Journal of Cellular Biochemistry 66:346–357 (1997)

Binding of SPAAT, the 44-Residue C-Terminal Peptide of α1-Antitrypsin, to Proteins of the Extracellular Matrix

Marilyn A. Niemann,[1] Joseph E. Baggott,[2] and Edward J. Miller[1]*

[1]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, Alabama 35294
[2]Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract SPAAT (short piece of α_1-antitrypsin [AAT]), the 44-residue C-terminal peptide of AAT, was originally isolated from human placenta [Niemann et al. (1992): Matrix 12:233–241]. It was shown to be a competitive inhibitor of serine proteases [Niemann et al. (in press): Biochem Biophys Acta]. The binding of SPAAT to one or more proteins of the extracellular matrix (ECM) was initially suggested on the basis of its recovery from tissue residues following a series of extractions designed to remove easily solubilized proteins [Niemann et al. (1992): Matrix 12:233–241]. Our binding studies with the model ECMs, Matrigel and Amgel, suggested that SPAAT might be bound by a specific collagen type as well as one or more non-collagenous ECM proteins. Individual ECM components were screened for their ability to bind SPAAT. When the four commonly occurring fiber-forming collagens (types I, II, III, and V) were evaluated, type III was found to be preferred. In addition, although SPAAT bound to preformed type III collagen fibers in a concentration dependent fashion, it did not bind to type III collagen molecules undergoing fibril formation. This is consistent with a physiological mode of interaction between SPAAT and type III collagen in vivo. Of the non-collagenous ECM macromolecules (laminin-1, fibronectin, entactin, and heparan sulfate) tested, laminin-1 was preferred. The binding of radiolabelled SPAAT to type III collagen and laminin-1 was competitively inhibited by unlabelled SPAAT as well as an unrelated protein, human serum albumin (HSA), to establish binding specificity. The kinetics of the release of the bound radiolabelled SPAAT were also examined to substantiate the non-covalent and reversible nature of this association. These results support the view that susceptible proteins of the ECM may actually be coated with SPAAT in vivo, possibly affording protection against inappropriate protease digestion. J. Cell. Biochem. 66:346–357, 1997. © 1997 Wiley-Liss, Inc.

Key words: α1-antitrypsin; collagen binding protein; laminin-1 binding protein; extracellular matrix binding protein; Matrigel; Amgel

Mutual interaction between cells and matrix provides a dynamic reciprocity for the regulation of cellular movement, proliferation, and differentiation [Bissel et al., 1982]. Modification of specific matrix constituents by bound proteins may change the effects of the matrix on cell function and may therefore play an important role in the control of cell-matrix interactions. These interactions can influence cellular protein synthesis [Madri and Williams, 1983], cell differentiation [Cambell et al., 1985], and morphology [Vlodavsky et al., 1980] which in turn may lead to the production of a different matrix particularly during tissue remodeling, wound healing, and organ regeneration [Majack et al., 1985; Kay et al., 1985]. In addition to their potential role in inflammation [Reich, 1978] and wound healing [Highsmith, 1981], bound proteins may also mediate diapedesis of cells through the vessel wall [Sheela and Barrett, 1982] as well as the breakdown of vascular tissue during metastasis [Knudsen et al., 1986].

The ECM may thus act as a storage depot [Huber and Weiss, 1989] for biologically active

Abbreviations: AAT, α1-antitrypsin; BSA, bovine serum albumin; buffer A, 0.2 M NaCl + 50 mM Tris, pH 7.4, + 1 mM $CaCl_2$ + 0.02% NaN_3; buffer B, 0.1 M NaCl + 50 mM Tris, pH 8.0, + 50 mM EDTA + 1% SDS; DW, distilled water; ECM, extracellular matrix; ELISA, enzyme linked immunoabsorbant assay; HNE, human neutrophil elastase; HSA, human serum albumin; Ig G, immunoglobulin G; MMP, matrix metalloproteinase; N-AAT, the N-terminal peptide of AAT after cleavage of SPAAT; OPD, O-phenylenediamine; PBS, 0.15 M phosphate buffered saline, pH 7.8; PG, proteoglycan; PROM, premature rupture of (fetal) membranes; SKALP, skin deprived anti-leukoproteinase; SLPI, secretory leukocyte proteinase inhibitor; SPAAT, the C-terminal 44-amino acid residue peptide of AAT.

Contract grant sponsor: National Institutes of Health, contract grant number DE-08520.

*Correspondence to: Edward J. Miller, Ph.D., Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, Birmingham, AL 35294.

Received 21 June 1996; accepted 16 April 1997

© 1997 Wiley-Liss, Inc.

molecules which are thereby stabilized and protected. This deposition may then produce a more localized and persistent effect when compared to the same molecules in the fluid-phase [Vlodavsky et al., 1991]. Enzymes, such as thrombin [Bar-Shavit et al., 1989], plasminogen [Knudsen et al., 1986] or plasmin, von Willerbrand factor [Wagner et al., 1984], and lipoprotein lipase as well as factors, such as granulocyte/macrophage-colony stimulating factor, interleukin-3, acidic and basic fibroblast growth factor [Bashkin et al., 1989] and osteogenin, have previously been reported to bind to various proteins of the ECM. Other examples include the binding of type I plasminogen activator inhibitor (PAI-1) to vitronectin [Declerck et al., 1988; Mimuro and Loskutoff, 1989] and heparan sulfate to type V collagen [LeBaron et al., 1989]. Even the parent protein of SPAAT, AAT, has been reported to bind saturably and tightly to an interstitial ECM secreted by rat heart smooth muscle cells via both covalent and noncovalent mechanisms [Rinehart et al., 1993].

SPAAT was initially isolated from an ECM residue following a series of extraction procedures designed to remove easily solubilized proteins [Niemann et al., 1992]. In addition to SPAAT, several other naturally occurring, relatively low molecular weight protease inhibitors, such as SLPI [Rice and Weiss, 1990], elafin [Wiedow et al., 1990]/SKALP [Molhuizen et al., 1993], and inhibitors from human articular cartilage [Andrews and Ghosh, 1990] as well as skin fibroblasts [Rao et al., 1995a] and umbilical vein endothelial cells [Rao et al., 1995b], have also been isolated from the ECM. We previously proposed that SPAAT may represent yet an additional example of a biologically active molecule for which the ECM provides sites of attachment [Niemann et al., 1992]. In this study we now examine the ability of SPAAT to bind to individual proteins of the ECM in order to more precisely define the specificity of this putative association.

