Active Site Blockade of Factor VIIa Alters Its Intracellular Distribution*

Factor VIIa binding to tissue factor on cell surfaces not only triggers the coagulation cascade but also induces various intracellular responses that may contribute to many pathophysiological processes. Active site-inhibited factor VIIa, similar to factor VIIa, binds to tissue factor on cell surfaces and subsequently gets internalized and degraded. At present, it is unknown whether factor VIIa and active site-inhibited factor VIIa undergo a similar intracellular processing. The data presented herein show that a fraction of both the internalized factor VIIa and active site-inhibited factor VIIa recycle back to the cell surface, the amount of active site-inhibited factor VIIa recycled back to the cell surface was substantially higher than that of factor VIIa. Furthermore, internalized factor VIIa and not active site-inhibited factor VIIa associates with nuclear fractions. Factor VIIa associated with the nuclear fraction was intact and functionally active. In contrast to factor VIIa, tissue factor is not found in the nuclear fraction. Additional studies show that the internalized factor VIIa specifically associates with cytoskeletal proteins, actin, and tubulin. In summary, the present data reveal that despite the common pathway of tissue factor-mediated processing, considerable differences exist in the trafficking of factor VIIa and active site-inhibited factor VIIa in fibroblasts.

The coagulation cascade is initiated by binding of coagulation factor VIIa (FVIIa) to its cell surface receptor tissue factor (TF) (1). The formation of TF-FVIIa complexes not only leads to activation of the coagulation pathway but may also induce other cellular responses (2–4). Since TF was found to express aberrantly in a number of diseases, such as septic shock, atherosclerosis, and cancer (5), it has been thought that the TF-FVIIa-induced cellular responses may contribute to pathogenesis of these diseases (6–9). In agreement with this, attention has been paid to the clotting as well as pathogenesis of these effects by competing with FVIIa for a limited number of TF sites expressed in vivo and thus blocking the generation of proteolytically active TF-FVIIa complexes, it is not clear whether this could alone explain prolonged and distinctive effects associated with FVIIa (10). It is conceivable that potential differences between FVIIa and FVIIai in their interaction with TF on cell surfaces and their subsequent internalization and intracellular trafficking may also play a role in mediating the protective effects of FVIIai. At present, it is unknown whether FVIIa and FVIIai undergo a similar cellular catabolism once they bound to TF on cells.

Radioligand binding studies with a human bladder carcinoma cell line (J82) showed that FVIIai associated with TF at a faster rate and dissociated from it at a slower rate compared with FVIIa (11). These studies also revealed that the affinity of FVIIai to nonfunctional (cryptic) TF was 5-fold higher than that of FVIIa, whereas both FVIIa and FVIIai bound to functional TF sites with the same high affinity. Differences between FVIIa and FVIIai for their affinity to TF was also evident in studies with relipidated TF, as shown first by Bach and colleagues (17) and extended by others (16, 18, 19). Our recent studies show that FVIIa and FVIIai bound to TF on cell surfaces was internalized in a time-dependent manner and a substantial portion of the internalized ligand recycles back to the cell surface as the intact protein (20, 21).

In the present study, we examined the pattern of endocytosis and intracellular distribution of FVIIa and FVIIai in fibroblasts to determine potential differences between FVIIa and FVIIai in their cellular processing. The data presented herein reveal that despite the common pathway of TF-mediated processing, considerable differences exist in the trafficking of FVIIa and FVIIai in fibroblasts. Compared with FVIIa, more internalized FVIIai recycles back to the cell surface. Furthermore, internalized FVIIai and not FVIIa is co-isolated with nuclei. Additional experiments revealed that FVIIai binds to cytoskeletal proteins actin and tubulin and this interaction may be responsible for FVIIai association with the nuclear fraction.

