL

This experiment was performed as a control on the same day and using the same cells as for the activation of protein C. The cells, grown to confluence in 35 mm-diameter Petri dishes, were washed three times with PBS and twice with Tyrode/albumin buffer. They were then incubated at 37 °C in 1 ml of Tyrode/albumin buffer with 50 μl of acetylated LDL labelled with 1′,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI-Ac-LDL) (200 mg/ml in 0.15 M-NaCl/0.05 M-Tris/2 μM-EDTA, pH 7.4) to reach a final DiI-Ac-LDL concentration of 10 μg/ml. Dishes were removed from the incubator after 30 and 180 min, washed three times with PBS and fixed in 3% paraformaldehyde. They were examined with a Nikon Diaphot TMD inverted microscope equipped with an epifluorescence attachment, using the standard rhodamine excitation/emission filter combination.

### Preparation of thrombin–colloidal-gold probes

Colloidal gold particles, averaging 15 nm in diameter, were prepared as described (Frens, 1973). Briefly, 165 μl

of $HAuCl_4$ was added to 10 ml of boiling water and stirred vigorously before the addition of 1 ml of freshly prepared 1% sodium citrate. After 5 min at 100 °C the solution became orange–red and was left to cool to room temperature. Human α-thrombin was dialysed for 4 h against 5 mM-sodium carbonate buffer, pH 7.5 at 4 °C. The thrombin (1000 NIH units in 5 ml of 5 mM-$Na_2CO_3$, pH 7.5) was added under stirring to 10 ml of colloidal gold in 5 mM-$Na_2CO_3$, pH 9.8. This amount of thrombin was determined by using the serial-dilution–electrolyte-flocculation test (Horisberger, 1979). The thrombin–colloidal-gold conjugate was then washed three times by centrifugation at 50000 *g* for 15 min in 30 ml of 5 mM-$Na_2CO_3$/0.01% Tween 20, pH 9.8. Amidolytic activity, as assayed using the chromogenic substrate S-2238, was 40% of that of the thrombin originally added. The ability of the thrombin–colloidal-gold conjugate to cause washed human platelet aggregation or clotting of human citrated plasma was reduced to 12% of that of the thrombin originally added. These values were comparable with those previously obtained by Handley & Chien (1985) or Liu *et al.* (1985).

### Labelling of endothelial cells with thrombin–colloidal-gold probes

This experiment was performed on the same day and by use of the same cells as those used for the activation of protein C. The cells, grown to confluence in 35 mm-diameter Petri dishes, were washed three times with PBS and twice with Tyrode/albumin buffer. The amount of thrombin–colloidal-gold possessing the plasma-clotting activity of 2 NIH units of thrombin was incubated with the cells for 90 s at 37 °C in 1 ml Tyrode/albumin buffer. The dishes were then washed three times in PBS and incubated in 1 ml of Tyrode/albumin at 37 °C for up to 180 min. They were then fixed by adding 1 ml of 2% glutaraldehyde/0.1 M sodium cacodylate/0.1 M-sucrose, pH 7.25, 0.510 osM, for 5 min at 37 °C, then 2 ml of pure fixative for 1 h at 37 °C and overnight at 4 °C. The dishes were washed in sodium cacodylate buffer, post-fixed in 1% $OsO_4$ in 0.1 M-sodium cacodylate, pH 7.4, for 1 h at room temperature, dehydrated in ethanol and embedded in Epon. Ultrathin sections, stained with lead citrate and uranyl acetate, were examined using a Siemens Elmiscope 10.

## RESULTS

### Activation of protein C on the surface of HSVECs

Monolayers of confluent fresh or fixed HSVECs were exposed for 5 min to 8.5 nM human α-thrombin and then washed. When protein C (120 nM, final concentration) was added thereafter, its activation could only originate from thrombin bound to thrombomodulin on the cell surface. Fig. 1 shows that, when the cells were left to incubate without protein C for as long as 90 min, thrombin–thrombomodulin complexes remained virtually unchanged, as can be judged from the constant rate of protein C activation on the surface of these cells. After longer times of incubation, protein C activation decreased, but 180 min after washing away the unbound thrombin, 56% of the original protein C activation was still observed (Fig. 1).