MATERIALS AND METHODS

Materials

[1-^{14}C]-acetic anhydride and Aquasol were purchased from NEN (DuPont, Boston, MA). Matrigel was obtained from Collaborative Biomedical Products (Becton Dickinson, Bedford, MA) and a connective tissue preparation termed "Amgel" was a generous gift of Drs. Linden Goodly and Gene Siegal (UAB, Pathology Dept.). Twenty-four-well membrane coated cell culture inserts of fibrillar (coated with 216 μg of protein/cm^2) and nonfibrillar (coated with 217 μg of protein/cm^2) type I collagen, type IV collagen (coated with 18 μg of protein/cm^2), fibronectin (coated with 39 μg of protein/cm^2), and laminin-1 (coated with 31 μg of protein/cm^2) as well as laminin-1 (coated with 8 μg of protein/cm^2) Biocoat cellware were also obtained from Collaborative Biomedical Products. Type IA crude bacterial collagenase, rabbit anti-human AAT polyclonal antibody, goat anti-rabbit Ig G antibody, and O-phenylenediamine (OPD) were purchased from Sigma (St. Louis, MO). Proteinase K was obtained from Boehringer Mannheim (Indianapolis, IN). SPAAT was synthesized by the UAB Protein Core Facility. Its purity was checked by gel permeation HPLC, amino acid analysis, amino acid sequence analysis, and mass spectroscopy. All other reagents used were of the highest analytical grade commercially available.

Collagen Preparation

Human types I, III, and V collagen were prepared from human placenta as previously described [Miller and Rhodes, 1982]. Bovine type II collagen was also prepared as previously described [Miller and Rhodes, 1982].

Radiolabelling

Human type III collagen was radiolabelled using the ^{14}C-acetylation method of Cawston and Barrett [1979]. SPAAT was also radiolabelled using a modification of this method. Briefly, SPAAT was dissolved in a minimum volume of 6 M guanidium chloride (2.33 mg/ml). The pH was adjusted to 9.0 by the addition of NaOH. Next, 100 μCi of [1-^{14}C]-acetic anhydride was added and incubated for 90 min maintaining a pH of 9.0 by adding NaOH when necessary. The reaction was stopped by adjusting the pH to 4.0 with glacial acetic acid and desalted into distilled water (DW) using a PD-10 (Pharmacia, Pistcataway, NJ) column. In subsequent experiments this stock ^{14}C-acetylated SPAAT solution was diluted immediately before use with the indicated buffer.

Binding of SPAAT to the Model ECMs, Matrigel and Amgel

Matrigel and a preparation of Amgel [Siegal et al., 1993] were used in a quantitative gel film assay based on the procedure of Johnson-Witt [1980]. Briefly, 250 μl aliquots of Matrigel (1 mg/ml, 5 mg/ml, and 16 mg/ml) or Amgel (0.5 mg/ml) were added to bovine serum albumin (BSA)-coated 24-well tissue culture plates maintained at a 45° angle. The plates were incubated at 37°C for 30 min and then dried overnight in the hood. The wells were rehydated and washed three times with 1 ml of DW before the addition of 500 μl of ^{14}C-acetylated SPAAT (approximately 100,000 cpm/45 μg) diluted in DW. The plates were again incubated at a 45° angle at 37°C for 3 h before washing with DW. The wells were enzymatically digested overnight at 37°C with 500 μl of bacterial collagenase (1 mg/ml in 0.2 M NaCl, 50 mM Tris-HCl, 1 mM $CaCl_2$, 0.02% NaN_3, pH 7.4, buffer A), followed by 500 μl of proteinase K (0.5 mg/ml in 0.1 M NaCl, 50 mM Tris-HCl, 50 mM EDTA, 1% SDS, pH 8.0, buffer B) to release bound ^{14}C-SPAAT. These supernatants plus an equal volume DW wash were transferred to scintillation vials and counted. The amount of radiolabelled SPAAT released was calculated by dividing this radioactivity by the specific activity of SPAAT.

Binding of SPAAT to Collagen

Initial screening. Four fiber forming collagens, types I, II, III, and V (1 mg/ml), were equilibrated in solvents that allowed native fiber-formation (0.15 M sodium phosphate, pH 7.4) or caused aggregation and/or precipitation (DW). To a 24-well tissue culture plate maintained at a 45° angle, 250 μl aliquots of each collagen preparation were added. The plate was incubated at this angle at 37°C for 3 h to polymerize the collagen in the phosphate buffer to a native gel, while the aggregated collagen in the DW remained in suspension. Maintaining the plate at a 45° angle, 100 μl of an appropriately diluted solution of ^{14}C-acetylated SPAAT (approximately 75,000 cpm/34 μg) was added to the lower side of each well such that only the collagen gel crescents of the experimental wells were covered, while SPAAT was permitted to mix with the aggregated collagen molecules in the control wells. The plate was incubated at 37°C for an additional 90 min at a 45° angle, then dried in the hood at room temperature (25°C). The next day the wells were washed with 1 ml of DW followed by three 1 ml aliquots of phosphate buffered saline (PBS) to remove unbound material and equilibrate the bound material with PBS. The remaining material in each well was digested overnight at 37°C with 500 μl of bacterial collagenase (1 mg/ml in buffer A). The digests were transferred to scintillation vials, each well was carefully washed with 500 μl of DW, and the entire 1 ml mixture counted. Any protein remaining in the wells was subsequently digested overnight at 37°C with 500 μl of proteinase K (0.5 mg/ml in buffer B). These digests were also collected, the wells washed, and counted as above. Counts were corrected for non-specific binding of ^{14}C-SPAAT to BSA-coated wells incubated in the same buffer. The amount of radiolabelled SPAAT added and released was calculated by dividing this radioactivity by the specific activity of SPAAT.

Binding assay. This assay was done using the following modifications of the above "initial screening" method. Briefly, type III collagen (1 mg/ml) was equilibrated in 0.15 M sodium phosphate, pH 7.4, buffer. Collagen fibers were formed and appropriately diluted solutions of ^{14}C-acetylated SPAAT were added to each gel crescent. The plate was incubated at 37°C for an additional 2.5 h, then washed with 1 ml of DW before being digested overnight at 37°C with 500 μl of bacterial collagenase (1 mg/ml in buffer A). The digests were transferred to scintillation vials, each well was washed with 500 μl DW, and the entire 1 ml mixture counted. The amount of radiolabelled SPAAT added and released was again calculated by dividing this radioactivity by the specific activity of SPAAT.

