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Tissue Plasminogen Activator Binding to the Annexin II Tail Domain

DIRECT MODULATION BY HOMOCYSTEINE*

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Tissue plasminogen activator binds to endothelial cells via the calcium-regulated phospholipid-binding protein annexin II, an interaction that is inhibited by the prothrombotic amino acid homocysteine. We sought to identify the tissue plasminogen activator binding domain of annexin II and to determine the mechanism of its modulation by homocysteine. Tissue plasminogen activator binding to immobilized annexin II was inhibited by intact fluid phase annexin II but not by its "core" fragment (residues 25–339). Two overlapping "tail" peptides specifically blocked 65–75% of binding. Localization of the tissue plasminogen activator binding domain was confirmed upon specific inhibition by the hexapeptide LCKLSL (residues 7–12). Expressed C9G annexin II protein failed to support tissue plasminogen activator binding, while binding to C133G, C262G, and C335G was equivalent to that of wild type annexin II. Upon exposure to homocysteine, annexin II underwent a 135 ± 4-Da increase in mass localizing specifically to Cys^9 and a 60–66% loss in tissue plasminogen activator-binding capacity (I_{50} = 11 μM). Upon treatment of cultured endothelial cells with [^{35}S]homocysteine, the dithiothreitol-sensitive label was recovered by immunoprecipitation with anti-annexin II IgG. These data provide a potential mechanism for the prothrombotic effect of homocysteine by demonstrating direct blockade of the tissue plasminogen activator binding domain of annexin II.

Tissue plasminogen activator (t-PA),[1] a M_r 68,000 glycoprotein synthesized and secreted by endothelial cells, activates plasminogen (PLG) to form the principal fibrinolytic protease, plasmin (1). Both PLG and t-PA bind to the surface of endothelial cells, a process that is mediated by the calcium-dependent, phospholipid-binding protein annexin II (Ann-II) (2). Assembly of a functional PLG-t-PA·Ann-II complex, which is associated with a 60-fold increase in catalytic efficiency of plasmin generation (3, 4), appears to require at least three independent binding domains of Ann-II. First, interaction of Ann-II with endothelial cell membrane phospholipid is mediated by the calcium-regulated juxtaposition of Lys^{130}–Thr^{134} with Asp^{161} within endonexin repeat II (5). Second, PLG binding requires the carboxyl-terminal Lys^{307} of processed Ann-II, which interacts specifically with "kringle"-associated lysine binding sites of PLG (2). Third, an independent domain of Ann-II binds t-PA, increasing its affinity for PLG and subsequent generation of plasmin (3, 4).

Cell surface plasmin generation is thought to contribute to the thromboresistance of endothelial cells (6). The specific interaction of t-PA with endothelial cells is inhibited by the thiol-containing amino acid, homocysteine (HC) (7), which is formed upon demethylation of dietary methionine. HC accumulates in several inborn errors of metabolism and in some nutritional deficiencies, and homocysteinemia has been identified as an independent risk factor for atherothrombotic vascular disease (8–10). Here, we postulated that HC selectively impairs the t-PA binding domain of Ann-II, thereby inhibiting vessel wall thromboresistance and predisposing to blood vessel occlusion (7). We sought to identify the t-PA binding domain of annexin II and to determine the molecular mechanism by which HC impairs its interaction with t-PA.

EXPERIMENTAL PROCEDURES

Materials—Bovine serum albumin, DL-homocysteine, L-cysteine, and α-chymotrypsin were purchased from Sigma. Peptides were synthesized and purified by reverse phase HPLC by Quality Control Biochemicals, Inc. (Hopkinton, MA).

Purified Proteins—Native Ann-II was purified from human placental membranes and characterized as described previously (4). Recombinant Ann-II was prepared from *Escherichia coli* transformed with the pET21b(+) vector containing the Ann-II cDNA as described previously (5). This construct lacked the codon for the initial methionine of Ann-II but added sequences encoding both an amino-terminal tag (MASMTGGQQMGRDP, designated −14 to −1) and a carboxyl-terminal tag (LEHHHHHH, designated +340 to +347). The integrity of the initial amino-terminal domain (MASMTGGQQMGRDPSTVHEILCK) was verified by automated amino acid sequencing at the Protein/DNA Technology Center at Rockefeller University, and the recombinant protein was characterized as described previously (5). Recombinant human t-PA was generously provided by Genentech. Amino-terminal lysine plasminogen was provided by Immuno (Vienna, Austria).

Preparation of the "Core" Fragment of Annexin II—Purified recombinant Ann-II (1 mg) was subjected to controlled proteolysis with α-chymotrypsin-agarose beads (30 units, 250 μg/ml) which had been washed three times in 10 ml of phosphate-buffered saline/5 mM dithiothreitol and resuspended as a 1:2 (v/v) slurry (11, 12). Proteolytic products were analyzed on Coomassie Blue-stained 15% SDS-PAGE and by Western blotting using polyclonal rabbit IgG directed against human placental Ann-II (4).

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[1] The abbreviations used are: t-PA, tissue plasminogen activator; Ann-II, annexin II; ESI, electrospray ionization, nAnn-II, native annexin II; PLG, plasminogen; rAnn-II recombinant annexin II; HC, homocysteine; HPLC, high pressure liquid chromatography; HUVEC, human umbilical vein endothelial cell(s).