EXPERIMENTAL PROCEDURES

Cell Culture—A human fibroblast cell line, WI-38, was obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Rockville, MD) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro, Herndon, VA), 1% penicillin (50 units/ml), and streptomycin (50 mg/ml) mixture, and 1% L-glutamine (2 mM). When cells reached near confluency, the culture medium was removed and the cells were washed with buffer A (10 mM Tris, pH 7.4, 50 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.5 mM EDTA, and 0.1% BSA).
Intracellular Distribution of FVIIa and FFR-FVIIa

Proteins—Recombinant human FVIIa was a gift from Novo Nordisk ( Bagsvaerd, Denmark). FVIIai was prepared by incubating recombinant human FVIIa with a peptidyl inhibitor, n-Phe-n-Phe-Arg chloromethyl ketone (30 μM) at 500 rpm for 30 min at 4°C to inhibit factor Xa generation assay after the addition of fresh FVIIa (10 nM) and expressed earlier (21). FFR-FVIIa. Factors X and Xa were obtained from Enzyme Research Laboratories (South Bend, IN). Preparation of monospecific, polyclonal rabbit anti-human TF IgG was described earlier (22). Monoclonal antibody against human TF (TF9–10H10) was provided by Dr. Wolfram Ruf (Scripps Research Institute, La Jolla, CA). α-Actin, β-tubulin, and other proteins were obtained from Sigma.

Radio labeling of Proteins—FVIIa and FFR-FVIIa were labeled by using IODO-GEN-coated tubes and Na125I according to the manufacturer’s (Pierce) technical bulletin and as described previously (23). Free iodine was removed by extensive dialysis against 10 mM Hepes, pH 7.5, 150 mM NaCl. The concentration of labeled proteins was determined spectrophotometrically at A254 using E1%1 cm of 13.8. Specific radioactivity of labeled proteins was 5–9 × 104 cpm/nM. SDS-polyacrylamide gel electrophoresis showed that the radiolabeled proteins were intact with no apparent degradation. 125I-Labeled FVIIa retained 80% or more of the functional activity of the unlabeled material (as measured for its ability to activate factor X in the presence of tissue factor).

Labeling of FVIIa, FFR-FVIIa, and Anti-TF Antibody with Fluorescent Proteins—FFR-FVIIa (80 μg/ml) was labeled with Alexa Fluor 568 (Molecular Probes, Eugene, OR) using slight modifications to a protocol recommended by the manufacturer for labeling of proteins with amine reactive probes. Briefly, FVIIa or FFR-FVIIa (3 mg/ml) was incubated in 0.1 M NaHCO3, pH 8.5, buffer with the fluorophore (0.5 mg/ml) for 15 min at room temperature. Free probe was removed by gel filtration on PD-10 column (Amersham Pharmacia Biotech). The labeled FVIIa retained about 70% of its original functional activity. TF monoclonal antibody (TF9 10H10) was labeled with fluorescein isothiocyanate according to the protocol recommended by the manufacturer.

Fluorescent Microscopy—WI-38 cells grown on glass coverslips were incubated with labeled FVIIa (or FFR-FVIIa) (1 μg/ml) and 10H10 antibody (1 μg/ml) for 2 h at 37°C in buffer B (buffer A containing 5 mM CaCl2 and 5 mM mg/ml bovine serum albumin), (TF9 10H10 antibody binds to TF without interfering FVIIa binding to TF.) Unbound FVIIa and the antibody were removed, cells were washed with 0.1 M NaHCO3, pH 8.5, buffer with the fluorophore (0.5 mg/ml) for 15 min at room temperature. Free probe was removed by gel filtration on PD-10 column (Amersham Pharmacia Biotech). The labeled FVIIa retained about 70% of its original functional activity. TF monoclonal antibody (TF9 10H10) was labeled with fluorescein isothiocyanate according to the protocol recommended by the manufacturer.

Tissue Factor-dependent Ligand Binding and Internalization Assays—Cell surface binding, internalization, and degradation of 125I-labeled FVIIa or 125I-labeled FFR-FVIIa were performed essentially as described previously (20). To determine TF-specific binding, internalization and degradation of FVIIa, parallel experiments were carried out in which the monolayers were first incubated for 30 min with rabbit anti-human TF (100 μg/ml) before reactive ligands were added to the cells. TF-specific binding and internalization were determined by subtracting the values from the corresponding values obtained in the absence of anti-TF antibody. Duplicate wells were used for all measurements and each experiment was repeated 3–4 times.