Fibril formation. This assay was also done using the following modifications of the above "binding assay" method. Briefly, ^{14}C-acetylated type III collagen was polymerized into native fibers for 2 h either in the absence of SPAAT and subsequently incubated with appropriately diluted solutions of SPAAT or in the presence of varying concentrations of SPAAT and subsequently incubated for 2 h without SPAAT. Enzyme-linked immunoabsorbant assays (ELISAs) were performed as previously described [Niemann et al., 1992] except that the wells were coated with 100 μl collagen (1 mg/ml). Briefly, after antigen coating and blocking, appropriately diluted (1/40,000) primary rabbit anti-human AAT polyclonal antibody was added to

each well. After subsequent incubation and washing appropriately diluted (1/16,000) secondary enzyme-linked goat anti-rabbit Ig G antibody was then added to each well. After binding and washing, color was finally developed in the appropriate wells by the addition of 0.2% OPD substrate solution. The color was stabilized by the addition of 4.5 M H_2SO_4 and read at A_{492}. To determine the amount of exogenous SPAAT that bound in each case the A_{492} was corrected for non-specific binding of SPAAT to BSA-coated wells. The A_{492} values were converted to micrograms by comparison with a standard curve. The wells were then digested with 500 µl bacterial collagenase (1 mg/ml in buffer A) to release the ^{14}C-acetylated type III collagen bound to the wells in each case (average approximately 2,600 cpm/well). By dividing this radioactivity by the specific activity of the protein, the amount of ^{14}C-type III collagen released was converted to micrograms.

Specificity. Radiolabelled SPAAT (approximately 50,000 cpm/50 µg) was added to each well of a 24-well tissue culture plate coated with a hydrated preformed gel crescent of type III collagen fibrils, then incubated for 0, 30 min, 1 h, 3 h, 6 h, and overnight (24–26 h) in the absence or presence of a ninefold molar excess of unlabelled SPAAT or an approximately equimolar amount of HSA. At each time point, the unbound supernatant radioactivity was removed and each well was washed with an equal volume of 0.15 M sodium phosphate, pH 7.4, buffer. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with 500 µl of bacterial collagenase (1 mg/ml in buffer A). This digest plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms.

Dissociation time-course. Hydrated preformed type III collagen fibril gel crescents were equilibrated with radiolabelled SPAAT (approximately 84,000 cpm/50 µg) by preincubating each well of a 24-well tissue culture plate for 3 h at 37°C. After 3 h the unbound supernatant radioactivity was removed and each well was washed with an equal volume of 0.15 M sodium phosphate, pH 7.4, buffer. About 22% of the total added radiolabelled SPAAT bound. Each day for 4 days, a fresh 500 µl aliquot of PBS, pH 7.4, was then added to each well and incubated for 0, 30 min, 1 h, 3 h, 6 h, and overnight (24 h). At each time point the radiolabelled SPAAT released into the supernatant buffer plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms.

Binding of SPAAT to Other Components of the ECM

Initial screening. Five commercially available 24-well ECM protein coated membrane inserts, fibrillar collagen, type I rat tail (insert pore size 1.0 µ); rat tail collagen, type I (insert pore size 3.0 µ); mouse collagen IV (insert pore size 3.0 µ); mouse laminin-1 (insert pore size 3.0 µ); and human fibronectin (insert pore size 3.0 µ), were evaluated for their ability to bind ^{14}C-acetylated SPAAT. Immediately before use the inserts were rehydrated according to the manufacturer's instructions with 0.15 M sodium phosphate, pH 7.4, for 30 min at room temperature. Radiolabelled SPAAT (approximately 26,000 cpm/37 µg) was added to each rehydrated insert, then incubated for 0, 30 min, 1 h, 3 h, 6 h, and overnight (24 h) at 37°C. At each time point, the unbound supernatant radioactivity was removed and each insert was washed with an equal volume of DW. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with 500 µl of proteinase K (0.5 mg/ml in buffer B). The digest plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the minimum number of SPAAT molecules bound per molecule of ECM protein (as reported by the manufacturer) at equilibrium (3 h) was estimated.

Binding assay. This assay was done using the following modifications of the above "initial screening" method. Briefly, a 24-well mouse laminin-1 Biocoat cellware plate was incubated with various concentrations of ^{14}C-acetylated SPAAT. The plate was incubated at 37°C for 2.5 h, washed with DW, and digested overnight with proteinase K. The digests plus an equal volume DW wash were transferred to scintillation vials and counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT added and released was converted to micrograms.

Specificity. Radiolabelled SPAAT (approximately 28,000 cmp/20 µg) was added to each well of a 24-well tissue culture plate coated with mouse laminin-1, then incubated for the

indicated times in the absence or presence of a ninefold molar excess of unlabelled SPAAT or an approximately equimolar amount of HSA. At each time point, the unbound supernatant radioactivity was removed and each well was washed with an equal volume of DW. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with 500 μl of proteinase K (0.5 mg/ml in buffer B). This digest plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms.

Dissociation time-course. Mouse laminin-1 was equilibrated with radiolabelled SPAAT (approximately 28,000 cpm/20 μg) by preincubating each well of a 24-well tissue culture plate overnight (approximately 18 h) at 37°C. The next day the unbound supernatant radioactivity was removed and each well was washed with an equal volume of DW. About 6% of the total added radiolabelled SPAAT bound. Each day for 4 days, a fresh 500 μl aliquot of PBS, pH 7.4, was then added to each well and incubated for 0, 30 min, 1 h, 3 h, 6 h, and overnight (24 h). At each time point the radiolabelled SPAAT released into the supernatant buffer plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms.

