Coagulation Factor XIa Cleaves the RHDS Sequence and Abolishes the Cell Adhesive Properties of the Amyloid β-Protein*

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Amyloid β-protein (Aβ) is the major constituent of senile plaques and cerebrovascular amyloid deposits in Alzheimer’s disease and is proteolytically derived from its transmembrane parent protein the amyloid β-protein precursor (AβPP). Although the physiological role(s) of secreted AβPPs are not fully understood, several potential functions have been described including the regulation of hemostatic enzymes XIa and IXa and a role in cell adhesion. In the present study, we investigated the proteolytic processing of AβPP by factor XIa (FXIa). Incubation of the human glioblastoma cell line U138 stably transfected to overexpress the 695 isoform of AβPP with FXIa (2.5–5 nM) resulted in proteolytic cleavage of secreted AβPP. Higher concentrations of FXIa (>25 nM) resulted in loss in cell adhesion. Coincubation of FXIa with purified, recombinant Kunitz protease inhibitor domain of AβPP blocked both the proteolytic processing of AβPP and the loss of cell adhesion. The RHDS cell adhesion site of AβPP resides within residues 5–8 of the Aβ domain. Incubation of synthetic Aβ1–40 peptide with increasing concentrations of FXIa resulted in cleavage of Aβ between Arg5 and His6 within the cell adhesion domain of the peptide. FXIa-digested Aβ1–40 or AβPP res res AβPP lost their abilities to serve as cell adhesion substrates consistent with cleavage through this cell adhesion site. Together, these results suggest a new potential biological function for FXIa in the modulation of cell adhesion. In addition, we have shown that FXIa can proteolytically alter Aβ and therefore possibly modify its physiological and perhaps pathological properties.

Deposition of the amyloid β-protein (Aβ)1 in senile plaques in the neuropil and in the walls of cerebral blood vessels is a pathologic feature of Alzheimer’s disease. Aβ is a 39–42-amino acid protein that is proteolytically derived from its transmembrane parent protein, amyloid β-protein precursor (AβPP) (1–4). AβPP is a multidomain protein that can be translated from alternatively spliced transcripts from a single gene located on chromosome 21 (5–11). The major mRNA species encode proteins of 695, 751, and 770 amino acids. The latter two isoforms contain a 56-amino acid domain that is homologous to Kunitz-type serine protease inhibitors (KPI) (9–11). These isoforms are identical to the cell secreted inhibitor identified as protease nexin-2 (PN-2) (12, 13). Secretory cleavage of AβPP occurs within the Aβ domain, and therefore, processing through this pathway precludes Aβ formation (14, 15).

Although the physiological roles of secreted AβPP are not fully understood, several potential functions have been ascribed to it. For example, several laboratories have provided evidence that AβPP can mediate both cell-cell and cell-surface adhesion in neural and non-neural cells (16–20). Recently, AβPP has been shown to be involved in regulating intracellular calcium levels in neurons, thus providing protection to these cells (21, 22). Both the cell adhesion and neuroprotective activities have been localized to the carboxy-terminal region of the secreted AβPP (17, 21, 22). In addition, growth-promoting activities of AβPP in non-neural and neural cells have been reported (23, 24). The region responsible for this autocrine activity has been identified as the sequence RERMS located in the middle portion of AβPP (24).

Secreted forms of AβPP that contain the KPI domain have been shown to inhibit several different serine proteases, which include trypsin, chymotrypsin, and coagulation Factors XIa and IXa (12, 25–29). Through inhibition of these latter two proteases, it has been suggested that the KPI-containing isoforms of AβPP may play a role in regulating hemostasis by acting as an anticoagulant (30). Factor XIa (FXIa) participates in the middle phase of the intrinsic pathway of blood coagulation by converting the zymogen Factor XI to the active serine protease Factor IXa. Factor IXa then converts Factor X to Factor Xa, the next serine protease to participate in the coagulation cascade ultimately leading to fibrin clot formation (31).

In the present study, we investigated the proteolytic processing of AβPP by FXIa. We report here that FXIa can cleave the RHDS sequence in the Aβ domain, altering the cell adhesive properties of both Aβ and secreted AβPP. These data suggest a new potential biological function for FXIa in the modulation of cell adhesion. These findings also indicate that the KPI domain of AβPP can regulate the activity of a protease that can process the Aβ domain, thus altering its potential physiological and pathological properties.