The amidolytic activity developed in the presence of cells and of protein C without thrombin was 16% of the activity observed in the presence of thrombin. In other

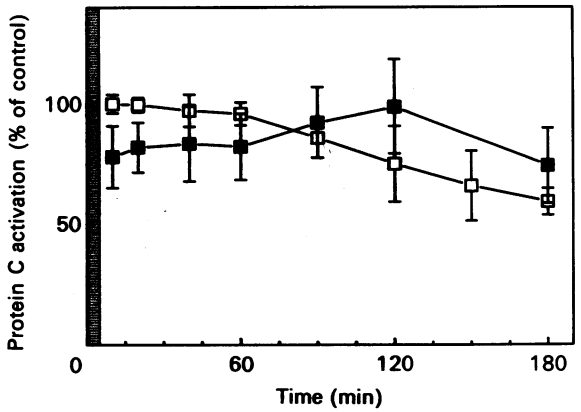


**Fig. 1. Activation of protein C in the presence of HSVECs in culture after exposure to thrombin**

Confluent monolayers [(374±57) × $10^3$ cells] of fresh (□) or fixed (■) HSVECs were exposed to 8.5 nM human α-thrombin for 5 min at 37 °C (stippled area). The monolayers were washed twice with cold Tyrode/albumin buffer and re-incubated at 37 °C in 1 ml Tyrode/albumin. After the various times indicated, human protein C (120 nM final concn.) was added and cells were incubated for a further 15 min at 37 °C. The reaction was terminated by adding 5 a.t.u. of hirudin, and the activated protein C generated was estimated. Activity is given as a percentage of the protein C activation observed at 5 min after washing away the thrombin. This '100% of control' corresponded to 3.8±0.9 pmol of activated protein C formed/min per ml per $10^6$ cells. Control incubation in the absence of protein C gave approx. 10% of the amidolytic activity obtained in the presence of protein C. Points correspond to means±S.E.M. for five different experiments.

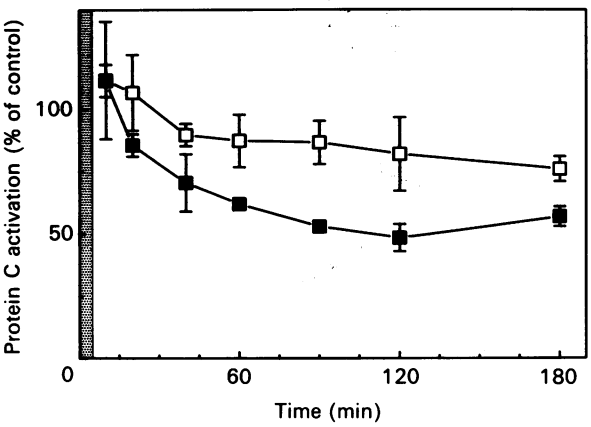


**Fig. 2. Activation of protein C in the presence of EA.hy 926 cells after exposure to thrombin**

Confluent monolayers of fresh (□) or fixed (■) EA.hy 926 cells [(1114±183) × $10^3$] were exposed to 8.5 nM human α-thrombin exactly as described in the legend to Fig. 1. The '100%' activity corresponds to 2.0±0.4 pmol of activated protein C formed/min per ml per $10^6$ cells. Control incubation performed in the absence of protein C gave approx. 15% of the amidolytic activity obtained in the presence of protein C. Points correspond to means±S.E.M. for three different experiments.