RESULTS

Binding of SPAAT to the Model ECMs, Matrigel and Amgel

The binding of SPAAT to the ECM was originally proposed on the basis of its recovery in tissue residues following a series of extractions designed to remove easily solubilized proteins [Niemann et al., 1992]. This proposal is supported by the data presented in Figure 1 comparing the protease released ^{14}C-SPAAT recovered from Matrigel and Amgel. In both these in vitro ECM models, the collagenous component(s) was clearly capable of binding exogenous SPAAT. Amgel (0.5 mg/ml), however, bound twice as much SPAAT released by collagenase as an equivalent amount of Matrigel (1 mg/ml). These results suggested that SPAAT may preferentially bind to one collagen type over another (see Table I). In addition, most of the radiolabelled SPAAT in the Amgel preparation was released by collagenase digestion, while substantial additional radiolabelled SPAAT was released from Matrigel preparations following subsequent proteinase K digestion. This result suggested that SPAAT may also bind to noncollagenous Matrigel protein(s).

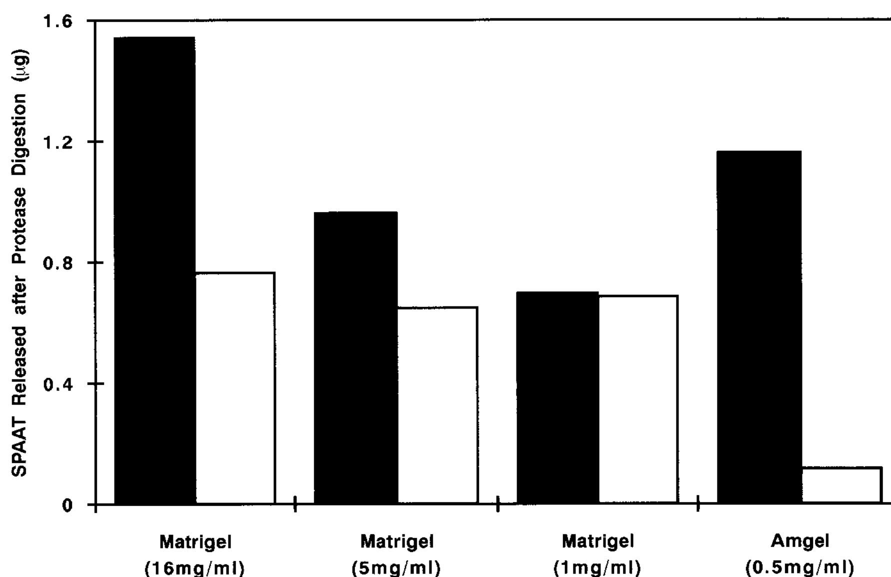

Fig. 1. Release of radiolabelled SPAAT bound to ECM preparations, Matrigel or Amgel, after protease digestion. The shaded bars represent bacterial collagenase digestion, while the open bars represent proteinase K digestion. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT released was converted to micrograms.

TABLE I. Binding of SPAAT to Individual Proteins of the ECM

Protein	No. of SPAAT molecules bound: molecules of protein at 3 h
Collagens	
I (nonfibrillar)	0.7
I (fibrillar)	0.9
III	3
IV	6
Glycoproteins	
Laminin-1	49
Fibronectin	3

The ability of five commercially available 24-well ECM protein coated membrane inserts, fibrillar collagen, type I rat tail (2.16×10^{-10} M); rat tail collagen, type I (2.17×10^{-10} M); mouse collagen IV (1.05×10^{-11} M); mouse laminin-1 (1.14×10^{-10} M); and human fibronectin (2.57×10^{-11} M), were compared with human type III (8.33×10^{-10} M) collagen for their ability to bind ^{14}C-acetylated SPAAT. Radiolabelled SPAAT (approximately 26,000 cpm/37 μg) was added to each rehydrated insert, then incubated at 37°C. At maximal binding (3 h), the unbound supernatant radioactivity was removed and each insert was washed with an equal volume of DW. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with either 500 μl of bacterial collagenase (types I, III, and IV collagen) or proteinase K (laminin-1 or fibronectin). By dividing this radioactivity by the specific activity of the peptide, the minimum number of SPAAT molecules bound per molecule of ECM protein (as reported by the manufacturer) was estimated. Additional experimental details are given in the Materials and Methods section.

Binding of SPAAT to Collagen

In order to further investigate this suspected preferential binding, the four commonly occurring fiber-forming collagens (types I, II, III, and V) were evaluated for their ability to bind SPAAT. The collagens were equilibrated in a solution which induced fiber formation or in a solution which caused aggregation and/or precipitation. All the tested collagen types bound collagenase-releasable ^{14}C-SPAAT (Fig. 2). Type III collagen fibers, however, had the highest affinity for ^{14}C-SPAAT of all the fibrillar collagens. There was essentially no difference observed for SPAAT binding among the amorphous precipitates of these collagens. Since subsequent overnight digestion with proteinase K released only negligible radioactivity (data not shown), the data indicate that SPAAT specifically binds to collagen in these experiments. As shown in Figure 3, several binding sites are apparent. Moreover, the binding of SPAAT to type III collagen is concentration dependent, although concentrations saturating all binding sites were not achieved. Furthermore, as shown in Figure 4, although SPAAT binds to preformed type III collagen fibers in a concentration dependent fashion, it does not bind to type III collagen molecules undergoing fibril formation. This would be consistent with the expected physiological mode of interaction between SPAAT and type III collagen in vivo.

Association time-course. The time-course of SPAAT binding to type III collagen fibrils was, as shown in Figure 5A, found to reach an equilibrium in vitro. This equilibrium was achieved around 3 h at which time about 28% of the total added radiolabelled SPAAT was bound or, as shown in Table I, one molecule of SPAAT per type III collagen chain or approximately three molecules of SPAAT per triple helical molecule of type III collagen. The half-life of the binding process was about 30 min. This binding was not affected by the presence of an approximately equimolar concentration of an unrelated protein, HSA, but was inhibited by about 29% in the presence of a ninefold molar excess of unlabelled SPAAT, suggesting that this binding is specific.

Dissociation time-course. The release time-course of the bound radiolabelled SPAAT to type III collagen was also examined. As shown in Figure 5B, the half-life of dissociation of SPAAT from type III collagen fibrils was rapid, about 15–20 min at 37°C. In addition, since about two-thirds of the bound radiolabelled SPAAT is dissociated by each wash, this association appears to involve a reversible binding phenomenon rather than a covalent or irreversible adsorption interaction.