**EXPERIMENTAL PROCEDURES**

Materials—Purified human FXIa was purchased from Enzyme Research Laboratories. Purified α-thrombin (3554 units/mg) was purchased from Calbiochem. Dulbecco’s modified Eagle’s medium, fetal bovine serum, and Genetin were obtained from Life Technologies, Inc. Aβ1–40 was synthesized and structurally characterized as described previously (32). The recombinant KPI domain of AβPP was expressed and purified as described (33). Purified secreted AβPP was prepared in a baculovirus system (34) and was kindly provided by Drs. L. Gregori and D. Goldgaber of the State University of New York at Stony Brook.
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The anti-FN-2/AβPP monoclonal antibody (mAb) P2–1 was prepared as previously reported (12). mAb 6E10 to Aβ was a generous gift from Dr. K. Kim. Hybrid nitrocellulose membranes, peroxidase-coupled secondary antibodies, and enhanced chemiluminescence reagents were purchased from Amersham Corp.

Cells—The human glioblastoma cell line U138 stably transfected to overexpress the 695-amino acid isoform of AβPP (U138/695) was generously provided by Dr. M. Murphy of the Salk Institute Biotechnology/Industrial Associates, Inc. These cells were kindly provided by Dr. M. Murphy of the Salk Institute Biotechnology/Industrial Associates, Inc. These cells were routinely cultured in Dulbecco's modified Eagle’s medium containing 2 mM glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin sulfate, nonessential amino acids, 10% fetal bovine serum, and 20 μg/ml of Geneticin.

Effects of Factor XIa on Proteolytic Processing of AβPP—U138/695 cells were grown to near confluency in 12-well tissue culture plates in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. At 85% cell density, the medium was changed to serum-free Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin, and then incubated in 1 ml of the same medium for 1 h. Then the medium was removed, replaced with 0.5 ml of serum-free medium and then incubated in 1 ml of the same medium for 3 h. In parallel experiments, cultures were treated with either 50 nM FXIa, 100 nM FXIa, and 100 nM KPI, or 100 nM thrombin for 18 h at 37°C. In parallel experiments, cultures were treated with either 50 nM FXIa, 100 nM FXIa, and 100 nM KPI, or 100 nM thrombin for 18 h at 37°C. The medium from control and treated cultures was collected, and the cells were solubilized as described previously (35). All samples were stored at −30°C until immunoblotting analysis.

Proteolytic Processing of Aβ by FXIa—Synthetic Aβ(1–40) (25 μM) was incubated with increasing concentrations of FXIa for 24 h at 37°C in serum-free medium. In a similar manner, Aβ(1–40) was also treated with either 50 nM FXIa, 100 nM KPI, or 50 nM FXIa and 100 nM KPI. Aliquots of the digested peptide were analyzed by immunoblotting as described below.

Immunoblotting—For analysis of secreted and cellular AβPP proteins, aliquots of culture medium samples or cell lysate samples were separated by electrophoresis on nonreducing SDS 10% polyacrylamide gels (36). For analysis of Aβ(1–40) peptides, samples were electrophoresed on Tris/Tricine/SDS 10–20% gradient polyacrylamide gels. The proteins were then transferred onto Hybrid membranes and probed using mAb P2–1 (5 μg/ml) or mAb 6E10 (1:3000) for detection of AβPP or Aβ, respectively. Bound mouse monoclonal antibody was detected using a peroxidase-coupled sheep anti-mouse IgG (1:1000). Immunoreactivity was detected by enhanced chemiluminescence followed by exposure to Kodak X-Omat film.

Amino Acid Sequence Analysis—Synthetic Aβ(1–40) (10 μg) was incubated with 100 nM FXIa at 37°C for 24 h. Aliquots of the digested Aβ(1–40) fragment was gel-purified by electrophoresis on a Tris/Tricine/SDS 10–20% polyacrylamide gel. The peptide was transferred to a polyvinylidene difluoride membrane and subjected to automated sequential Edman degradation analyses using on a 475A protein sequence.