Cysteine Mutant Constructs—Using site-directed mutagenesis by overlap extension as described previously (2, 13), the four cysteine residues of Ann-II (Cys^9, Cys^{133}, Cys^{262}, and Cys^{335}) were mutated individually to glycine residues. The pCMV5 expression vector containing the wild type cDNA for human Ann-II served as template. For the C9G mutation, initial primer pairs consisted of 5′-CCGCGTTACATAACTTA-3′ (sense) with 5′-GAGCTTGCCCAGGATTT-3′ (antisense) and 5′-AAATCCTGGGCAAGCTC-3′ (sense) with 5′-AAAGTCGACATTTCTGGACGCTCA-3′ (antisense). For C133G, initial primer pairs consisted of 5′-AAAAGATCTCCAGCTTCCTTCAAA-3′ (sense) with 5′-TCTGGAGCCGATGATCT-3′ (antisense) and 5′-AGATCATCGGCTCCAGA-3′ (sense) with 5′-AAAGTCGACATTTCTGGACGCTCA-3′ (antisense). For the C262G mutant, initial primer pairs consisted of 5′-AAAAGATCTCCAGCTTCCTTCAAA-3′ (sense) with 5′-CTGAATGCCCTGAACCA-3′ (antisense) and 5′-TGGTTCAGGGCATTCAG-3′ (sense) with 5′-AAAGTCGACATTTCTGGACGCTCA-3′ (antisense). For the C335G mutant, initial primer pairs consisted of 5′-AAAAGATCTCCAGCTTCCTTCAAA-3′ (sense) with 5′-TCCACCACCCAGGTACA-3′ (antisense) and 5′-TGTACCTGGGTGGTGGA-3′ (sense) with 5′-CCCGTCTCTACCAAAAA-3′ (antisense). Secondary reactions were carried out with primers 5′-AAAAGATCTCCAGCTTCCTTCAAA-3′ (sense) and 5′-AAAGTCGACATTTCTGGACGCTCA-3′ (antisense). All polymerase chain reactions were performed by preincubating the reactants at 94 °C × 90 s and consisted of 30 cycles of 94 °C × 30 s, 51–60 °C × 60 s, and 72 °C × 90 s and were followed by a 5-min incubation at 72 °C. Polymerase chain reaction products were ligated directionally into pCMV5 using *Bgl*II and *Sal*I restriction sites. All constructs were sequenced completely to verify their integrity. Plasmids were propagated in HB101 *E. coli*.

Transient Transfection Assays—Renal epithelial 293 cells were propagated in RPMI 1640 medium containing 20% fetal bovine serum in 24-well plates to a density of $1.0–1.5 \times 10^5$ cells/well. Cells were transferred to serum- and antibiotic-free (basal) medium for 24 h. Each well was rinsed once and then treated with 0.4 μg of DNA/2.4 μl of lipofectamine (Life Technologies, Inc.) in 0.2 ml of basal medium. After 24 h, the transfection medium was replaced with RPMI 1640, 20% fetal bovine serum. t-PA binding capacity was studied after an additional 24 h of incubation. Transfection efficiency was optimized using the β-galactosidase expression construct (pSV-βgal) and assay system according to the manufacturer's instructions (Promega). Ann-II mRNA levels in transfected cells were estimated by a ribonuclease protection assay.

Mass Spectrometry—Recombinant human Ann-II (2.8 mg/ml) was dialyzed (18 h, 4 °C, 1:1000 (v/v)) against phosphate-buffered saline, divided into two equal aliquots, and treated with HC (5 mM) or buffer. Following adjustment of the pH to 7.4 with NH_4HCO_3, the tubes were incubated at 37 °C, 3 h and stored at 4 °C until analyzed. The integrity of HC-treated recombinant Ann-II (rAnn-II) was evaluated by SDS-polyacrylamide gel electrophoresis, which showed no alteration in electrophoretic mobility. Using electrospray ionization (ESI), molecular mass spectra were derived from multiply charged ions observed for both HC-treated and untreated Ann-II as described previously (14). The purified protein was diluted 1:10 with 50% methanol, 50% water (v/v), pH 3.0, and 5 μl of analyte solution injected into a Finnigan-MAT TSQ-700 triple quadrupole instrument. In some experiments, both treated and untreated rAnn-II (40 pmol) were digested with trypsin, and the resulting peptides were analyzed by on-line liquid chromatography-ESI-mass spectrometry and by liquid chromatography-ESI-tandem mass spectrometry, using a Finnigan-MAT LCQ ion trap mass spectrometer. Liquid chromatography was performed with an HPLC system (Ultrafast Microprotein Analyzer, Microchrom BioResources, Inc.), using a reverse phase (C18) column (5 μm, 1 × 150 mm). Chromatographic separation was performed using a linear gradient from 5 to 65% of buffer B (5% water containing 0.09% trifluoroacetic acid, 95% acetonitrile) in buffer A (5% acetonitrile in 0.1% trifluoroacetic acid) over 35 min at a flow rate of 50 μl/min.

Preparation of L-[^{35}S]Homocysteine—L-[^{35}S]Homocysteine was prepared from L-[^{35}S]methionine as described by Ewadh *et al.* and Mudd *et al.* (15, 16). Briefly, L-[^{35}S]methionine (7.6 μmol; Amersham Pharmacia Biotech catalog number SJ123) was refluxed with 100 μl of HI and 2 μl of H_3PO_3 under N_2 (100 °C, 8 h) (16). The resulting yellow solution (homocysteine thiolactone) was cooled to 21 °C (30 min) and then dried under N_2. The amber residue was dissolved in 30 μl of H_2O and incubated at 21 °C (3 min) following the addition of 20 μl of 2.5 N NaOH to generate the free sulfhydryl (16, 17). The pH was adjusted to 7.4 with 5× phosphate-buffered saline (pH 5.0), and the molar quantity of free sulfhydryl was estimated using 5,5′-dithiobis-(2-nitrobenzoic acid) (Ellman's reagent; Pierce catalog number 22582) according to the manufacturer's instructions. Free sulfhydryl content was calculated based upon the molar absorptivity of Ellman's reagent using the formula $E = A/(b)(c)$, where E is the molar absorptivity of 5,5′-dithiobis-(2-nitrobenzoic acid) (14,150), A is the observed absorbance at 412 nm, b is the spectrophotometric cell path length, and c is the sulfhydryl concentration in mol/liter. Values for c were determined using a homocysteine standard curve. On a molar basis, mean recovery of free sulfhydryl was 105.4 ± 9.8% (S.E., $n = 3$) of the starting methionine.