Binding of SPAAT to Other Components of the ECM

In order to quantitate as well as compare and contrast the binding of other ECM macromolecules with that observed for type III collagen, the SPAAT binding ability of several major ECM glycoproteins (laminin-1, type IV collagen, and fibronectin) was evaluated in an assay system similar to that used above for type III collagen. As can be seen in Table I, SPAAT preferentially bound to laminin-1. Furthermore, as shown in Figure 3, several binding sites are again apparent. In addition, this bind-

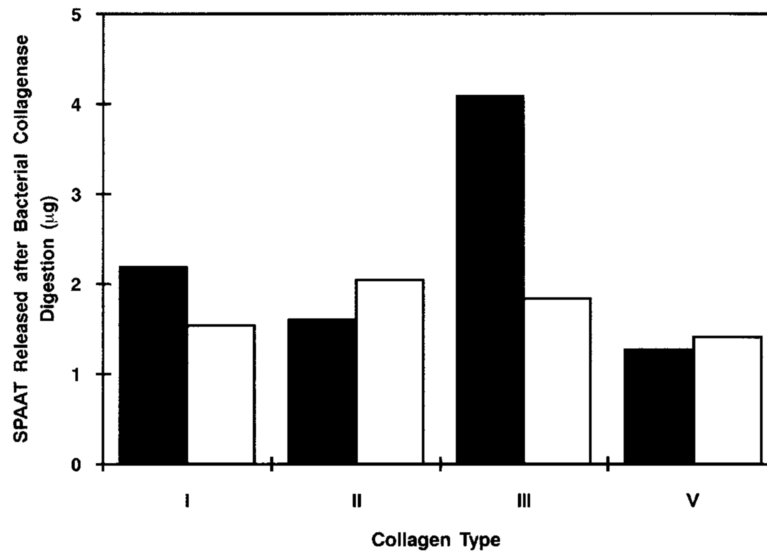

Fig. 2. Release of radiolabelled SPAAT bound by the fibrillar collagens after bacterial collagenase digestion. The shaded bars represent the amount of radiolabelled SPAAT released from fibers, while the open bars represent the amount of radiolabelled SPAAT released from amorphous DW precipitates. Counts were corrected for non-specific binding of radiolabelled SPAAT to BSA-coated wells incubated in the same buffer. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT released was converted to micrograms. Additional experimental details are given in the Materials and Methods section.

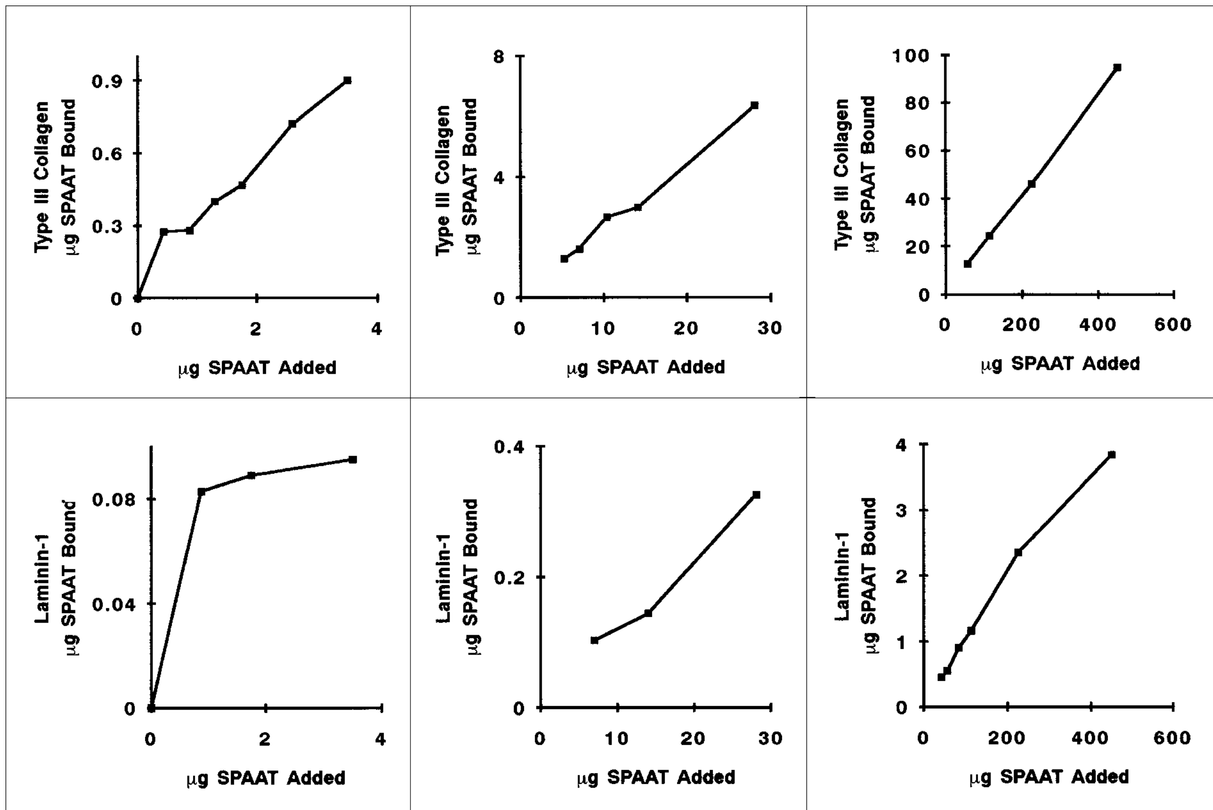

Fig. 3. Dose-response binding curve of SPAAT to type III collagen (upper panels) and laminin-1 (lower panels). Type III collagen fibrils and laminin-1 coated plates were incubated with the indicated amounts of SPAAT, then digested with bacterial collagenase or proteinase K, respectively. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT added and released was converted to micrograms. Additional experimental details are given in the Materials and Methods section.