Determination of Tissue Factor and FVIIa Functional Activity Associated with Cells—Control and treated cells were washed four times with ice-cold buffer B and the cell surface TF activity was assayed in a factor Xa generation assay after the addition of fresh FVIIa (10 nM) and FX (175 nM) (23). The functional activity of FVIIa in nuclear fractions was also determined in factor X activation assay by providing exogenous TF. Briefly, suspension of nuclei (10 mg/ml) before radioactive ligands were added, was also determined. Briefly, wells in a 96-well microplate were coated with DNA (0.6 μg), which was digested by restriction endonuclease (ALU1, DPN, and NDE) for 1 h at 37°C and the mixture was electrophoresed on a 4–15% gradient gel. Electrophoresis was performed for 60 min at 30 V, and the radioactive bands were visualized with a gel scanner. As a variant of this assay, binding of FVIIa or FFR-FVIIa to immobilized purified actin or tubulin was also determined. Briefly, wells in a 96-well microplate were coated with actin, tubulin, or fibronectin (as a control) (100 μl of 10 μg/ml in 0.1 M NaHCO3 buffer, pH 9.4) overnight at 4°C and then blocked with buffer A containing 10 mg/ml BSA. 125I-FVIIa or 125I-FFR-FVIIa (10 nM), with or without 50-fold molar excess of unlabeled FVIIa (or FFR-FVIIa), was added to the wells and incubated for 60 min at 37°C. FVIIa and FFR-FVIIa bound to the immobilized ligand was determined as the above.

RESULTS

Differences in Intracellular Distribution of FVIIa and FFR-FVIIa—Our recent studies show that incubation of 125I-FVIIa or 125I-FFR-FVIIa with WI-38 fibroblasts resulted in a similar TF-mediated cell surface binding, internalization, and degradation of these molecules (20). However, a closer look into the binding kinetics of these ligands suggest that, despite the common mechanism of cell association, the steady-state distribution of FVIIa and FFR-FVIIa between cell surface and internalized pools may differ, especially at 10–30 min intervals at which time more FFR-FVIIa appears to be associated with the cell surface, as compared with FVIIa. To verify and confirm this observation, we analyzed data from more than 10 experiments and presented them in a normalized form, where the amount of internalized ligand was taken as a 100%. The results, as depicted in Fig. 1, show that the amount of FFR-FVIIa associated with the cell surface was substantially higher than the amount of FVIIa associated with the cell surface. It is possible that a factor in incubation and/or higher affinity of FFR-FVIIa, compared with FVIIa, for cytosolic TF (16) may be responsible for this difference. If so, the difference between FVIIa and FFR-FVIIa associated with the cell surface would be reduced if the cells contain a higher percentage of TF as an active TF. Indeed, the cells that supported 70 to 90% of maximal FVIIa binding during the first 10 min of the incubation period, which indicates that most of the TF is in active form (23), showed a
negligible difference between FVIIa and FFR-FVIIa binding and internalization measured at 10 min (Fig. 2). However, the data depicted in Fig. 2 also show that the ratio of internalized/surface bound ligand for FFR-FVIIa is not changed over a period of 1 h, whereas the amount of internalized FVIIa was clearly increasing during this period. These data suggest that FVIIa, but not FFR-FVIIa, accumulates intracellularly. Although the data presented in Figs. 1 and 2 are consistent with our earlier observation (20), i.e. a higher percentage of FVIIa was internalized (in relative to the surface bound) compared with FFR-FVIIa, the extent of internalization of both FVIIa and FFR-FVIIa was much higher in the present study compared with the earlier study. Variation in cell batches and culture media may be responsible for this difference.