Metabolic Labeling of HUVEC—Confluent HUVEC (~5×10^6 cells) were pretreated with cycloheximide (100 μM, 2 h, 37 °C) to inhibit *de novo* protein synthesis and then treated with L-[^{35}S]HC (~6 μmol) for 18 h. Cells were harvested by gentle scraping and lysed by three cycles of freeze-thaw in the presence of protease inhibitors (21 μM leupeptin, 15 μM pepstatin A, and 1 mM phenylmethylsulfonyl fluoride), and the 25,000 × *g* supernatant was immunoprecipitated with monoclonal anti-Ann-II IgG (2.5 μg/ml, 4 °C, 2 h) followed by Protein G-Sepharose beads (4 °C, 2 h). The beads were washed five times with 100 mM Tris base (pH 8.0) and resuspended in one-fifth volume of 1.25% SDS, 12.5 mM Tris, 1.25 mM EDTA, 6.25% sucrose (w/v), and 12.5 μg/ml bromphenol blue with or without 40 mM dithiothreitol (100 °C, 5 min). Final immunoprecipitates were collected as the 500 × *g* supernatant and resolved by 12% SDS-polyacrylamide gel electrophoresis. Gel fluorography was performed as described previously (5).

RESULTS

t-PA Binds to the Amino Terminal Domain of Annexin II—To determine the ligand binding properties of rAnn-II, microtiter plate wells were coated with either rAnn-II or native Ann-II (nAnn-II) and probed with either ^{125}I-t-PA or ^{125}I-PLG in a range of concentrations (Fig. 1). Parameters for ^{125}I-t-PA binding to nAnn-II and rAnn-II were essentially equivalent (K_d of 47 and 42 nM; B_{max} of 300 and 272 fmol/well, respectively) (Fig. 1*A*), suggesting that t-PA binding of Ann-II does not require post-translational modification of the parent protein. Binding capacity of rAnn-II for Lys-plasminogen, on the other hand, was significantly less than that of nAnn-II (K_d of 117 and 150 nM; B_{max} of 85 and 271 fmol/well) (Fig. 1*B*) possibly because plasminogen binding is thought to require proteolytic processing for generation of a carboxyl-terminal lysine residue (Lys^{308}) in mammalian cells (18). Interaction of rAnn-II with the endothelial cell surface was equivalent to that of nAnn-II, as described previously (5). These experiments justified the use of rAnn-II to investigate the nature of the t-PA binding domain.

rAnn-II was subjected to controlled α-chymotryptic cleavage for the purpose of localizing t-PA binding within a specific domain of the protein (Fig. 2). Amino terminal "tail" (3-kDa) and carboxyl-terminal core (33-kDa) fragments were generated by α-chymotryptic cleavage between residues Tyr^{24} and Gly^{25} and separated by ion exchange chromatography (12) (Fig. 2*A*). In solid phase radioligand binding experiments (Fig. 2*B*), excess quantities of both intact rAnnII and unlabeled t-PA blocked binding of ^{125}I-t-PA to nAnn-II by ~55%. The purified core fragment, however, was completely ineffective in blocking binding. These data suggested that the t-PA binding domain of Ann-II was contained within the amino-terminal tail region.

To examine the role of specific linear amino acid sequences within the tail region of Ann-II, synthetic peptides were employed in a competitive binding strategy (Fig. 3). Three overlapping dodecapeptides corresponding to residues 2–13 (STVHEILCKLSL), 8–19 (LCKLSLEGDHST), and 14–25 (EGDHSTPPSAYG) were tested for their ability to inhibit binding of t-PA to native Ann-II (Fig. 3*A*). While peptide 14–25 showed only minimal inhibitory activity, peptides 2–13 and 8–19 were both effective in blocking binding of the radiolabeled ligand (I_{50} = 317 ± 60 μM (S.E., $n = 3$ experiments)) with maximal inhibition observed at 1 mM concentration. To test whether the region of overlap (LCKLSL, residues 8–13) might contribute directly to t-PA binding, the LCKLSL hexapeptide as well as a control (LGKLSL) were examined (Fig. 3*B*). While LGKLSL blocked less than 15% of total binding of ^{125}I-t-PA to

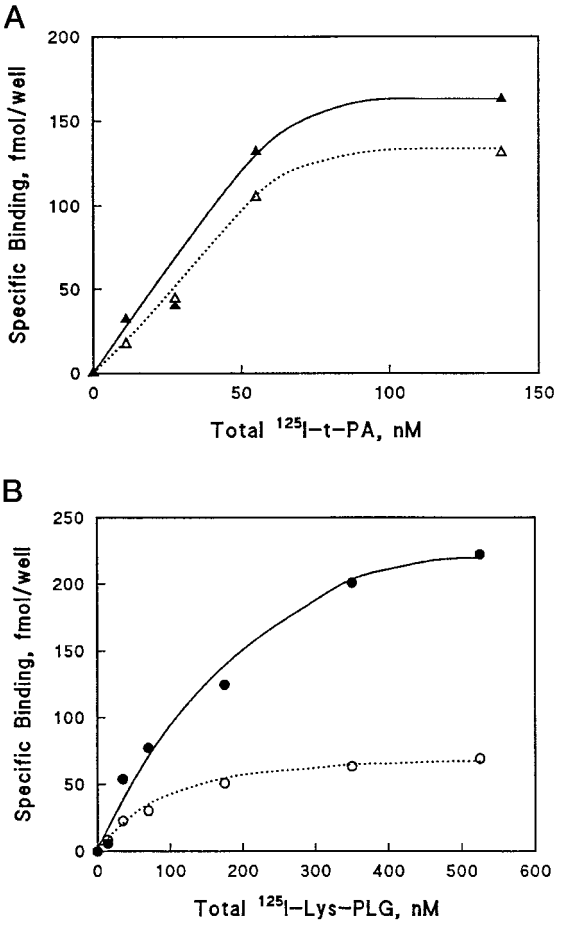

FIG. 1. **Ligand binding properties of native and recombinant Ann-II.** Wells of a 96-well Nunc microtiter plate were coated with either nAnn-II (▲, ●) or rAnn-II (△, ○; 5 μg/ml; 4 °C, 18 h) and washed three times with Tris-buffered saline containing 0.02% Tween 20 (*TBS/Tw*). Following incubation with either ^{125}I-t-PA (▲, △; 0–138 nM; 1 h, 37 °C) (*A*) or ^{125}I-PLG (●, ○; 0–525 nM; 1 h, 37 °C) (*B*), free radioactivity was sampled. The wells were washed rapidly three times, and bound ligand was solubilized in 1% SDS, 0.5 M NaOH, 0.1 M EDTA (1 h, 58 °C) (4, 26).