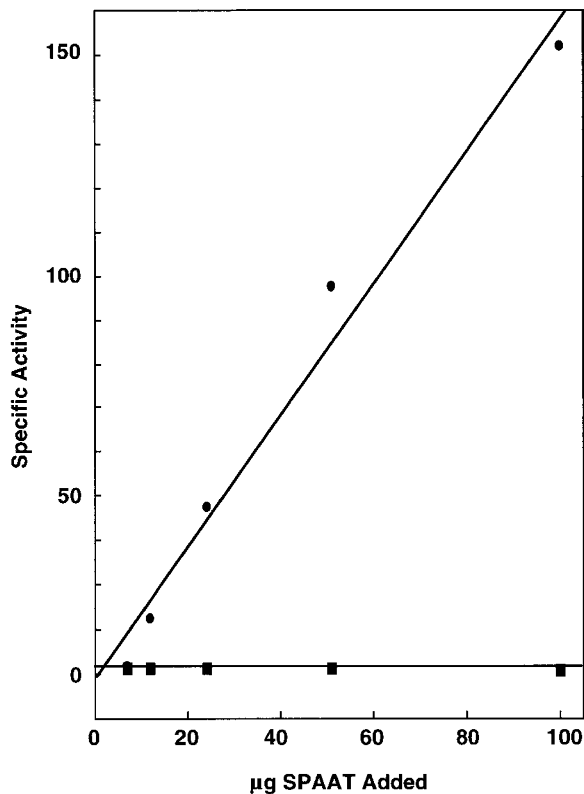

Fig. 4. Binding of SPAAT to type III collagen during and after fiber formation. ^{14}C-acetylated type III collagen was polymerized into native fibers in the absence of SPAAT and subsequently incubated with SPAAT (●) or polymerized into native fibers in the presence of SPAAT and subsequently incubated without SPAAT (■). ELISAs were performed as previously described [Niemann et al., 1992] except that the wells were coated with 100 µl of collagen (1 mg/ml). To determine the amount of exogenous SPAAT that bound in each case A_{492} was corrected for non-specific binding of SPAAT to BSA-coated wells. The A_{492} values were converted to micrograms by comparison with a standard curve. The wells were then digested with 500 µl bacterial collagenase to release the ^{14}C-acetylated type III collagen bound to the wells in each case. By dividing this radioactivity by the specific activity of the protein, the amount of ^{14}C-type III collagen released was converted to micrograms. The data are expressed as specific activity (µgSPAAT/µg type III collagen $\times 10^{-5}$). Additional experimental details are given in the Materials and Methods section.

ing, like that to type III collagen, also appears to be concentration dependent, although again concentrations saturating all binding sites were not achieved.

Association time-course. The time-course of SPAAT binding to laminin-1, as shown in Figure 6A, was found, like type III collagen, to reach an equilibrium in vitro. This equilibrium was similarly achieved around 3 h at which time about 13.5% of the total added radiolabelled SPAAT was bound or approximately 49 molecules of SPAAT per molecule of laminin-1. SPAAT also appeared to bind more rapidly to laminin-1 than type III collagen fibrils with a half-life of about 10 min. This binding was inhibited by about 65% in the presence of a ninefold molar excess of unlabelled SPAAT. Binding, however, was also inhibited by about 30% in the presence of an approximately equimolar concentration of an unrelated protein, HSA. These results suggest that a substantial portion of labelled SPAAT binding is nonspecific.

Dissociation time-course. The release time-course of the bound radiolabelled SPAAT to laminin-1 was also examined. As shown in Figure 6B, the half-life of dissociation of SPAAT from laminin-1 was considerably slower, about 3–4 h at 37°C, than type III collagen. In addition, this linear slow rate of decay continued for 48 h (in the case of laminin-1) rather than 3 h (in the case of type III collagen) before dropping down to essentially equilibrium levels, suggesting that although the binding of SPAAT to laminin-1, like type III collagen, is noncovalent and reversible, it is released more slowly from laminin-1 than type III collagen fibrils.

DISCUSSION

The Matrigel and Amgel preparations used in our SPAAT binding assays (Fig. 1) have been reported to represent reasonably complete in vitro models of ECMs. Matrigel was originally prepared from the mouse Engelbreth-Holm-Swarm (EHS) sarcoma, which arose spontaneously in C57BL/6 mice [Orkin et al., 1977], and biochemically, structurally, and biologically resembles normal basement membrane [Yurchenco and Schittny, 1990; Timpl, 1989]. It is composed of approximately 60% laminin-1, 30% type IV collagen, 6% entactin, and 2% heparan sulfate proteoglycan (PG) [Kleinman et al., 1986]. Amgel, on the other hand, is a human-derived ECM from normal amnionic membrane. It consists predominantly of type I (460 µg/ml) and type IV (380 µg/ml) collagen as well as entactin (250 µg/ml), laminin-1 (130 µg/ml), and tenascin (75 µg/ml) [Siegel et al., 1993].

The in vitro data presented here demonstrate that SPAAT binds preferentially (Table I) and in a dose dependent manner (Figure 3) to individual ECM proteins. The specificity of this interaction between SPAAT and the ECM proteins, type III collagen and laminin-1, was demonstrated by competition experiments using ex-

cess unlabelled SPAAT (Fig. 5A and 6A). In addition, since a substantial fraction of the bound radiolabelled SPAAT can be displaced by a relatively modest excess of unlabelled fluid-phase peptide (Figs. 5A and 6A), the association of SPAAT with these ECM proteins is more consistent with such a receptor-ligand equilibrium than with an irreversible adsorption process. The observation that a substantial amount of this SPAAT also remained bound to these ECM proteins in the presence of HSA (Figs. 5A and 6A) suggests that this binding may occur in vivo. Finally, the non-covalent and reversible nature of the association of SPAAT with these ECM proteins was suggested by its release time-course (Figs. 5B and 6B). These data thus support our previous proposal that SPAAT may represent an additional example of a biologically active molecule for which the ECM provides sites of attachment [Niemann et al., 1992].