Internalized FFR-FVIIa Recycles Back to Cell Surface Faster than FVIIa—To elucidate the mechanism of distinct intracellular distribution of FVIIa and FFR-FVIIa, recycling of these ligands after internalization was studied. For these experiments, WI-38 cells were incubated with 125I-FVIIa or 125I-FFR-FVIIa (10 nM) for 60 min at 37 °C to allow the internalization. Thereafter, the cell surface-associated ligand was removed by washing the cells with 0.1 M glycine, pH 2.4, followed by buffer A. Then, the monolayers were overlaid with buffer B and allowed to stand at 37 °C for 30 min. At the end of 30 min, the amount of cell surface-associated, dissociated (both intact and degraded), and the remaining internalized ligand were determined. As shown in Fig. 3, more internalized FFR-FVIIa returns back to the cell surface as compared with FVIIa. Despite the higher amount of FFR-FVIIa on the cell surface, the amount of dissociated FFR-FVIIa into overlying incubation medium was slightly lower than that of FVIIa. This is an expected finding since FFR-FVIIa was shown to dissociate from cell surface TF at a slower rate than that of FVIIa (16). Determination of internalized ligand in totality will not provide information on a possible distinct intracellular compartmentalization of FVIIa and FFR-FVIIa, because internalization is an integral parameter, which includes ligands in endosomal, lysosomal, cytosolic, nuclear, and other compartments. Therefore, to reveal potential differences in intracellular distribution of internalized FVIIa and FFR-FVIIa, we compared the amounts of FVIIa and FFR-FVIIa associated with the nuclear fraction. In contrast, only 7–8% of internalized FFR-FVIIa was associated with the nuclear fraction (Fig. 4). A comparison of time-dependent FVIIa association with the cells and the nuclear fractions revealed that a steady state condition for the cell surface binding was reached within the first 60 min of FVIIa incubation with the cells as shown in our earlier studies (20, 23), whereas the amount of FVIIa associated with the nuclear fraction was increased steadily until the end of experiment duration (4–6 h). When cells were incubated with FVIIa or FFR-FVIIa on ice instead of 37 °C to block endocytosis, but allow the binding of FVIIa or FFR-FVIIa to cells, the amount of radioactivity associated in the nuclear fraction was minimal for both FVIIa and FFR-FVIIa, i.e. 2–4% of the total cell associated radioactivity. These results suggest that the endocytosed FVIIa, but not FFR-FVIIa is accumulated intracellularly in the form, which is associated with nuclei or co-isolating with nuclei. To exclude the possibility of contamination of nuclear fractions with other cell organelles/membranes during isolation, additional experiments were carried out in which nuclear
fractions were further purified by ultracentrifugation. In these experiments, nuclear fractions isolated from the homogenates of the cells incubated with FVIIa or FFR-FVIIa were applied on the top of the sucrose barrier, consisting of 2M sucrose supplemented with 20% glycerol. Measurement of the radioactivity in nuclear fractions (bottom fractions) revealed a 4-fold higher amount of 125I-FVIIa in these fractions (normalized to total radioactivity present in the remaining fractions) compared with FFR-FVIIa. These results confirmed the translocation of internalized FVIIa but not FFR-FVIIa to nuclear fractions.

**TF Is Not Associated with the Nuclear Fraction—**WI-38 cells were incubated with unlabeled FVIIa (10 nM) for 4 h at 37 °C and nuclear fractions were prepared as described above. Samples were subjected to SDS-PAGE followed by Western blot analysis with anti-TF antibody. As shown in Fig. 5, TF content in the nuclear fraction was less than 1% of cellular TF antigen (see lane 2). This observation also rules out the possibility that the nuclear association of FVIIa observed in the above experiments is the result of contaminating plasma membrane or endosomes in nuclear fractions. These results also suggest that FVIIa is dissociated rapidly from TF if it was internalized as FVIIa-TF complexes.

**FVIIa Translocated into the Nuclear Fraction Is Intact and Functionally Active—**Since FVIIa and not FFR-FVIIa translocates into the nuclear fraction, we hypothesized that the FVIIa active site is required to interact with a binding protein, such as Kunitz-type inhibitors like amyloid precursor protein (APP). Such an interaction may be responsible for FVIIa association with the nuclear fraction. It has been shown that a part of APP in neural cell lines was associated with detergent-insoluble cytoskeleton (24), which could associate with nuclei. If FVIIa interacts with such a protein, then the nuclear-associated FVIIa would be in catalytically inactive form. Therefore, we have tested the proteolytic activity of FVIIa associated with the nuclear fractions. Contrary to our assumption, FVIIa associated with the nuclear fraction, based on its ability to activate factor X in the presence of TF, is functionally fully active (Table 1). Additional experiments were performed to test whether the FVIIa associated with nuclei was intact. Trichloroacetic acid (12% w/v) precipitation of SDS-solubilized nuclear fractions showed that 80–90% of radioactivity of this fraction can be precipitated, suggesting that FVIIa associated with the nuclei was intact. Indeed, SDS-PAGE electrophoresis of 125I-FVIIa associated with nuclear fractions showed that the apparent molecular weight of 125I-FVIIa in nuclear fraction was similar to that of native FVIIa, i.e. 50,000 (Fig. 6). A faint band seen at 32 kDa (difficult to visualize) may represent the heavy chain of FVIIa, probably derived by intracellular reduction. In addition, a substantial amount of FVIIa is in complex with high molecular weight material, which failed to enter into the separating gel. The intensity of both the radioactive bands increased with the increased times of 125I-FVIIa incubation with cells. Overall, the data coupled with the above observations provide strong evidence for a time-dependent translocation of FVIIa molecules into the nuclear fraction.