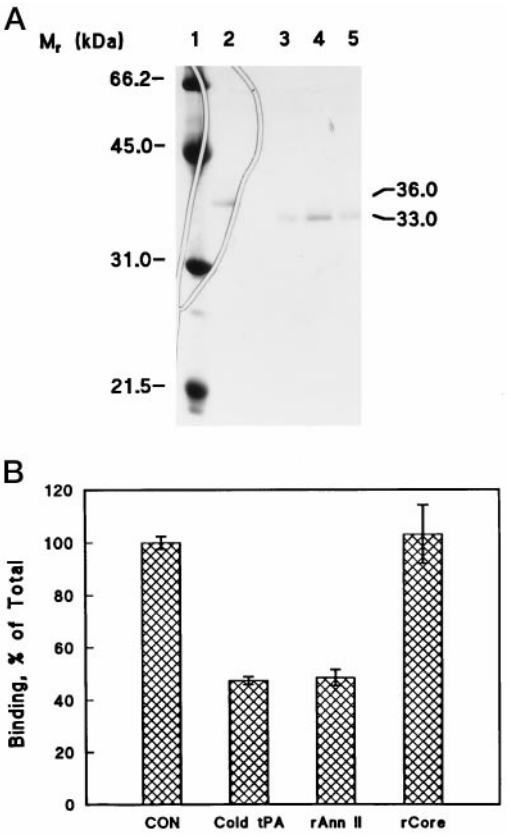

FIG. 2. **Role of the core domain of Ann-II in t-PA binding.** *A*, isolation of core fragment of Ann-II. rAnn-II (1 mg, *lane 2*) was incubated with 250 μg/ml α-chymotrypsin beads, and cleavage products were evaluated by 15% SDS-polyacrylamide gel electrophoresis. The 33-kDa chymotryptic core fragment was recovered in the flow-through of the digest applied to a DE52 ion exchange column (*lanes 3–5*). Molecular mass markers are shown in *lane 1*. *B*, effect of core fragment on binding. Nunc Maxisorp plates were coated with nAnn-II as described in the legend to Fig. 1, washed, and incubated with ^{125}I-t-PA (10 nM), either alone (*CON*) or in the presence of a 50-fold excess of unlabeled t-PA, intact rAnn-II, or core fragment of rAnn-II (1 h, 37 °C). Bound and free radioactivity were quantified as described in Fig. 1. Shown are mean values for one experiment representative of three. *Error bars* indicate standard deviation for three determinations for the experiment shown.

rAnn-II, LCKLSL was strongly inhibitory, blocking 95% of binding at a concentration of 1 mM and displaying an I_{50} of 208 ± 15 μM (S.E., n = 4 experiments). In addition, while LGKLSL had no effect on binding of ^{125}I-t-PA to HUVEC, LCKLSL inhibited all but ~7% of binding and displayed an I_{50} of ~ 1040 ± 40 μM (average ± range, n = 2 experiments) (Fig. 3*C*). Thus, binding of t-PA to both Ann-II and the HUVEC surface appears to require the LCKLSL sequence, and specifically Cys^9.

Cysteine 9 Is Essential for t-PA Binding to Annexin II—To examine further the potential role of Cys^9 in t-PA binding to Ann-II on cell surfaces, transient transfection of mutant Ann-II constructs were carried out in 293 cells (Fig. 4). Mammalian expression plasmids (pCMV5), containing either the wild type Ann-II cDNA or cDNAs in which Cys^9, Cys^{133}, Cys^{262}, or Cys^{335} were individually mutated to Gly residues, were created. By ribonuclease protection assay, all four mutant mRNAs, as well as the wild type construct, were found to be expressed at 8–12 times the nontransfected control (Fig. 4*A*). In radioligand assays, specific binding of ^{125}I-t-PA to 293 cells transfected with the C133G, C262G, or C335G constructs did not differ significantly from that observed with the wild type expression construct (Fig. 4*B*). However, binding of ^{125}I-t-PA to the C9G-transfected cells, was dramatically reduced almost to the level of nontransfected or mock-transfected cells. These data indicate that Cys^9, which resides within the putative t-PA binding domain (LCKLSL), contributes to the ligand-receptor binding interaction.

Homocysteine Interacts Directly with the t-PA Binding Domain of Annexin II—The thiol-containing amino acid HC has been previously shown to impair binding of t-PA to its endothelial cell receptor, Ann-II (7). Because the presence of Cys^9 appears to be critical for t-PA binding, we elected to determine whether HC might directly modify the t-PA binding domain of Ann-II. Electrospray ionization mass spectrometry was employed to determine the molecular masses for untreated rAnn II (theoretical mass = 40,914 Da) and HC-treated rAnn II (Fig. 5). Spectra for the untreated protein revealed a major peak at 40,919 ± 4 Da (mean ± S.E., n = 4), while the HC-treated protein showed a mass of 41,054 ± 4 Da (Fig. 5). These data indicate an increment of 135 ± 4 mass units, suggesting the formation of an Ann-II-HC adduct with 1:1 stoichiometry (theoretical increment of 133.2 Da).

