Interaction of the Fibrinolytic Receptor, Annexin II, with the Endothelial Cell Surface

ESSENTIAL ROLE OF ENDONEXIN REPEAT 2*

(Received for publication, April 24, 1996)

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Endothelial cells express a surface co-receptor for plasminogen and tissue plasminogen activator (t-PA) which we recently identified as annexin II (Hajjar, K. A., J acovina, A. T., and Chacko, J. (1994) J. Biol. Chem. 269, 21191-21197). This protein enhances the catalytic efficiency of t-PA-dependent plasmin generation by 60-fold (Cesarman, G. M., Guevara, C. A., and Hajjar, K. A. (1994) J. Biol. Chem. 269, 21198-21203). Here, we demonstrate that annexin II is constitutively translocated to the endothelial cell surface within 16 h of biosynthesis, and that cell surface annexin II comprises 4.3 ± 1.0% of the total cellular pool. Exogenous 125I-annexin II bound to EGTA-washed endothelial cells with high affinity (Kd 49 nm) and in a calcium-dependent (I50 = 3 μm) phospholipid-sensitive manner. Peptides KASMKGLGTDE and YDSMKKGKTRDK, mimicking the calcium-binding "endonexin" motif (KGXGT) of annexin II, blocked its interaction with endothelial cells. Recombinant annexin II, bearing the calcium-binding site substitution DI63A of core repeat 2, failed to compete with binding of the wild type protein to the cell surface, while E246A and D321A mutants, corresponding to core repeats 3 and 4, behaved as effective competitors. These data suggest that translocated annexin II interacts with cell surface phospholipid via a high affinity calcium-dependent binding site that includes residues 118-122 (KGLGT) and the coordinating Asp161 of core repeat 2. Thus, calcium-regulated expression of annexin II on the endothelial cell surface may play a central role in control of plasmin-mediated processes.

*This work was supported in part by National Institutes of Health Grants HL 42493 and HL 46403, and by an American Heart Established Investigatorship (to K. A. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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∥The abbreviations used are: t-PA, tissue plasminogen activator; HBS, HEPES-buffered saline; HUVEC, human umbilical vein endothelial cells; IPB, immunoprecipitation buffer; PLG, plasminogen, PBS, phosphate-buffered saline; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.

genesis, and activation of growth and differentiation factors (1). Recently, we identified annexin II as an endothelial cell surface co-receptor for plasminogen (PLG) and t-PA (3). When overexpressed in a renal epithelial cell line, this protein was translocated to the cell surface, conferred the capacity to bind both t-PA and PLG, and enhanced cell surface plamin generation (3). Upon treatment of human endothelial cells with either EGTA, polyvalent anti-annexin II IgG, or antisense oligonucleotides directed against annexin II mRNA, binding of both t-PA and PLG decreased by approximately 50%, suggesting that annexin II accounts for about one-half of available endothelial cell binding sites for these ligands (3). Purified annexin II, moreover, specifically enhanced the catalytic efficiency of t-PA-dependent PLG activation by 60-fold in a cell-free system (4).

The annexins represent a family of some 13 calcium-dependant phospholipid-binding proteins, at least 10 of which occur in mammals (5, 6). All annexins possess a variable amino-terminal "tail" domain followed by a conserved "core" region that imparts membrane-binding capability (7). The latter consists of either four or eight homologous "endonexin" motifs which contain the general consensus sequence (L/M)-K-G-T-(D/E) (8).

Recent crystallographic studies have served to identify potential calcium-dependent phospholipid-binding sites of annexin V and annexin I (9, 10). The pentapeptide sequence K-G-X-G-T-(38 residues)-(D/E) (8).

In the present study, we examined the mechanism by which annexin II associates with the endothelial cell surface. The data indicate that this interaction is an equilibrium-based, calcium-dependent binding event, with an absolute requirement for endonexin repeat 2. These findings suggest that calcium-mediated binding of annexin II to the cell membrane may play a central role in the control of cell surface plasminogen activation.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, EGTA, CaCl2•H2O, lactic dehydrogenase assay kit (no. 340-LD), MgCl2, phosphatidic acid (P9511), 21652

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phosphatidylcholine (P2772), phosphatidylethanolamine (P7943), phosphatidylinositol (P8443), and phosphatidylserine (P7769) were obtained from Sigma. MnCl₂, NaCl, SrCl₂, H₂O, ZnSO₄, 7H₂O, and CuSO₄·5H₂O were purchased from Fisher. p-Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine were obtained from Bio-Rad. Tris base was purchased from Polyscience. Na₂HPO₄ from ICN, and o-Val-Leu-Lys-aminofluoromethylcoumarin from Enzyme Systems Products. Phosphatidylinositol-specific phospholipase C was obtained from Boehringer Mannheim. Anti-annexin I, II, IV, VI, and p11 monomodal IgG were from Zymed.