**Cytosolic and Nuclear Targets of FVIIa—**The biochemical data show that the internalized FVIIa translocates to the nuclear fraction. Although these data have been consistent with both crude and purified nuclei preparations, it is not possible to differentiate whether FVIIa is translocated into nuclei or associated with nuclei. We have examined these possibilities by microscopy as well as potential interaction of FVIIa with DNA and cytoskeleton proteins.

Our initial attempts to monitor intracellular distribution of FVIIa and FFR-FVIIa by immunostaining with monospecific, polyclonal antibodies against FVIIa were not successful. Therefore, in later studies, we have used FVIIa and FFR-FVIIa conjugated with fluorophores. In these studies, WI-38 cells were treated with fluorophore-labeled FVIIa (or FFR-FVIIa) (10 nM) for 2 h, surface bound FVIIa was removed by low pH buffer wash and the cells were analyzed by fluorescence microscopy. A punctate staining of FVIIa was seen in cytosolic compartment. Fluorescence was also evident in perinuclear compartment. In contrast, no fluorescence was found in nuclei (data not shown). Although it is difficult to contrast the staining pattern of FVIIa from FFR-FVIIa, it appears that more FVIIa was accumulated intracellularly compared with FFR-FVIIa.

The DNA binding properties of FVIIa were tested using human DNA isolated from hepatic cells as described under “Experimental Procedures.” We are unable to obtain any evidence for FVIIa binding to DNA. These data suggest that it is unlikely that FVIIa is a DNA-binding protein.
To test FVIIa binding to cytosolic proteins, we used methanol fixed and permeabilized WI-38 cells grown in 96-well microtiter plates. Binding characteristic of FVIIa to the permeabilized cells was different from that of FVIIa binding to intact cells. For example, FVIIa binding to intact WI-38 cells was mainly through its binding to cell surface TF, as evidenced by the observation that anti-TF IgG blocked the FVIIa binding to intact cells by about 70–80%. In contrast, only less than 50% of the FVIIa binding to permeabilized cells was blocked by anti-TF IgG. In contrast, addition of a 50-fold molar excess of unlabeled FVIIa fully blocked (by 80% or more) the binding of radiolabeled FVIIa to permeabilized cells (data not shown). These data suggest that FVIIa may interact specifically with one or more intracellular proteins.

Actin, Tubulin, and β-Casein Inhibit FVIIa Binding to Permeabilized Cells—To identify intracellular FVIIa-binding sites, [125I]-FVIIa (10 nM) was incubated with methanol-fixed cells pretreated with anti-TF antibody. Different concentrations (0–20 μM) of purified actin, tubulin, along with other cytosolic, extracellular, and plasma proteins (cytochrome C, ovalbumin, myoglobin, thyroglobulin, fibronectin, gelatin, IgG, glucose oxidase, β-casein, chicken albumin, DNase I) were included in the buffer as potential competitors. We found that two of the tested proteins, actin and tubulin, had the most profound effect on FVIIa binding to permeabilized/fixed cells (Fig. 7). Both the proteins inhibited FVIIa binding to permeabilized cells in a dose-dependent manner. β-Casein also inhibited FVIIa binding to permeabilized cells, but a 10- or more-fold higher concentration of the protein is required to get the same level of inhibition that was observed with actin or tubulin. Other proteins tested had no effect on FVIIa binding to permeabilized cells (data not shown).