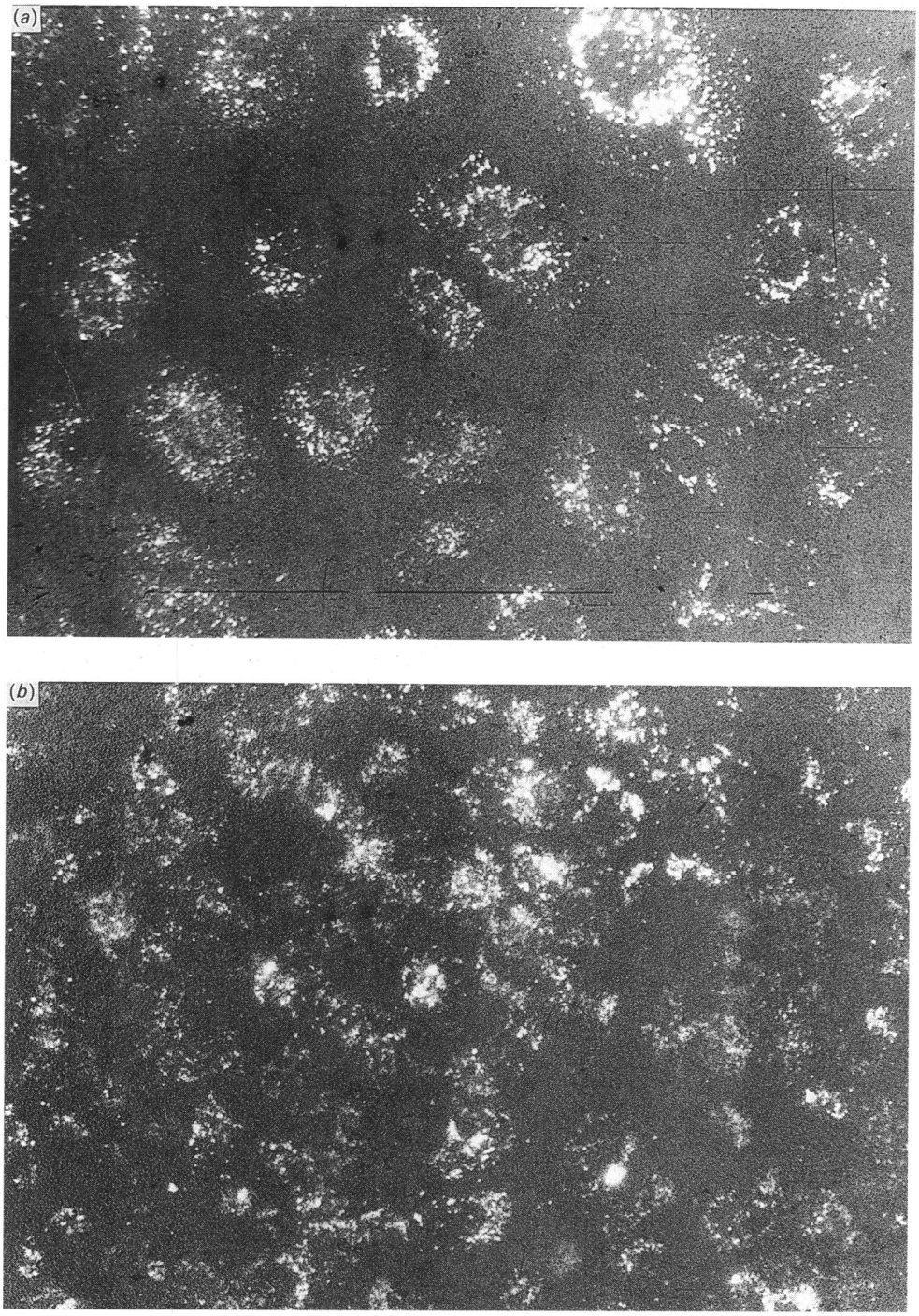


**Fig. 3. Labelling of HSVECs and EA.hy 926 cells with DiI-Ac-LDL**

Fluorescence micrographs of HSVECs (*a*) or EA.hy 926 cells (*b*) incubated for 180 min with 10 mg of DiI-Ac-LDL are shown. Cells were revealed by using the standard rhodamine excitation/emission filter set. Cells that were fixed with 3% paraformaldehyde before incubation with DiI-Ac-LDL showed only background fluorescence (result not shown). Magnification × 360.

control experiments, where either protein C, thrombin and protein C, or cells were absent from the system, amidolytic activity was never higher than 15% of the values observed with the full reagent combination.