Purified Proteins—Native annexin II was purified from human placental membranes and characterized as described previously (4). This protein reacted strongly with antibody to annexin II, but not annexins I, IV, or VI. Purified annexin II was specifically found to be free of plasminogen activator activity in a fluorogenic assay as described previously (4). Purified recombinant wild type and mutant annexin II (D161A, E264A, and D321A) were a generous gift from Dr. Volker Gerke (University of Munster, Munster, Germany). These have been extensively characterized in previous studies (12, 13).

Recombinant Annexin II—Using the wild type pCMV5-Ann-II plasmid (3) as a template, polymerase chain reactions were carried out using 26-mer oligonucleotide forward and reverse primers and polymerase. Primers (5'-AAAAACTCGAGGTCATCTCCACCACA-3') corresponded, respectively, to bases 52–66 and 1052–1066, numbered according to Gerke (University of Munster, Munster, Germany). These have been extensively characterized in previous studies (12, 13).

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of alkaline phosphatase-conjugated goat anti-rabbit IgG (1:500, 30 min, 4°C). The wells are washed again five times (PBS, 0.5% Tween 20), and incubated with 1 mg/ml of annexin II (200 nM) at 4°C. The reaction mixture consisted of 7.5 μM purified native annexin II, 1.3 μM EDTA, 22 units/ml lactoperoxidase, 70 μM H2O2 in PBS. The reaction was stopped with excess KI, and bound 125I was separated from unbound on a Sephadex G-25 (PD-10) column. The structural and functional integrity of labeled annexin II was verified by SDS-PAGE/autoradiography and by plasmin generation assay (4).

Binding Assays—Confluent HUVEC monolayers (P2–4) in 24-well plates were equilibrated to 4°C (5 min), washed twice with IB(5) incubation buffer 5: 11 mM Hepes, 137 mM NaCl, 4 mM KCl, 3 mM CaCl2, 1 mM MgCl2, 1 mM glucose, 5 mg/ml bovine serum albumin), and incubated at 4°C with anti-annexin II (100 nM), EGTA eluates from a fresh, intact umbilical vein (lane 3), and authentic native annexin II (lane 4) were probed with monoclonal anti-annexin II (lane 1), anti-annexin I (lane 2), anti-annexin IV (lane 3), anti-annexin VI (lane 4), or anti-p11 (lane 5). C, cell specificity. EGTA eluates of HUVEC were probed with monoclonal anti-annexin II (lane 1), anti-annexin I (lane 2), anti-annexin IV (lane 3), anti-annexin VI (lane 4), or anti-p11 (lane 5).

RESULTS

Expression of Annexin II on the Endothelial Cell Surface—In previous studies, we and others have demonstrated the presence of annexin II on the surface of cultured human umbilical vein endothelial cells using a variety of methods including flow cytometry, immunoprecipitation of surface labeled protein, and radioantibody binding analyses (3, 24, 25). In the present study, our goal was to characterize the mechanism by which annexin II, a protein lacking a classical transmembrane domain, associates with the endothelial cell surface. To test the potential role of calcium-regulated phospholipid binding domains of annexin II, HUVEC were treated with (Fig. 2, panels B and D) or without (panels A and C) 10 mM EGTA and then incubated at 4°C with anti-annexin II (panels A and B) or anti-annexin VI (panels C and D) monoclonal antibody followed by fluorescein isothiocyanate-conjugated goat anti-mouse IgG at 4°C. While cells treated with anti-annexin II in the absence of EGTA displayed positive immunofluorescence (panel A), cells pretreated with EGTA showed a 7-fold reduction in cell surface immunofluorescence (panel B) (mean relative fluorescence 1.10 and 0.14, respectively). Control HUVEC treated with anti-annexin VI showed low level fluorescence regardless of whether EGTA treatment was included in the protocol (panels C and D) (mean relative fluorescence 0.34 and 0.14, respectively). These data indicate that annexin II is expressed on the endothelial cell surface in a calcium-dependent manner.