Cell Attachment Assay—35-mm plastic dishes were coated by evaporation overnight at 37°C with the appropriate substrate diluted in 0.1 × phosphate-buffered saline. The substrates were used were 2 μg Aβ(1–40), 400 ng FXIa; 65 ng KPI; 2 μg Aβ(1–40), and 65 ng KPI; 400 ng FXIa and 65 ng KPI; 2 μg Aβ(1–40) digested with 400 ng FXIa for 48 h at 37°C; 2 μg Aβ(1–40) digested with 400 ng FXIa and then incubated with 65 ng KPI for 2 h at 37°C; 10 μg of purified AβPP695; 10 μg of AβPP695 digested with 200 ng of FXIa then incubated with 65 ng KPI for 2 h at 37°C; and 10 μg of AβPP695 digested with 200 ng of thrombin. Exponentially dividing U138/695 cells were collected and suspended in serum-free medium. A 2-ml suspension containing 3.4 × 10⁵ cells was pipetted into the coated dishes and incubated for 1 h at 37°C. Nonadherent cells were removed by two gentle washes with serum-free culture medium. Adherent cells were then photographed using an Olympus phase contrast microscope. Cells were counted from 3–5 fields for each condition tested.

RESULTS

FXIa Induces Proteolytic Processing of Secreted AβPP in Cultured U138/695 Cells—We examined the effects of FXIa on AβPP processing in cultured U138/695 cells that have previously been shown to overproduce the AβPP695 isoform by 80-fold over untransfected glioblastoma cells (35). Because AβPP695 lacks the KPI domain, the effects of FXIa could be tested without intrinsic inhibition by the PN-2 form of AβPP. Proteolytic processing of secreted AβPP was observed at 2.5 nM FXIa as evidenced by the appearance of a truncated secreted AβPP of ~85 kDa in the culture media (Fig. 1A). At 50 nM FXIa, >90% of the secreted AβPP was cleaved. With higher concentrations of FXIa, additional cleavage sites were utilized as suggested by the appearance of additional smaller AβPP fragments. Cellular AβPP was not affected (data not shown).

It was noted that the ~85-kDa truncated secreted AβPP was very similar in size to that we and others have shown to be generated by thrombin proteolysis (35, 37, 38). Therefore, we compared the effects of 50 nM FXIa and 100 nM thrombin on AβPP proteolysis in U138/695 cells (Fig. 1A). The amino acid sequence at the thrombin cleavage site in AβPP was determined to be EPR with cleavage occurring on the carboxyl side of the arginine residue (37). This is the same sequence as the synthetic chromogenic substrate used to assay FXIa activity in vitro (39). Based on sequence similarity and the size of the truncated secreted AβPP protein, the initial cleavage site utilized by FXIa is most likely the same as the thrombin cleavage site located at Arg¹⁰–Ile¹¹ of the AβPP695 sequence. FXIa cleavage of secreted AβPP695 could be inhibited when FXIa was preincubated with 100 nM KPI (Fig. 1B).

Factor Xla Alters Cell Adhesion of U138/695 Cells—In the course of studying the effects of increasing concentrations of FXIa on the proteolytic processing of AβPP695, we observed that the cells lost adherence after 18 h when treated with 5–25 nM FXIa. Many of the cells that remained were rounded and no longer showed their characteristic elongated morphology. After an 18-h incubation with 50 nM FXIa, less than 20% of the cells

Fig. 1. Immunoblot analysis of secreted AβPP in cultured medium from U138/695 cells incubated with FXIa. A, near confluent cultures of U138/695 cells were incubated with increasing concentrations of FXIa for 18 h. The concentrations of FXIa used were none (lane 1), 2.5 nM (lane 2), 5 nM (lane 3), 25 nM (lane 4), 50 nM (lane 5), 250 nM (lane 6), and 100 nM thrombin (lane 7). B, near confluent cultures of U138/695 cells were incubated alone (lane 1) or with 50 nM FXIa (lane 2), 50 nM FXIa and 100 nM KPI (lane 3), 100 nM KPI (lane 4), or 100 nM thrombin (lane 5) for 18 h. Aliquots of culture medium were analyzed for secreted AβPP by SDS-PAGE and subsequent immunoblotting with mAb P2–1 as described under "Experimental Procedures."
remained adhered to the culture dish (Fig. 2B). In parallel experiments, similar concentrations of thrombin (Fig. 2E) and coagulation Factors IXa and Xa (data not shown) had no effect on cell adhesion. In addition, these effects of FIXa treatment could be inhibited by the addition of 100 nM KPI to the FIXa prior to incubation with the cells (Fig. 2D). Similar but less robust losses in cell adhesion were observed in the untransfected parental glioblastoma cell line, a neuroblastoma cell line, and cultured cerebrovascular smooth muscle cells (data not shown).