For further analysis, untreated and HC-treated rAnnII were subjected to tryptic digestion (Fig. 6). Upon examination of the resultant peptides, a complete shift in the molecular mass of a single 11-amino acid peptide (DPSTVHEILCK, peptide −2 to +10; theoretical mass 1241.2) from 1241.2 ± 0.4 (Fig. 6*A*) to

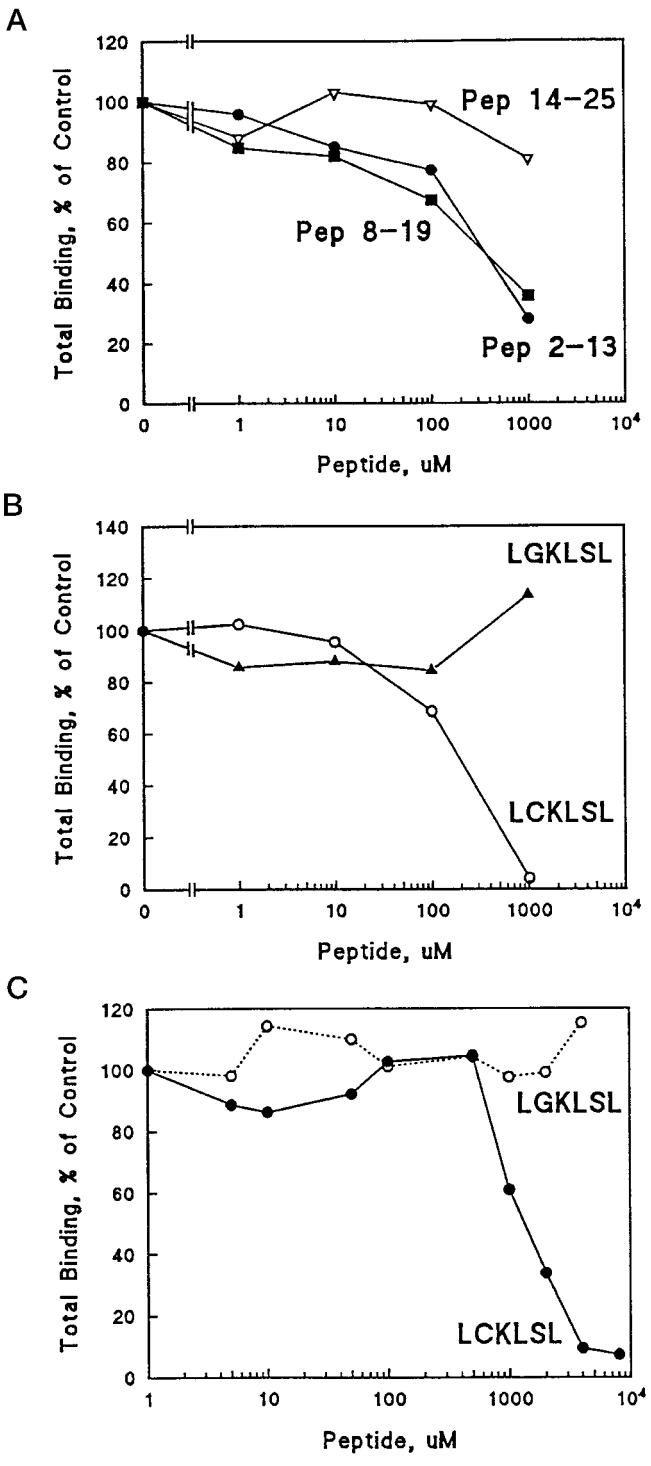

FIG. 3. **Effect of Ann-II tail peptides on binding of t-PA to Ann-II and HUVEC.** For *A* and *B*, microtiter plate wells were coated with rAnn-II (10 μg/ml; 37 °C; 2 h), washed three times, passivated with IB(5) (37 °C; 2 h), and exposed to ^{125}I-t-PA (100 nM; 37 °C; 2 h) in the presence or absence of acetyl-STVHEILCKLSL, acetyl-LCKLSLEGDHST, or acetyl-EGDHSTPPSAYG (*A*), representing residues 2–13, 8–19, and 14–25 or LCKLSL or LGKLSL (*B*), representing residues 8–13 of Ann-II. *Panels A* and *B* show one experiment representative of three and four, respectively. *C*, confluent fourth-passage HUVEC, cultured as described previously (3), were washed three times, passivated with IB(5) (37 °C; 2 h), washed, and exposed to ^{125}I-t-PA (20 nM) in the absence or presence of hexapeptide LCKLSL or LGKLSL. *Panel C* shows one experiment representative of two.