To further characterize anti-annexin II cross-reactive material on the surface of endothelial cells, EGTA eluates of HUVEC monolayers were analyzed by Western blotting (Fig. 3). As an index of cell lysis, EGTA eluates were assayed for lactate dehydrogenase activity. Lactate dehydrogenase activity in cell surface eluates did not differ from that in cell-free controls or in HBS cell surface eluates (9.0 ± 2.4 units/ml, 8.9 ± 2.9 units/ml, and 8.8 ± 2.6 units/ml, respectively, S.D., n = 3). Lactate dehydrogenase values were routinely less than 1% of the maximal release induced by freeze-thaw cell lysis (975 ± 47 units/ml, S.D., n = 3). Similarly, release of 51Cr from EGTA-treated endothelial cells did not differ from the spontaneous rate of release (12.4 ± 0.8 versus 9.8 ± 3.4% of maximal, respectively, S.D., n = 3). These data indicated that the presence of annexin II in EGTA eluates was not the result of cell lysis. EGTA eluates (Fig. 3A, lane 2) and HBS eluates (lane 1) from HUVEC contained a ~36-kDa protein which reacted specifically with a monoclonal antibody directed against annexin II. This band co-migrated with authentic native annexin II (lane 4). A similar band was observed in eluates of fresh, intact

![Fig. 2. Flow cytometric analysis of cultured endothelial cells.](http://www.jbc.org/)

![Fig. 3. Immunoblot analysis of Ca2+-dependent cell surface proteins.](http://www.jbc.org/)
umbilical vein (lane 3), suggesting that cultured HUVEC accurately reflect expression of annexin II on the surface of blood vessels in situ. When EGTA eluates of HUVEC were tested for reactivity with monodonal antibodies directed against annexins I, IV, VI, or p11, an annexin II-associating protein, no reactivity was observed (Fig. 3, lanes 2–5), although the same samples did react with anti-annexin II (Fig. 3, lane 1). In addition, while EGTA eluates from human smooth muscle cells (Fig. 3, lane 2), like those of HUVEC (lane 1), showed a 36-kDa cross-reactive band, eluates from HepG2 hepatoma cells and renal epithelial 293 cells did not (lanes 3 and 4).

Finally, phosphatidylinositol-specific phospholipase C (26) failed to release annexin II from HUVEC as judged by immunoblot analysis (data not shown). This result contrasted with the release of the urokinase receptor, a known glycosylphosphatidylinositol-linked protein (27) which was easily detected in the phosphatidylinositol-specific phospholipase C supernatant. Together these data suggest that HUVEC, endothelial cells in situ, and smooth muscle cells all express annexin II as a peripheral membrane protein linked to the cell surface through a calcium-dependent mechanism.

To determine whether cell surface annexin II was synthesized by the endothelial cell, metabolic labeling studies were carried out (Fig. 4). HUVEC were treated for 16 h with [35S]methionine and [35S]cysteine and then surface-eluted with HBS with (panel A) or without (panel B) 10 mM EGTA. While whole cell lysates from both groups of cells contained numerous labeled bands upon SDS-gel fluorography (lanes 1 and 5), only the EGTA eluates (lane 2), and not HBS eluates (lane 6), contained labeled bands. When the same eluates were immunoprecipitated with polyclonal anti-annexin II IgG conjugated to Sepharose beads, a ∼36-kDa band was recovered from EGTA eluates (lane 4), but not from the HBS samples (lane 8). This band was not recovered upon precipitation with preimmune IgG-conjugated beads (lanes 3 and 7). These data demonstrate that cell surface annexin II is synthesized by cultured endothelial cells and translocated to the cell surface within 16 h.

To quantify the relative amounts of annexin II both on the cell surface and within the cell, a competitive ELISA was developed (20). A standard curve was linear at concentrations of purified native annexin II up to ∼250 nM (r = 0.98) and displayed a lower detection limit of ∼0.5 ng/well. While cell lysates from ∼4 x 10⁷ HUVEC contained annexin II in a concentration of 2.5–5.3 μM, EGTA eluates contained 70–190 nM annexin II. HBS eluates, prepared in parallel, routinely showed no detectable antigen. EGTA-elutable cell surface annexin II represented 4.3 ± 1.0% (mean ± S.E., n = 4) of total cellular annexin II.