We previously proposed [Niemann et al., 1992] that AAT is secreted and remains in the extracellular fluids or is deposited in the ECM. Cleavage then occurs as the result of the activity of a tissue enzyme, possibly a matrix metalloprotease (MMP). The larger N-terminal peptide is released, while SPAAT remains bound to, or is deposited on one or more biologically susceptible proteins of the ECM, such as collagen and/or laminin-1. In these locations SPAAT might play an important role in the protection

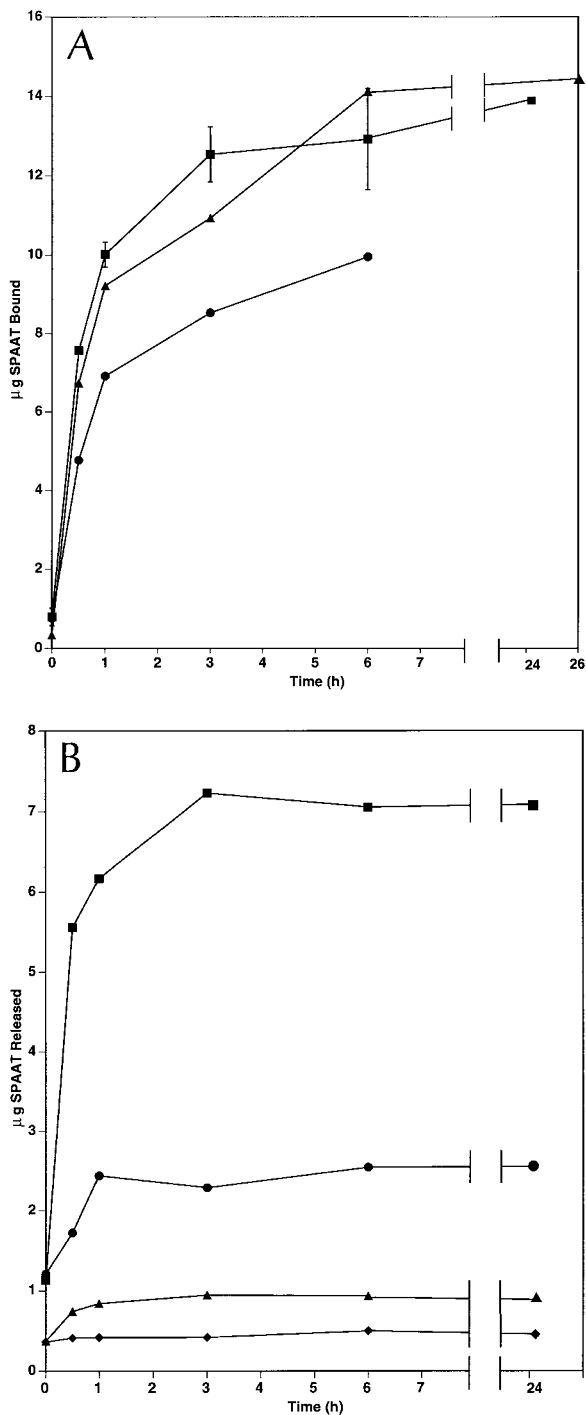

Fig. 5. SPAAT binding to type III collagen fibrils. A: Specificity. Radiolabelled SPAAT (approximately 50,000 cpm/50 µg) was added to each well of a 24-well tissue culture plate coated with a hydrated preformed crescent of type III collagen fibrils, then incubated for the indicated times in the absence (■) or presence (●) of a ninefold molar excess of unlabelled SPAAT or an approximately equimolar amount of HSA (▲). At each time point, the unbound supernatant radioactivity was removed and each well was washed with an equal volume of 0.15 M sodium phosphate, pH 7.4, buffer. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with 500 µl of bacterial collagenase. This digest plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms and plotted. Uncompeted radiolabelled SPAAT binding was assayed in duplicate. The error bars represent the range of these two averaged values. B: Decay. Hydrated preformed type III collagen fibril gel crescents were equilibrated with radiolabelled SPAAT (approximately 84,000 cpm/50 µg) by preincubating each well of a 24-well tissue culture plate for 3 h at 37°C. After 3 h the unbound supernatant radioactivity was removed and each well was washed with an equal volume of 0.15 M sodium phosphate, pH 7.4, buffer. A fresh 500 µl aliquot of PBS, pH 7.4, was then added to each well and incubated for the indicated times. At each time point the radiolabelled SPAAT released into the supernatant buffer plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms and plotted. The above procedure was repeated for 4 days until the released radioactivity had fallen to essentially background levels. Day 1 (■), day 2 (●), day 3 (▲), day 4 (◆). Additional experimental details are given in the Materials and Methods section.

of these proteins from inappropriate enzyme digestion.

Previous immunohistochemical studies indicated that AAT and/or possibly its cleavage fragments, including SPAAT, may be present in a variety of human tissues, such as stomach [Kittas et al., 1982b], pancreas [Ray et al., 1977], small intestine [Nielsen, 1984; Geboes et al., 1982], and gastric carcinomas [Ray et al., 1982; Tahara et al., 1984; Kittas et al., 1982a; Wittekind et al., 1986]. Subsequently, AAT has also been reported to biochemically bind saturably and tightly to an interstitial ECM secreted by rat heart smooth muscle cells via both covalent and noncovalent mechanisms [Rinehart et al., 1993]. This binding was unaffected by the prior removal of ECM glycopropteins by digestion with trypsin which would be consistent with a collagen (type I and/or type III) binding. These authors further reported endogenous proteolytic activity associated with their cell culture-derived interstitial ECM which could cleave ECM-bound AAT into low molecular weight fragments. This activity was inhibited by the MMP inhibitors, 1,10-phenanitroline and doxycycline. Such cleavage generated a higher molecular weight enzyme-AAT (possibly N-AAT) complex as well as lower molecular weight fragments of AAT (possibly SPAAT). In addition, only half of the total ECM-bound AAT