### Activation of protein C on the surface of EA.hy 926-cell-line monolayers

The EA.hy 926 cell line has been established through the fusion of human umbilical-vein endothelial cells with the permanent cell line A 549 (Edgell *et al.*, 1983). It has been shown to possess various characteristics of human endothelial cells, such as the synthesis of von Willebrand factor, prostaglandin $I_2$ and thrombomodulin (Suggs *et al.*, 1986). The presence of thrombin–thrombomodulin complexes on the surface of these cells was monitored after a short exposure to thrombin, followed by washing, exactly as for HSVEC. The ability of membrane bound thrombin to activate protein C

remained constant at approx. 75–90% of the original value, even when the cells were incubated for as long as 180 min in the absence of protein C (Fig. 2).

The amidolytic activity detected in the presence of cells and of protein C without thrombin was 13% of the activity observed in the presence of thrombin. In other control experiments, where either protein C, thrombin and protein C, or cells, were absent from the system, amidolytic activity was never higher than 15% of the values observed with the full reagent combination.

### Internalization of fluorescent acetylated LDL

Cells were incubated with DiI-Ac-LDL in the same buffer (Tyrode/albumin) as used for the protein C experiments. Even as soon as 30 min, both cell types were capable of internalization of DiI-Ac-LDL and were brightly stained after 180 min of incubation (Fig. 3). When cells were fixed with 3% paraformaldehyde, no intracellular staining was observed (results not shown).

### Ultrastructural studies with thrombin–colloidal-gold probes

Cells were incubated with the thrombin–colloidal-gold probe under exactly the same experimental conditions as those used for the study of $\alpha$-thrombin interaction with thrombomodulin. Membrane labelling was still observed after 180 min, both as random labelling and in clusters (results not shown). Intracellular labelling was limited to 26% of total label (as determined after counting the beads in 122 different frames). Incubation of the cells with the thrombin–colloidal-gold probe resulted in a morphological change similar to that observed with thrombin.

## DISCUSSION

Maruyama & Majerus (1985) used a monoclonal antibody (IgG-TM) to measure thrombomodulin on cultured umbilical-vein endothelial cells and the cell line A549. Using this antibody, they were able to show that exposure of these cells to thrombin decreased thrombomodulin on the cell surface. Radioactive thrombin was shown to be internalized and degraded. In experiments similar to those reported here, 20 min after excess thrombin was washed off, A549 cells lost their capacity to activate protein C. These data were interpreted as evidence that the thrombin–thrombomodulin complex was cleared from the cell surface through internalization of thrombomodulin with a half-time ($t_{\frac{1}{2}}$) of less than 15 min. In our study, the stability of the capacity to activate protein C on the surface of HSVEC or EA.hy 926 cells over a 180 min period does not suggest that the thrombin–thrombomodulin complexes are internalized.

The characteristics of protein C activation by thrombin bound to the surface of paraformaldehyde-fixed cells were similar to those observed for non-fixed cells (Figs. 1 and 2), although, as previously demonstrated (Cazenave *et al.*, 1985), fixation leads to a 10–25% decrease in the ability of cells to activate protein C. It was thus necessary to check that the non-fixed cells used in our experiments were still capable of endocytosis. Acetylated low-density lipoproteins (Ac-LDL) are taken up by endothelial cells by a receptor-mediated mechanism at an accelerated rate compared with other cell types (Voyta *et al.*, 1984). Fluorescent-labelled Ac-LDL (DiI-Ac-LDL) was taken up by both HSVECs and EA.hy 926 cells (Fig. 3). This uptake was already noticeable after 30 min of incubation with the cells, and led to strong intracellular fluorescence after 180 min. Fixed cells showed no intracellular labelling (results not shown). Thus, in the incubation conditions for the functional activation of protein C used here, receptor-mediated endocytosis of acetylated LDL was still possible.