Equilibrium Binding of Purified Annexin II to Cultured Endothelial Cells—To further characterize the interaction between annexin II and the endothelial cell surface, direct binding studies using radiolabeled native annexin II were conducted (Fig. 5). In time course studies, binding of a fixed concentration of [125I]-annexin II (8 nM) to EGTA-washed HUVEC reached a steady state at approximately 60 min. At least 60% of total binding was reversible upon “infinite dilution” of the unbound ligand (Fig. 5A), indicating a dynamic equilibrium between bound and unbound ligand. In addition, EGTA-treated endothelial cells bound native annexin II in a dose-dependent and apparently saturable fashion (Fig. 5B). Binding was maximal at an input dose of ∼50 nM, and approached a plateau at 75–100 nM. Scatchard analysis (28) (Fig. 5B, inset) suggested a single saturable site with Kd, 49 ± 9 nM (S.E., n = 3), and Bmax, 1,637,000 ± 403,000 (S.E., n = 3), indicating a high affinity, high capacity interaction.
Annexin II may be subject to at least four types of post-translational modification including myristoylation (29), glycosylation (30), phosphorylation (31), and proteolytic cleavage (3). Interestingly, both native and recombinant human annexin II produced in *E. coli* interacted with HUVEC with comparable affinity (Fig. 6), suggesting that cell surface binding does not require post-translational modification. Both labeled and unlabeled recombinant annexin II competed with labeled native protein for binding to HUVEC (I50; 10- and 12-fold molar excess, respectively) (Fig. 6A). Similarly, both unlabeled native and recombinant annexin II competed with the labeled recombinant protein for binding to HUVEC (I50; 5- and 8-fold molar excess, respectively) (Fig. 6B). In all cases, 60–80% of total binding was inhibited in the presence of excess competing protein. Interestingly, unlabeled recombinant annexin I also competed with native 125I-annexin II for cell surface binding (I50; 7-fold molar excess; data not shown).

Binding of Annexin II to the Cell Surface’s Calcium-dependent—One of the salient properties of annexin II is its capacity for calcium-dependent binding to phospholipid-containing membranes (5, 6). This process is enhanced by the annexin II-binding protein, p11, and reduced upon phosphorylation of annexin II by p60src (32). We examined the mechanism of annexin II surface association in more detail. As shown in Fig. 7, binding was calcium-dependent, with maximal specific binding observed at pCa 4.0–4.5 (10–30 μM), and a nadir at pCa 6.5–8.0 (10–300 μM) (Fig. 7A). Half-maximal binding was observed at pCa ~5.5 (~3 μM), consistent with the involvement of high affinity calcium-binding sites residing within domains 2, 3, and 4 (Kd ~10 μM) (13). In the presence of other divalent cations such as Mg2+, Sr2+, Zn2+, and Cu2+, however, specific binding was no more than 16% of that observed in the presence of Ca2+ (Fig. 7B). These data indicate a specific requirement for calcium in a manner that implicates one or more high affinity endonexin repeats of the core region of annexin II.

Since calcium-binding sites regulate interactions between the annexins and cellular membranes, one would expect binding to be inhibitable by anionic phospholipid. Binding of 125I-annexin II to HUVEC was blocked in a dose-dependent manner in the presence of vesicles composed of anionic phospholipid in a 1:1 molar ratio with phosphatidylcholine (Table I). Vesicles containing phosphatidylserine or phosphatidic acid blocked approximately 50% of specific binding of native annexin II to HUVEC at concentrations of 9 and 43 μM, respectively. At a maximum dose of 125 μM, these vesicles blocked between 66 and 94% of specific annexin II binding. Phosphatidylinositol

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**Fig. 6.** Cold competition analyses. A, 125I-labeled native annexin II. Confluent HUVEC monolayers were incubated (60 min, 4°C) with 125I-labeled native annexin II (5 nM), purified from human placenta, in the presence of increasing concentrations (0–500 nM) of unlabeled native annexin II (●) or unlabeled recombinant annexin II (○). B, 125I-labeled recombinant annexin II. HUVEC were incubated (60 min, 4°C) with 125I-labeled recombinant annexin II (5 nM), purified from transformed bacterial lysates, in the presence of increasing concentrations (0–500 nM) of unlabeled native annexin II (●) or unlabeled recombinant annexin II (○).