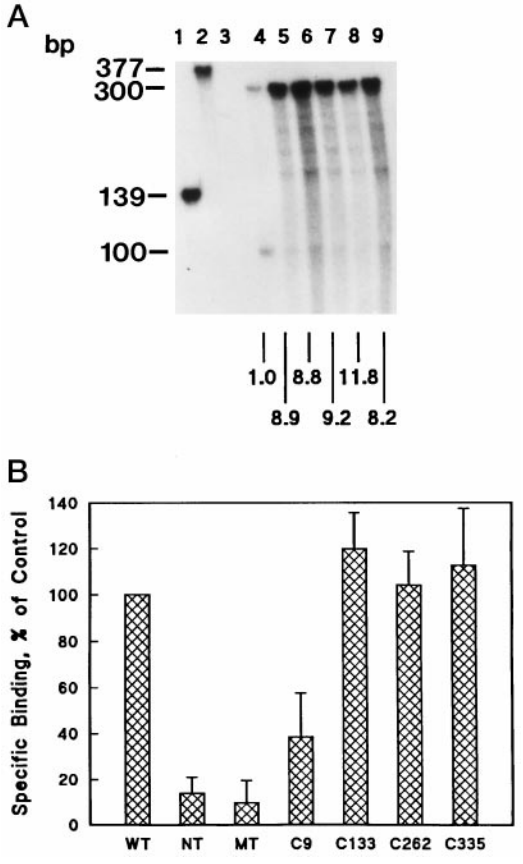

FIG. 4. **Transfection of 293 cells with cysteine mutant Ann-II constructs.** Renal epithelial 293 cells, propagated as described previously (2), were transfected with plasmids containing either wild type or mutant Ann-II cDNAs as described under "Experimental Procedures." *A*, ribonuclease protection assay. Expression of Ann-II mRNA was verified by ribonuclease protection assay of lysates from nontransfected cells, wild type transfectants, and C9G, C133G, C262G, and C335G transfectants (*lanes 4–9*, respectively). Glyceraldehyde-3-phosphate dehydrogenase and Ann-II riboprobes before (*lanes 1* and *2*) and after (*lane 3*) RNase treatment are shown. *Numbers below* each *lane* indicate the relative abundance of the 300-base pair Ann-II protected fragment after normalization to the 100-base pair glyceraldehyde-3-phosphate dehydrogenase protected fragment. *B*, binding of ^{125}I-t-PA to transfected cells. Transfectants were equilibrated to 4 °C (5 min), washed three times in IB(5), and exposed to ^{125}I-t-PA (20 nM, 4 °C, 30 min). Bound and free radioactivity were sampled as described in Fig. 1. Shown are mean values for three separate experiments for specific binding to wild type (*WT*); nontransfected (*NT*); mock-transfected (*MT*); and C9G, C133G, C262G, and C335G transfectants. *Error bars* indicate S.E. (n = 3).

1373.9 ± 0.4 Da (Fig. 6*B*) was noted. This 132.7 ± 0.5-Da increase in molecular mass demonstrated that HC interacts with peptide −2 to +10 within the tail region of recombinant Ann-II (theoretical mass increase = 133.2 Da).

Peptide −2 to +10 (DPSTVHEILCK) of rAnn-II was further examined by tandem mass spectrometry to identify the precise site of HC adduction (Fig. 7). For the doubly charged ions y_{10}^{2+} (PSTVHEILCK) and y_9^{2+} (STVHEILCK), an increment in m/z of 66.5 was observed over the corresponding fragments from the unmodified peptide. For the series of singly charged ions y_9^+ (STVHEILCK) through y_2^+ (CK), an increment in m/z of 133 was noted. Because interactions between thiols and lysine have not, to our knowledge, been reported, these data strongly indicate that HC formed a disulfide bond specifically with Cys[9].

Homocysteine Specifically Impairs the Binding Capacity of Annexin II for t-PA—To determine whether HC directly interferes with the ability of Ann-II to bind t-PA, a series of binding experiments was conducted (Fig. 8). ^{125}I-rAnn-II, untreated or

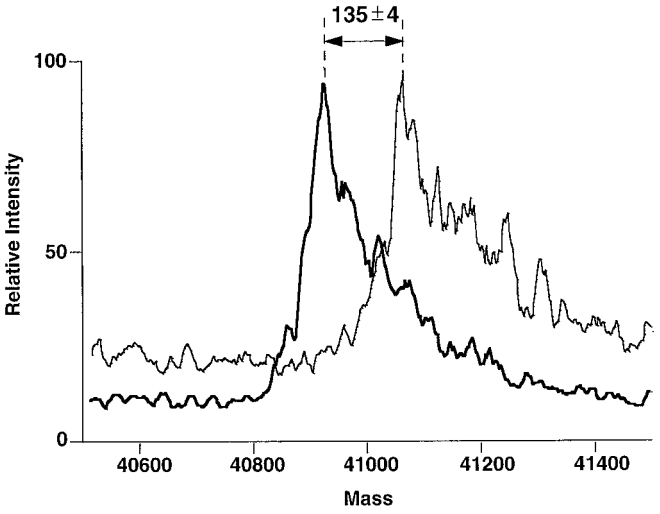

FIG. 5. **ESI-mass spectrometry analysis of untreated and HC-treated Ann-II.** rAnn-II (70 μM) was dialyzed against 100 volumes of phosphate-buffered saline (18 h, 4 °C) and either left untreated (*thick line*) or treated with 5 mM HC (*thin line*) (3 h, 37 °C, pH 8). Proteins (5 μl) were analyzed by ESI-mass spectrometry.

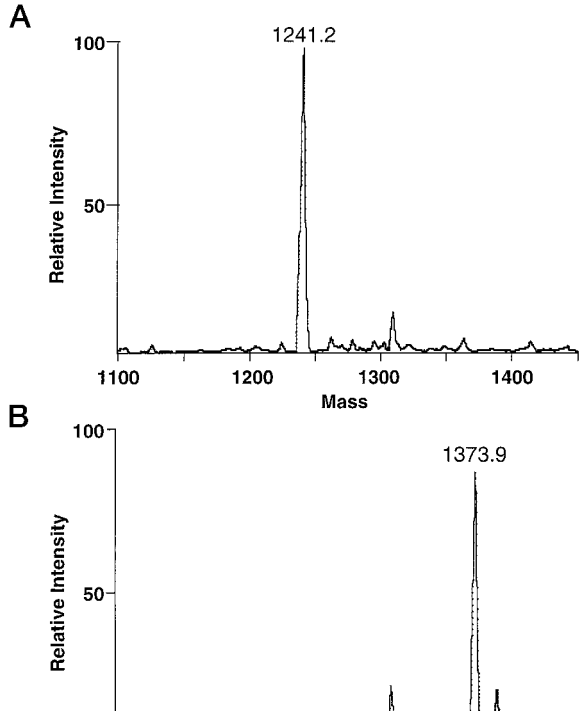

FIG. 6. **ESI-mass spectrometry analysis of peptide 12–22 from untreated and HC-treated rAnn-II.** Untreated (*A*) or HC-treated (*B*) Ann-II (40 pmol) was subjected to digestion with trypsin at a 5:1 molar ratio of Ann-II to trypsin (4 h, 37 °C) and then analyzed by on-line liquid chromatography-ESI-mass spectrometry.