**FXIa Cleaves the Cell Adhesion RHDS Sequence of Aβ—**A cell adhesion domain has been localized to the RHDS sequence of the Aβ domain located at the carboxyl terminus of secreted AβPP (18). To determine if disruption of this domain by FIXa is responsible for the loss of adherence observed in the FIXa-treated U138/695 cells, we examined the effect of FIXa on a synthetic Aβ1–40 peptide. With increasing FIXa concentrations, we detected a decrease in Aβ immunoreactivity using mAb 6E10 (Fig. 3A). At 50 nM FIXa, >80% of Aβ immunoreactivity was lost. In parallel experiments, neither thrombin nor KPI-treated FIXa had any effect on Aβ immunoreactivity (Fig. 3B).

To further identify the site of FIXa cleavage within Aβ1–40, 10 μg of the peptide was digested with 100 nM FIXa for 48 h. The digested peptide was analyzed on a Tris/Tricine/SDS 10–20% polyacrylamide gel. The Coomassie-stained gel revealed a truncated ~3.4-kDa Aβ fragment (Fig. 4). In parallel experiments, the truncated Aβ peptide was transferred to a polyvinylidene difluoride membrane and subjected to amino-terminal sequence analysis. The resulting sequence derived from five cycles of sequential Edman degradation is shown in Table I. The amino-terminal sequence of the truncated Aβ peptide identified the FIXa cleavage between Arg5-His6 within the RHDS cell adhesion domain.

**FXIa Cleavage of the RHDS Sequence Abolishes the Cell Adhesion Domain**—A truncated 3.4-kDa Aβ1–40 peptide was produced by FIXa (Fig. 4). In parallel experiments, neither thrombin nor KPI-treated FIXa had any effect on Aβ immunoreactivity (Fig. 3B). To determine if disruption of the cell adhesion domain by FIXa is sufficient to promote cell adhesion, we compared untreated and FIXa-digested Aβ1–40 as substrates in cell-surface adhesion. The U138/695 cells showed enhanced binding to the plastic culture dish treated with Aβ1–40 by nearly 20-fold over cells plated on 0.1% phosphate-buffered saline-treated salinized-treated plastic dishes (Fig. 5A). However, when FIXa-digested Aβ1–40 was used as a substrate, cell adherence was abolished (Fig. 5A). In control experiments, the numbers of cells adhered to FIXa, KPI, or FIXa and KPI closely resembled the numbers of cells on phosphate-buffered saline-treated plastic (Fig. 5A). Similar to Aβ1–40, cells adhered to purified AβPP, but not FIXa-digested AβPP, showed enhanced binding to the plastic culture dish treated with Aβ1–40 (Fig. 5B). It is noteworthy that cells adhered to thrombin-digested AβPP, but not FIXa-digested AβPP, showed enhanced binding to the plastic culture dish treated with AβPP (Fig. 5B). No significant differences in numbers of adhered cells using fibronectin or FIXa-treated fibronectin were observed in parallel experiments (data not shown).

**DISCUSSION**

The present studies show that FIXa, a target protease for inhibition by the KPI domain of AβPP, proteolytically cleaves secreted AβPP from U138/695 cells. Initial cleavage at low FIXa concentrations (~2.5 nM) occurred at the site previously reported for thrombin cleavage at Arg510-Ile511 of AβPP, It is noteworthy that the amino acid sequence at the thrombin cleavage site in AβPP is Glu508-Pro509-Arg510, the same sequence as the synthetic chromogenic substrate used to assay FIXa activity (22). The present studies show that FIXa cleaves the cell adhesion RHDS sequence of Aβ, a cell adhesion domain that has been localized to the RHDS sequence of the Aβ domain located at the carboxyl terminus of secreted AβPP (18). To determine if disruption of this domain by FIXa is responsible for the loss of adherence observed in the FIXa-treated U138/695 cells, we examined the effect of FIXa on a synthetic Aβ1–40 peptide. With increasing FIXa concentrations, we detected a decrease in Aβ immunoreactivity using mAb 6E10 (Fig. 3A). At 50 nM FIXa, >80% of Aβ immunoreactivity was lost. In parallel experiments, neither thrombin nor KPI-treated FIXa had any effect on Aβ immunoreactivity (Fig. 3B).

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Amino acid sequence analysis of FXIa-cleaved AP$_{1-40}$

| Residue | AP$_{1-40}$ | FXIa-cleaved AP$_{1-40}$ |
|---------|-------------|-------------------------|
| 1       | D           | A                       |
| 2       | A           | E                       |
| 3       | E           | F                       |
| 4       | F           | R                       |
| 5       | R           | H                       |
| 6       | H           | D                       |
| 7       | D           | S                       |
| 8       | S           | G                       |
| 9       | G           | Y                       |

*An ~3.4-kDa FXIa-cleaved AP$_{1-40}$ fragment was prepared and subjected to five cycles of amino-terminal amino acid sequence analysis as described under “Experimental Procedures.”