**Fig. 7.** Divalent cation dependence of annexin II binding to HUVEC. Binding studies were conducted as described under “Experimental Procedures.” A, calcium titration. Confluent HUVEC were incubated with 125I-labeled native annexin II (329,000 cpm/pmol, 8 nM, 60 min, 4°C) in the presence of graded concentrations of Ca2+ in HBS containing 5 mg/ml bovine serum albumin (pCa 4.0–8.0). Free Ca2+ was estimated using a calcium-specific electrode as described previously (15). B, divalent cation specificity. Confluent HUVEC were exposed to 125I-labeled native annexin II (40 nM, 247,000 cpm/pmol, 60 min, 4°C) in HBS containing 5 mg/ml bovine serum albumin and 3 mM concentrations of CaCl2, MgCl2-4H2O, SrCl2-4H2O, ZnSO4-7H2O, or CuSO4-7H2O.
was somewhat less effective, inhibiting little more than 60% of specific binding with an \( I_{50} \) of 85 \( \mu \)M. The uncharged phospholipid, phosphatidylcholine, was the least effective, inhibiting only 26% of annexin II binding at doses of 100–150 \( \mu \)M. These results agree reasonably well with the reported \( K_{d} \) (1.8 \( \mu \)M) for annexin II and phosphatidylserine-containing vesicles in the presence of micromolar \( Ca^{2+} \) (6) and suggest that annexin II can target cell surface phospholipid with greatest affinity for phosphatidylserine.

**Table I**

| Phospholipid | \( I_{50}^a \) | S.E. | n | Maximum inhibition | S.E. | n |
|--------------|--------------|-----|---|-------------------|-----|---|
| PC           | 85.2         | 18.8| 5 | 60.4              | 2.9 | 5 |
| PI           | 43.0         | 9.8 | 3 | 66.3              | 4.8 | 3 |
| PA           | 8.8          | 2.3 | 5 | 93.4              | 4.2 | 5 |

\( ^a \) \( I_{50} \) is defined as that concentration of phospholipid required to inhibit 50% of annexin II binding.

**Discussion**

This study demonstrates for the first time that cell surface association of the fibrinolytic receptor, annexin II, is a high affinity binding event that specifically requires endonexin repeat 2. The data indicate that annexin II is synthesized by cultured endothelial cells and translocated within hours to the cell surface in a calcium-dependent interaction. By quantitative ELISA, cell surface annexin II appears to represent approximately 4–5% of the total endothelial cell pool. Cell surface annexin II was not detected on hepatoma cells or renal epithelial cells, but did appear on the surface of human smooth muscle cells.

As a class, the annexins were originally identified in intracellular locations and shown to be involved in membrane fusion events (5, 6). Recently, however, several annexins have been found to be localized on cell surfaces or within extracellular compartments (33). Annexin II, for example, has been identified on the surface of human colon adenocarcinoma cells (34), murine large cell lymphoma cells (35), and endothelial cells (3, 25). Annexin II, as well as annexin V, is released in micromers from chondrocytes during bone mineralization (36–38), and from skin keratinocytes (39). Annexin I is selectively secreted in high concentrations by the human prostate (40) and by inflammatory cells (41). Annexin V is found in plasma, amniotic fluid, and post-culture medium from endothelial cells and endometrium (42). The specific stimuli for these redistribution events have yet to be determined, and their elucidation may provide insight into the regulation of pericellular protease activity.