FVIIa Binds to Immobilized Actin and Tubulin in the Ca²⁺-Dependent Mode—To confirm the above data that FVIIa can bind to cytoskeletal proteins, we investigated the FVIIa binding to immobilized actin, tubulin, and fibronectin (as a control) in the presence and absence of calcium ions. The data revealed that both FVIIa and FFR-FVIIa specifically bound to the immobilized actin and tubulin (data not shown) but not to fibronectin. However, the amount of FFR-FVIIa bound to the immobilized actin and tubulin was substantially lower (less than 20%) than FVIIa bound to these immobilized ligands. FVIIa binding to actin and tubulin is fully dependent on calcium ions as no FVIIa bound to these ligands in the absence of calcium.

**FIG. 6.** SDS-polyacrylamide gel electrophoresis of [125I]-FVIIa or [125I]-FFR-FVIIa with the nuclear fraction. Confluent monolayers of WI-38 cells were incubated with [125I]-FVIIa or [125I]-FFR-FVIIa (10 nM) at 37 °C for varying times. At specific time intervals, nuclear fractions were isolated and subjected to nonreducing SDS-PAGE, followed by autoradiography. The arrow indicates the boundary between the stacking and separating gels. It should be noted that the amount of protein loaded on SDS-PAGE for [125I]-FFR-FVIIa samples was three times more than was loaded for [125I]-FVIIa samples (to determine whether there was a time-dependent increase in [125I]-FFR-FVIIa in the nuclear fraction).

**FIG. 7.** Inhibition of FVIIa binding to permeabilized fibroblasts by actin and tubulin. Methanol fixed monolayers of WI-38 cells were incubated with [125I]-FVIIa (10 nM) in the presence of varying concentrations of actin, tubulin, or β-casein for 1 h at 37 °C. At the end of 1 h, unbound FVIIa was removed and the cell associated radioactivity was determined by eluting the bound FVIIa with trypsin-EDTA solution and counting the radioactivity as described under “Experimental Procedures.”
ated with the nuclear fraction, one could hypothesize that the molecular events leading eventually to cytosolic translocation and nuclear association of FVIIa can be mediated by the active site interactions of FVIIa, which is blocked in FFR-FVIIa. However, at present, it is unclear how FVIIa’s active site could influence its intracellular trafficking. At least two possibilities exist. It is possible that some of the signaling events induced by FVIIa via a proteolysis-dependent signaling pathway, a pathway that cannot be activated by FFR-FVIIa, may modulate intracellular vesicle trafficking of the internalized molecules. A number of recent studies show that FVIIa and not FFR-FVIIa induces various signaling responses, such as calcium mobilization (25), p44/42 MAP kinase activation (26), and gene induction (27, 28). It has been shown recently that the binding of FVIIa, but not FFR-FVIIa, to TF in human fibroblasts induced the production of inositol 1,4,5-triphosphate and activation of phospholipase C (29). Phospholipase activation may potentially increase endosomal leakage or “vesicular escape” of the endocytosed ligand into cytosol (30). We have attempted to test whether FVIIa protease-induced signaling may be responsible for differences in intracellular distribution of FVIIa and FFR-FVIIa. For this, fibroblasts were treated with thrombin, PAR1, or PAR2 peptide agonists, which activate PAR1 or PAR2-mediated signaling pathways, the presumed pathway(s) activated by FVIIa (31, 32). However, these treatments did not abolish the differences in intracellular trafficking of FVIIa and FFR-FVIIa (data not shown).

The other possibility is that the active site of FVIIa is required for a complex formation with a specific cell surface or intracellular protein, such as Kunitz-type inhibitors, like TFPI, APP. Participation of TFPI is unlikely, because extracellularly added TFPI or TFPI-Xa did not increase nuclear association of FVIIa (data not shown) despite the increased internalization of the inhibitory complex as observed in previous studies (20). Furthermore, treatment of cells with anti-TFPI IgG failed to abolish the association of FVIIa with the nuclear fraction. It was shown that APP could bind FVIIa and TF-FVIIa (33); and at least in neuronal cells, a fraction of APP is associated with cytoskeleton and co-isolates with detergent-insoluble fraction (24). However, the observation that FVIIa associated with nuclear fraction is functionally fully active makes it unlikely that the internalized FVIIa is complexed with APP, since APP binding to FVIIa at its active site renders FVIIa inactive.