Handley & Chien (1985) have shown that, after 2 h of incubation of thrombin–colloidal-gold probes with porcine aortic endothelial cells, internalization in coated vesicles and lysosomes was limited to 25% of cell-associated gold probes. We have obtained similar results here using HSVEC. Although it is difficult to quantify exactly the amount of internalized probe, there was definitely not endocytosis of as much as 50–60% of bound thrombin. Such a high degree of internalization should have been observed if thrombin bound to thrombomodulin (which should represent 50–60% of total thrombin-binding sites) was internalized within 30 min (Maruyama & Majerus, 1985).

In previous reports a linear rate of protein C activation was observed for at least 1 h on the surface of human umbilical-vein endothelial cells (Esmon & Owen, 1981; Owen & Esmon, 1981). In these experiments, protein C was present during the whole incubation period. Maruyama & Majerus (1987) have demonstrated that protein C can inhibit the endocytosis of thrombin–thrombomodulin complexes. However, the protocol used in our study excludes this possibility, as cells were incubated with bound thrombin for various times in the absence of protein C. Protein C was added at the end of the incubation period only, in order to reveal the amount of thrombin–thrombomodulin complexes remaining on the cell surface, and could thus not interfere with the eventual endocytotic process.

The amino acid sequence of thrombomodulin shows a striking similarity to that of the LDL receptor (Jackman *et al.*, 1986, 1987; Suzuki *et al.*, 1987; Wen *et al.*, 1987). The epidermal-growth-factor (EGF)-like domain in the LDL receptor would be involved in the acid-dependent conformational changes necessary for the recycling of the receptor in endosomes (Davis *et al.*, 1987*a*). A critical tyrosine residue at position 807 in the cytoplasmic tail of the LDL receptor is essential for the clustering in coated pits and subsequent internalization of the receptor (Davis *et al.*, 1987*b*). Both the EGF-like domain and the cytoplasmic domain of thrombomodulin show similarities to those of the LDL receptor. This has been put forward to support the finding that thrombomodulin could remove thrombin bound at the cell surface through receptor-mediated endocytosis. However, the primary binding site of thrombin on thrombomodulin has been localized in the fifth and sixth EGF-like structures (Kurosawa *et al.*, 1988), whereas in the LDL receptor LDL binds to the *N*-terminal domain before it is internalized (Brown & Goldstein, 1986). If the interaction of thrombin with thrombomodulin involves different acceptor domains from the binding of LDL to its receptor, this could suggest that postbinding fate of both ligands would be also different, despite the common structures of their receptor molecules. Results reported here show that, in cells still capable of endocytosis of LDL, thrombin–thrombomodulin complexes remain functionally unchanged on the surface. It is very well possible that the internalization of radiolabelled thrombin or of thrombin–colloidal-gold probes could be mediated

through the attachment of the proteinase to a membrane component other than thrombomodulin. This possibility is further supported by the finding that IgG-TM, the monoclonal antibody used to label thrombomodulin in studies reported by Maruyama & Majerus (1985, 1987), could also recognize two additional proteins, distinct from thrombomodulin, which were expressed in a human endothelial-cell cDNA library (Wen *et al.*, 1987).

We thank Mrs. M.-R. Neumann for her help in cell culture, Dr. J. N. Mulvihill for reviewing the English of the manuscript before its submission, Mrs. M.-O. Bernhard and M. Voyat for typing the manuscript. This work was supported by a research grant (N° 85-3-21-5-E) from Caisse Nationale d'Assurance Maladie des Travailleurs Salariés.