FIG. 5. Effects of FXIa on the ability of AP$_{1-40}$ and APβPP$_{695}$ to promote cell adhesion of U138/695 cells. A suspension of U138/695 cells (3 × 10^5) in serum-free culture medium were incubated at 37 °C for 1 h on 35-mm plastic dishes coated with (A) nothing (lane 1), 400 ng FXIa (lane 2), 65 ng KPI (lane 3), 400 ng FXIa and 65 ng KPI (lane 4), 2 μg AP$_{1-40}$ (lane 5), 2 μg AP$_{1-40}$ and 65 ng KPI (lane 6), 2 μg AP$_{1-40}$ digested with 400 ng FXIa (lane 7), or 2 μg AP$_{1-40}$ digested with 400 ng FXIa then incubated with 65 ng KPI (lane 8) or coated with (B) 10 μg of APβPP$_{695}$ (lane 1), 10 μg of APβPP$_{695}$ digested with 200 ng of FXIa (lane 2), or 10 μg of APβPP$_{695}$ digested with 200 ng of thrombin (lane 3). Nonadherent cells were removed as described under “Experimental Procedures.” Values are presented as the means ± S.D. percentage of adherent cells counted in 3–5 fields of view compared with AP$_{1-40}$ or APβPP$_{695}$.

FXIa activity in vitro (39). Additional cleavage sites were utilized at higher concentrations of FXIa (Fig. 1A). Several potential functions have been ascribed to secreted APβPP$_{695}$, including cell adhesion, growth supportive activity, and neuroprotection (16–24). Proteolytic processing of APβPP$_{695}$ by FXIa may disrupt one or more of these properties.

Reduced cell adhesion to the substratum was observed in FXIa-treated U138/695 cells. This may have resulted from disruption of the cell adhesion domain by FXIa. A cell adhesion site in APβPP has been localized within the amino-terminal region of the APβ domain, which is present in secreted forms of the protein (18). To test this hypothesis, synthetic AP$_{1-40}$ was digested with increasing concentrations of FXIa. The loss of APβ immunoreactivity observed in Fig. 3 was consistent with disruption of the mAb GE10 epitope, which has been mapped within the amino-terminal region of APβ (40). Accompanying the loss of APβ immunoreactivity, we observed the appearance of a truncated APβ peptide when treated with FXIa (Fig. 4). Amino-terminal sequence analysis of the first five amino acids of this truncated peptide yielded the APβ sequence HDGY (Table I), consistent with cleavage through the cell adhesion domain RHDS. It is noteworthy that the RHDS cleavage site in the APβ domain shows homology to the factor IX activation site for FXIa (32). Because cleavage through the RHDS sequence may disrupt the cell adhesion properties of the APβ domain, we compared the abilities of AP$_{1-40}$ and FXIa-cleaved AP$_{1-40}$ to serve as cell adhesion substrates. AP$_{1-40}$ promoted adhesion of U138/695 cells to the substrate by nearly 20-fold over buffer-treated substratum (Fig. 5). However, FXIa-cleaved AP$_{1-40}$ lost its ability to serve as cell adhesive substrate. Similarly, FXIa diminished the ability of secreted APβPP$_{695}$ to promote cell adhesion. These results suggest that FXIa could disrupt adhesion of cells that may use this region of the APβ domain as an extracellular matrix protein.

Together, these findings have identified a new potential function for FXIa in the modulation of cell adhesion. This suggests that in addition to a function in coagulation, FXIa may participate in other roles of wound repair and tissue remodeling at sites of vascular injury. It is noteworthy that platelets activated by physiological agonists release large amounts of PN-2/APβPP (27, 41). Therefore, PN-2/APβPP released by platelets at sites of vascular damage may participate in both the regulation of coagulation and cell adhesion. Through its intimate interactions with PN-2/APβPP, FXIa may also be involved in both of these processes. The present data also show that the KPI domain of PN-2/APβPP can regulate the activity of a target protease that possesses the ability to modulate another potential biological function on a distal region of the protein. Moreover, these findings demonstrate that the KPI domain of PN-2/APβPP can regulate the activity of a protease that can cleave the APβ peptide. Proteolytic processing of this nature could alter possible physiologic and perhaps pathologic properties of APβ.

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