In the present study, several lines of evidence support an annexin II-cell surface interaction which is absolutely dependent upon micromolar free \( Ca^{2+} \). First, surface-associated immunoreactive material was efficiently eluted upon chelation of extracellular \( Ca^{2+} \) with EGTA (Fig. 2). Second, 36-kDa cross-reactive material that comigrated with authentic annexin II was recovered in EGTA eluates of endothelial cells both in vitro and in situ (Fig. 3). Third, metabolically labeled annexin II was
Ca\(^{5.5}\) or half-saturating calcium concentration (pEGTA-treated endothelial cells was demonstrated (Fig. 5). The 4). Fourth, calcium-dependent rebinding of annexin II to eluted from HUVEC cell surface with EGTA, but not HBS (Fig. 4). Fourth, calcium-dependent rebinding of annexin II to EGTA-treated endothelial cells was demonstrated (Fig. 5). The half-saturating calcium concentration (pCa 5.5 or \(-3 \mu M\)) agreed closely with that of the high affinity calcium-binding sites within repeats 2, 3, and 4 of the core region of annexin II (K\(_d\) 5–10 \(\mu M\) Ca\(^{2+}\)) (13), and with the calcium dependence of annexin II binding to phosphatidylserine-containing vesicles (K\(_d\) 2–5 \(\mu M\) Ca\(^{2+}\)) (32, 43). Since plasma free Ca\(^{2+}\) is \(-1 \mu M\), this interaction would be supported in vivo.

To further define the cell surface binding domain of annexin II, two types of experiments were conducted. In the first, peptides mimicking the four potential endonexin repeats of annexin II were tested for their ability to compete with the wild type protein for binding to the cell surface. Because only the mutant corresponding to repeat 2 failed to block binding (Fig. 9), the data suggest that repeat 2 plays a dominant role in mediating the cell surface interaction. This result is also consistent with our previous observation that cell surface annexin II is subject to proteolytic cleavage at Lys\(^{307}\)–Arg\(^{308}\). This modification creates a carboxyl-terminal lysine residue, enabling binding of plasminogen via lysine-binding “kringle” structures (3). Cleavage at Lys\(^{307}\)–Arg\(^{308}\) would release a carboxyl-terminal fragment (Arg\(^{308}\)–Asp\(^{328}\)) that contains Asp\(^{321}\), thereby inactivating the Ca\(^{2+}\)-binding site of repeat 4. Thus, carboxyl-terminal modification of annexin II is consistent with the hypothesis that repeat 2 is primarily responsible for Ca\(^{2+}\)-mediated cell surface binding of annexin II.

The binding isotherm depicted in Fig. 5 indicates a high affinity interaction between annexin II and the endothelial cell surface (K\(_d\) 49 \(\mu M\)). The dissociation constant is reasonably close to K\(_d\) values reported for the interaction of annexin V with platelets (7 \(\mu M\)) (44), ovarian carcinoma cells (9 \(\mu M\)) (45), or endothelial cells (16 \(\mu M\)) (46). The slightly higher affinity for annexin V, compared with annexin II, is consistent with the former’s relatively more avid binding to phospholipid (47). The preferential inhibition of binding of annexin II to endothelial cells by anionic phospholipid over neutral phospholipid most likely reflects greater affinity for phosphatidylserine over phosphatidylcholine.

In summary, the present data indicate that expression of annexin II on the endothelial cell surface results from translocation of newly synthesized annexin II, and its calcium-dependent association with outer leaflet phospholipid. This interaction requires the high affinity calcium-binding site of repeat 2. Although the mechanism of annexin II transport by the endothelial cell is unknown, several other proteins including fibroblast growth factor-1, interleukin-1\(\beta\), thioredoxin, and lectin L-29 that lack typical signal peptides, are known to undergo leaderless secretion in response to specific stimuli (48–52).

Highly regulated, nonclassical release of these proteins may represent a newly recognized form of host defense (33, 53). Since annexin II represents a major fibrinolytic receptor that

![Fig. 8. Effect of mimetic peptides on binding of annexin II to HUVEC.](Image 70x387 to 294x736)

![Fig. 9. Inhibition of annexin II binding to HUVEC by mutant annexin II proteins.](Image 317x548 to 563x736)

endothelial cell surface annexin II
co-binds plasminogen and tissue plasminogen activator and augments the efficiency of plasmin generation, it is reasonable to hypothesize that regulation of secretion and/or binding of annexin II to cell surfaces may play a central role in the control of plasmin-mediated processes.

Acknowledgments—We thank Dr. Volker Gerke for the generous gift of mutant recombinant annexin II, and Bernice Salkin for expert secretarial assistance.

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J. Biol. Chem. 1996, 271:21652-21659.
doi: 10.1074/jbc.271.35.21652

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