## REFERENCES

Brown, M. S. & Goldstein, J. L. (1986) Science **232**, 34–47
Cazenave, J.-P., Freyssinet, J.-M., Klein-Soyer, C., Gauchy, J., Shah, A. & Duclos, B. (1985) Thromb. Haemostasis **54**, 119 (abstr.)
Davis, C. G., Goldstein, J. L., Thomas, C. S., Anderson, R. G., Russel, D. W. & Brown, M. S. (1987*a*) Nature (London) **326**, 760–765
Davis, C. G., van Driel, I. R., Russel, D. W., Brown, M. S. & Goldstein, J. L. (1987*b*) J. Biol. Chem. **262**, 4075–4082
Edgell, C. J. S., McDonald, C. C. & Graham, J. B. (1983) Proc. Natl. Acad. Sci. U.S.A. **80**, 3734–3737
Esmon, C. T. (1987) Science **235**, 1348–1352
Esmon, C. T. & Owen, W. G. (1981) Proc. Natl. Acad. Sci. U.S.A. **78**, 2249–2252
Frens, G. (1973) Nature (London) Phys. Sci. **241**, 20–22
Freyssinet, J.-M. & Cazenave, J.-P. (1988) in Protein C and Related Proteins (Bertina, R. M., ed.), pp. 91–105, Churchill-Livingstone, Edinburgh
Freyssinet, J.-M., Grunebaum, L., Wiesel, M.-L., Cazenave, J.-P., Fritsche, R., Greber, S., Lampart, A. & Spaethe, R. (1985) in Protein C: Biochemical and Medical Aspects (Witt, I., ed), pp. 107–116, Walter de Gruyter, Berlin
Freyssinet, J.-M., Gauchy, J. & Cazenave, J.-P. (1986) Biochem. J. **238**, 151–157
Handley, D. A. & Chien, S. (1985) Eur. J. Cell Biol. **39**, 391–398
Horisberger, M. (1979) Biol. Cell **36**, 253–258
Jackman, R. W., Beeler, D. L., Van De Water, L. & Rosenberg, R. D. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8834–8838
Jackman, R. W., Beeler, D. L., Fritze, L., Sopp, G. & Rosenberg, R. D. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6425–6429
Jaffe, E. A. (1980) Transplant. Proc. **12** suppl. **1**, 49–53
Kurosawa, S., Stearn, D. J., Jackson, K. W. & Esmon, C. T. (1988) J. Biol. Chem. **263**, 5993–5996
Klein-Soyer, C., Beretz, A., Millon-Collard, R., Abecassis, J. & Cazenave, J.-P. (1986) Thromb. Haemostasis **56**, 232–235
Liu, C. Y., Handley, D. A. & Chien, S. (1985) Anal. Biochem. **147**, 49–56
Maruyama, I. & Majerus, P. W. (1985) J. Biol. Chem. **260**, 15432–15438
Maruyama, I. & Majerus, P. W. (1987) Blood **69**, 1481–1484
Owen, W. G. & Jackson, C. M. (1973) Thromb. Res. **3**, 705–714
Owen, W. G. & Esmon, C. T. (1981) J. Biol. Chem. **25**, 5532–5535
Owen, W. G., Goeken, J. A. & Lollar, P. (1986) in Vascular Endothelium in Hemostasis and Thrombosis (Gimbrone, M. A., ed.), pp. 57–69, Churchill–Livingstone, Edinburgh
Salem, H. H., Maruyama, I., Ishii, I. & Majerus, P. W. (1984) J. Biol. Chem. **259**, 12246–12251
Savion, N., Isaacs, J. D., Gospodarowicz, D. & Shuman, M. A. (1981) J. Biol. Chem. **256**, 4514–4519
Suggs, J. E., Madden, M. C., Friedman, M. & Edgell, C. J. S. (1986) Blood **68**, 825–829
Suzuki, K., Kusumoto, H., Deyashiki, Y., Nishioka, J., Maruyama, I., Zushi, M., Kawahara, S., Honda, G., Yamamoto, S. & Horiguchi, S. (1987) EMBO J. **6**, 1891–1897
Voyta, J. C., Via, D. P., Butterfield, C. E. & Zetter, B. R. (1984) J. Cell Biol. **99**, 2034–2040
Wen, D., Dittman, W. A., Ye, R. D., Deaven, L. L., Majerus, P. W. & Sadler, J. E. (1987) Biochemistry **26**, 4350–4357
Willems, C., Astaldi, G. C. B., De Groot, P. G., Janssen, M. C., Gonsalvez, M. D., Zeijlemaker, W. P., van Mourik, J. A. & Van Aken, W. G. (1982) Exp. Cell Res. **139**, 191–197

Received 22 July 1988/11 October 1988; accepted 17 October 1988