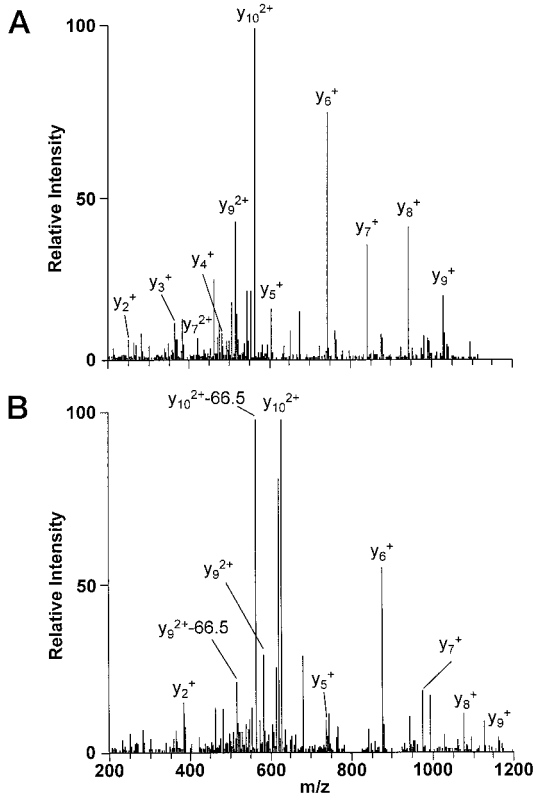

FIG. 7. **Liquid chromatography-ESI-tandem mass spectrometry analysis of peptide 12–22 of Ann-II.** Peptide 12–22 from either untreated (*A*) or HC-treated (*B*) Ann-II was analyzed by tandem mass spectrometry. The fragmentation pattern indicates that HC formed a disulfide bond with cysteine 9.

treated with either HC (Fig. 8*A*) or L-cysteine (Fig. 8*B*) (5–50 μM) for 3, 6, or 12 h, was added to t-PA immobilized on microtiter plate wells. After incubation at 4 °C, bound and free radioactivity were quantified in the presence and absence of excess unlabeled ligand (rAnn-II). Exposure of ^{125}I-rAnn-II to HC (*panel A*), but not L-cysteine (*panel B*), was associated with a time- and dose-dependent decrease in specific binding. In three separate experiments, the maximal inhibitory effect of HC (60–66%) was observed after a 12-h incubation at 50 μM. Interestingly, the I_{50} for this effect was ~11 ± 3 μM (S.E., n = 3), a value that approximates the upper limit of normal HC concentrations in plasma (8). Maximal inhibition of binding to t-PA after 3 and 6 h of HC preincubation was 20 and 30%, respectively. These data indicate that pathophysiologic concentrations of HC may, over time, significantly impair the t-PA binding capacity of rAnn-II.

[^{35}S]HC Metabolically Labels Annexin II in Cultured Endothelial Cells through a Disulfide Linkage—To ascertain whether endothelial cell Ann-II can be metabolically labeled with [^{35}S]HC and whether such labeling results from disulfide bond formation, lysates from HUVEC, pretreated with cycloheximide and incubated overnight with [^{35}S]HC, were immunoprecipitated with a monoclonal anti-Ann-II IgG (Fig. 9). Washed precipitates were analyzed in the presence and absence of the reducing agent, dithiothreitol. In the absence of dithiothreitol, anti-Ann-II IgG precipitated a prominent band with an apparent molecular mass of ~36 kDa. This band disappeared completely when the sample was reduced in the presence of dithiothreitol. These data indicate that HC labeling of Ann-II occurs in cultured endothelial cells and that adduct formation requires disulfide bond formation.

DISCUSSION

The present data identify a linear sequence of amino acids required for binding of t-PA to Ann-II, its major endothelial cell receptor. The t-PA binding domain of Ann-II was localized to the 23-amino acid amino-terminal tail region of the molecule and more specifically to the hexapeptide sequence LCKLSL, residues 8–13. Although the carboxyl-terminal core portions of the annexin family of proteins share roughly 40–50% homology at the amino acid level (19), the tail domains show limited homology and are thought to confer functional diversity (20).

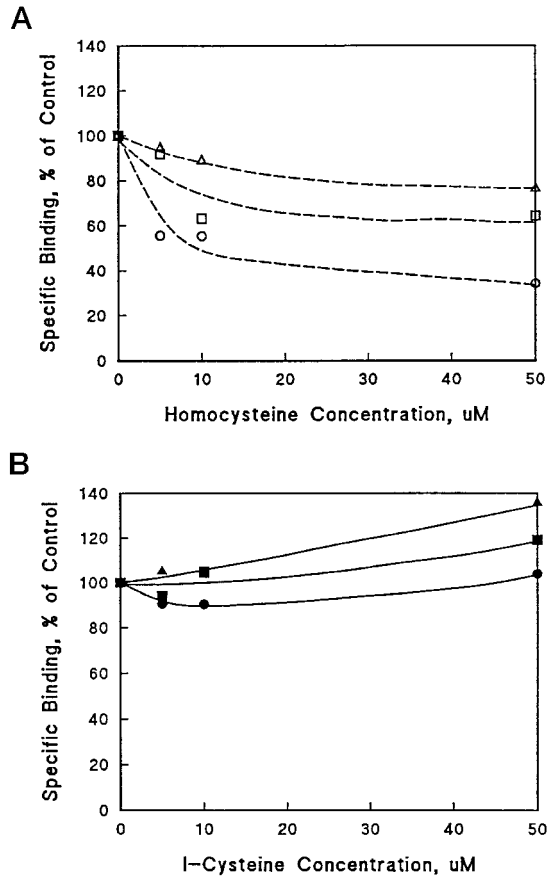

FIG. 8. **Effect of HC on t-PA-binding capacity of Ann-II.** Microtiter plate wells were coated with t-PA (100 μg/ml in carbonate buffer, 4 °C, 18 h) and washed three times. ^{125}I-rAnn II (125,860 cpm/pmol) (5) was treated with HC (*A*) or L-cysteine (*B*) in a range of concentrations (5–50 μM) for 3 (△, ▲), 6 (□, ■), or 12 h (○, ●). Treated or untreated ^{125}I-rAnn II (150 nM) was added to washed t-PA-coated wells. Following incubation (4 °C, 30 min), unbound radioactivity was sampled, the wells were washed three times, and bound radioactivity was solubilized. Specific binding was calculated as that portion inhibited in the presence of a 100-fold molar excess of unlabeled Ann II. *Panels A* and *B* show one experiment representative of three.