It was shown that fibroblast growth factor receptors, internalized upon their complex formation with fibroblast growth factor, are anchored to cytoskeleton and co-isolate with nuclei (34). By contrast to these receptors, FVIIa receptor, i.e., TF, was not associated with the nuclear fraction. These data suggest that the internalized FVIIa-TF complex dissociates quickly, and the dissociated FVIIa and TF follow different routes of intracellular trafficking. This notion is consistent with our earlier observations that showed almost all the internalized TF rapidly recycles back to the cell surface whereas a majority of the internalized FVIIa is degraded (20, 21).

A possible mechanism for FVIIa association with the nuclear fraction could be the cytosolic translocation of endocytosed FVIIa, followed by its direct binding to one or more nuclear proteins or protein-associated cytoskeletal structures, which associate with nuclei. Confocal microscopy studies suggest that the latter possibility is probably true since no clear signal of fluorescence was observed in nuclei of fibroblasts that were incubated with FVIIa tagged with a fluorophore. However, at present we cannot completely eliminate the possibility that a fraction of internalized FVIIa enters into nuclei since we have observed moderate to minimal levels of FVIIa fluorescence in some experiments and detection limits of internalized FVIIa by microscopy could be lower than that could be achieved with a radioligand.

Despite the physiological importance of cytosolic translocation of exogenous proteins and demonstration that some exogenous proteins can translocate across the plasma membrane and exert their effects in nuclei or cytosol, the pathways leading to cytosol are not completely understood. It was shown that some endocytosed viral proteins, like adenovirus pentone base (35), could disrupt the endosomal membrane and reach cytosol by “endosomal escape.” Sequence analysis did not show similarity of FVIIa to any of the peptides capable of disrupting the endosomal membranes, thus it is unlikely that this mechanism may be involved in the cytosolic or nuclear translocation of FVIIa. Lysosomal membranes can be disrupted, probably upon activation of cytosolic phospholipases (30). The plasma membrane ruffling and actin cytoskeleton reorganization in dendritic cells results in macropinocytosis and cytosolic delivery of antigen (36, 37). Because FVIIa, and not FFR-FVIIa, may induce phospholipase C, activation of phosphatidylinositol 3-kinase and forming of filopodia, lamellopodia, and membrane ruffles in fibroblasts (29, 38), the possibility does exist that these processes lead to cytosolic translocation and nuclear association of FVIIa. Whether these mechanisms are involved in FVIIa translocation need to be studied.

To identify the possible targets of FVIIa in the cytosol, we tested FVIIa binding to major cytosolic proteins. Our data clearly show that FVIIa can interact with ATP, GTP-bound proteins such as actin and tubulin, respectively; or phosphoserine-containing proteins, as exemplified by interaction with β-casein. Although apparent affinity constants for these interactions are in the micromolar range, which is 3 orders of magnitude higher as compared to FVIIa interaction with TF, the abundance of binding sites (for example, millimolar concentration of actin versus picomolar concentration of TF), can make these interactions physiologically important. The possible interaction of FVIIa with cytoskeletal proteins could be potentially important. In vascular damage, plasma factor VII(a) becomes exposed not only to its cell surface receptor TF, but also to cytosolic proteins of damaged cells. One consequence of these interactions is possible accumulation of FVIIa at the site of injury, which may not only participate in the coagulation process but also in processes other than coagulation. FVIIa bound to proteins associated with extracellular matrix can ligate cellular TF, which could lead to TF-tail dependent signaling (2).

In conclusion, the present studies show clear differences between FVIIa and FFR-FVIIa in their recycling and intracellular compartmentalization, and suggest that the internalized FVIIa could specifically bind to cytoskeletal proteins. The mechanism by which FVIIa translocates across the membrane, the role of FVIIa’s active site in this mechanism, the nature of FVIIa binding to cytoskeletal proteins, and the physiological consequences of this interaction remain to be investigated in future studies.

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