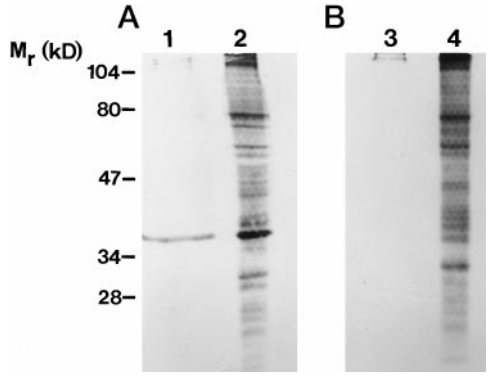

FIG. 9. **Metabolic labeling of endothelial cell Ann-II by L-[^{35}S]HC.** HUVEC were pretreated with cycloheximide (100 μM, 2 h) and incubated with L-[^{35}S]HC (345 μM, 18 h). Cells were washed and lysed, and the postnuclear supernatant was immunoprecipitated with monoclonal anti-Ann-II IgG as described previously (5). Whole cell lysates (*lanes 2* and *4*) as well as immunoprecipitates (*lanes 1* and *3*) were analyzed under nonreducing (*A*) or reducing (*B*) conditions by 12% SDS-polyacrylamide gel electrophoresis fluorography as described (5).

Indeed, our previous studies suggest that, of annexins I, II, IV, and VI, only Ann-II possesses t-PA binding capacity.[2] The present data, in addition, provide further evidence that the t-PA binding domain of Ann-II is independent of its PLG binding site, the latter having been localized to the extreme carboxyl terminus of the processed protein (2, 18).

The tail domain of Ann-II has previously been implicated in binding of the Ann-II light chain (p11) to form the Ann-II heterotetramer, a complex thought to be involved in intracellular membrane-bridging events (21). Interestingly, p11 is known to bind within the first 9 residues of the Ann-II tail (STVHEILCK), and Cys^9 has been localized within the Ann-II-p11 interface by fluorescence quenching studies (22). The carboxyl-terminal region of p11 includes the sequence CRDGK (residues 61–65) (23), which closely mimics a portion of the "finger" domain of t-PA (CRDEK, residues 6–17 (RDEKTQMIYQQ)) (24), previously reported to mediate binding of t-PA to HUVEC (25). Thus, it is possible that t-PA and the light chain of Ann-II, p11, bind to closely related regions of Ann-II and interact with Ann-II in a mutually exclusive or co-competitive fashion.

Peptides mimicking this region of Ann-II effectively blocked binding of t-PA to the purified recombinant protein. There is, further, ample precedent for the observation that this effect was observed at relatively high peptide concentrations (I_{50} = 200–1000 nM) compared with the apparent K_d for the intact protein (K_d = 10–20 nM) (5, 25). Potential explanations include the possibility that a 6–12-residue peptide might not assume a conformation identical to that of the parent protein or that additional vicinal residues might contribute to full binding capacity. In addition, peptides might interact nonspecifically with other cell-associated proteins or tissue culture surfaces. However, in the context of mutational experiments showing the requirement for Cys^9 for t-PA binding to Ann-II and the direct blockade of this residue by homocysteine, it seems reasonable to conclude that the LCKLSL region of the Ann-II tail plays a role in t-PA binding.

Our data indicate that the thiol-containing amino acid, HC, can inhibit t-PA binding to annexin II by directly complexing with Cys^9 within its putative hexapeptide binding domain (LCKLSL). This interaction with HC appears to be disulfide-mediated and unique among the four cysteine residues of Ann-II. Although blockade of Cys^9 of Ann-II impairs its ability to bind t-PA, there is no evidence for a disulfide linkage between t-PA and annexin II within the time frame of this experiment (26). This is perhaps a reflection of the fact that the single unpaired cysteine of t-PA (Cys^{83}) is located not within the "finger"-based Ann-II binding module (residues 6–17) but rather some distance away within the epidermal growth factor domain (27).

Elevated plasma HC has been identified as an independent risk factor for atherosclerotic cardiovascular, cerebrovascular, and peripheral vascular disease and is further associated with deep vein thrombosis and thromboembolism (8, 9, 28, 29). Because it is not a dietary constituent, the sole source of HC in human tissues is methionine. Classical homocystinuria results from deficiency of the pyridoxal-5′-phosphate (vitamin B_6)-dependent enzyme, cystathionine β-synthase, which condenses homocyst(e)ine with serine to form cystathionine, a precursor of cysteine (30, 31). However, genetic deficiencies of the enzymes 5-methyltetrahydrofolate-homocysteine methyltransferase and 5,10-methylenetetrahydrofolate reductase, which participate in the remethylation of HC and regeneration of methionine, are also causes of homocyst(e)inemia (32).

There is general agreement in the literature that elevated plasma HC levels may not only lead to endothelial cell dysfunction (7, 33–41) but also impose an independent risk of cardiovascular disease similar to that of smoking or hyperlipidemia

[2] K. A. Hajjar, unpublished observations.

(42). Normal adult males and females have HC levels of 6–10 and 8–12 μM, respectively (8), and levels exceeding 14 μM are associated with increased risk in nearly all studies (43). Untreated patients with homocystinuria may have plasma levels as high as 400 μM (10). One recent study found a strong, graded association between plasma HC levels from 9 to 20 μM and overall mortality in patients with angiographically confirmed coronary artery disease (44). Similarly, the risk of extracranial carotid artery stenosis for elderly men and women was 2-fold higher for individuals with plasma HC levels exceeding 14.4 μM compared with those with levels less than 9.1 μM (45). Our data suggest that HC at levels of $\sim$ 11 μM can inhibit $\sim$50% of t-PA binding to Ann-II and that higher levels can block as much as 66%. This loss of fibrinolytic potential could play an etiologic role in life-threatening vascular disorders.

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