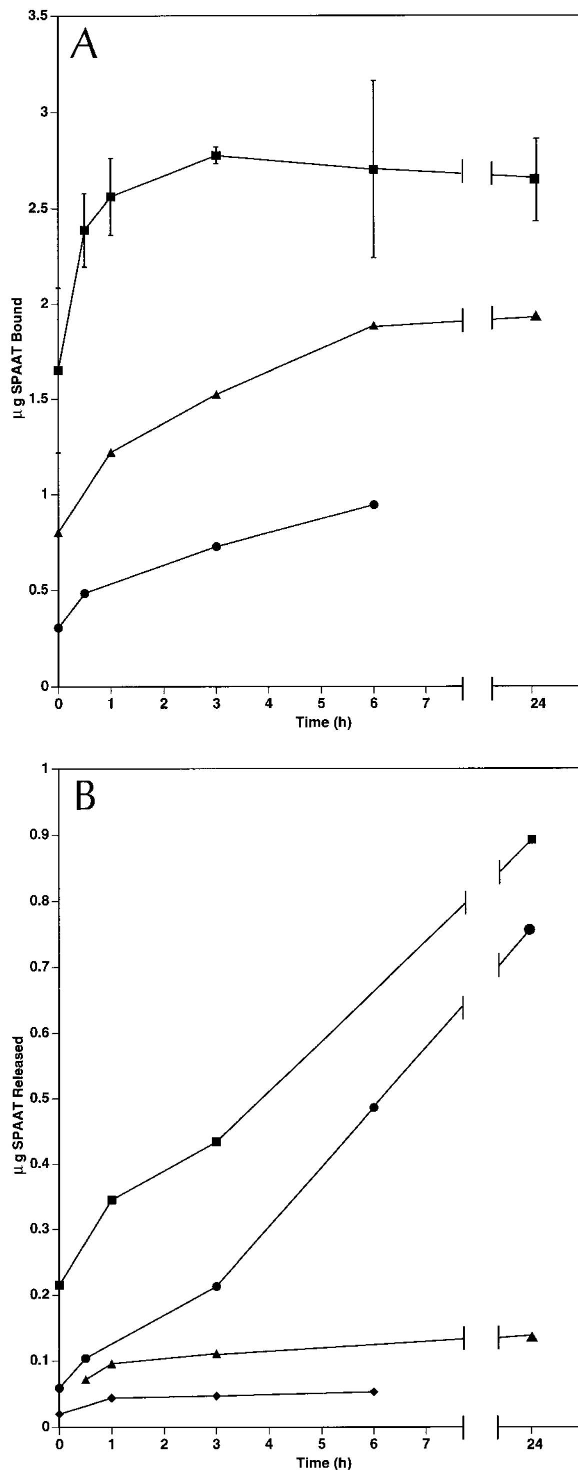

Fig. 6. SPAAT binding to laminin-1. **A:** Specificity. Radiolabelled SPAAT (approximately 28,000 cpm/20 µg) was added to each well of a 24-well tissue culture plate coated with mouse laminin-1, then incubated for the indicated times in the absence (■) or presence (●) of a ninefold molar excess of unlabelled SPAAT or an approximately equimolar amount of HSA (▲). At each time point, the unbound supernatant radioactivity was removed and each well was washed with an equal volume of DW. The remaining bound SPAAT radioactivity was then released by overnight digestion at 37°C with 500 µl of proteinase K. This digest plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT bound was converted to micrograms and plotted. Uncompeted radiolabelled SPAAT binding was assayed in duplicate. The error bars represent the range of these two average values. **B:** Decay. Laminin-1 was equilibrated with radiolabelled SPAAT (approximately 28,000 cpm/20 µg) by preincubating each well of a 24-well tissue culture plate coated with mouse laminin-1 overnight (approximately 18 h) at 37°C. The next day the unbound supernatant radioactivity was removed and each well was washed with an equal volume of DW. About 6% of the total added radiolabelled SPAAT bound. A fresh 500 µl aliquot of PBS, pH 7.4, buffer was then added to each well and incubated for the indicated times. At each time point the radiolabelled SPAAT released into the supernatant buffer plus an equal volume DW wash was counted. By dividing this radioactivity by the specific activity of the peptide, the amount of radiolabelled SPAAT released was converted to micrograms and plotted. The above procedure was repeated for 4 days until the released radioactivity had fallen to essentially background levels. Day 1 (■), day 2 (●), day 3 (▲), day 4 (◆). Additional experimental details are given in the Materials and Methods section.

retained its irreversible, stoichiometric ability to inhibit human neutrophil elastase (HNE) suggesting that as much as 50% of the bound AAT may be cleaved in situ to generate ECM-bound SPAAT.

More recently, another study demonstrated that human stromelysin-3 produced by stromal fibroblasts immediately surrounding invasive breast cancer cells cleaved AAT within its reactive-site loop between ALA-350 and MET-351 to generate SPAAT [Pei et al., 1994]. Such a cellular localization of this enzymatic activity would be consistent with a host defense response to stop the spread of the cancer cells by coating surrounding protease sensitive-ECM proteins with an inhibitor such as SPAAT. Consequently our current working hypothesis is that ECM-bound AAT is cleaved by stromelysin-3 in situ to generate ECM-bound SPAAT and that this ECM-bound SPAAT protects susceptible ECM proteins, particularly the basement membrane proteins, laminin-1 and type IV collagen, from inappropriate enzyme digestion. Various pathophysiological conditions in addition to tumor metastasis, such as emphysema, premature rupture of fetal membranes (PROM), and inflammatory diseases like rheumatoid arthritis and periodontal disease, therefore, might be exacerbated by an inadequate supply of tissue-bound SPAAT. The use of specific competitive peptide inhibitors of migration (that can be manufactured and purified in large quantities), like SPAAT, could therefore theoretically provide a rational basis for therapy of disorders involving aberrant invasion, such as the prevention of metastatic seeding of cancer cells after surgical removal of the primary tumor [Humphries et al., 1986].

The use of SPAAT as a therapeutic agent, moreover, has many attractive features. It is a derivative of a naturally occurring protein thereby minimizing the risk of potential immunological complications even at relatively high doses. The normal serum concentration of AAT is 2.5 ± 1 mg/ml [Brantly et al., 1988] and may be elevated three- to fourfold during an acute phase response [Schreiber, 1987; Kushner, 1988]. In addition, SPAAT can be synthesized readily and inexpensively by chemical, rather than recombinant, methods ensuring purity and allowing administration of the agent free of potentially toxic biological contaminants. Lastly, the affinity exhibited by SPAAT for components of the ECM, particularly laminin-1, suggests that the peptide might have a lengthy biological half-life thus obviating the requirement for frequent administration.

ACKNOWLEDGMENTS

The UAB Protein Synthesis Core Facility was supported by grant CA-13148 from the NCI, NIH. We thank James R. Deatherage for technical assistance in obtaining the non-collagenous ECM glycoprotein data and Connie Richardson for her secretarial assistance. An abstract of this work was presented at the 1995 American Society for Cell Biology annual meeting.

REFERENCES

Andrews JL, Ghosh P (1990): Low molecular weight serine proteinase inhibitors of human articular cartilage. Arthritis Rheum 33:1384–1393.

Bar-Shavit R, Elder A, Vlodavsky I (1989): Binding of thrombin to subendothelial extracellular matrix. J Clin Invest 84:1096–1104.

Bashkin P, Doctron S, Klagsbrum M, Svahn CM, Folkman J, Vlodavsky I (1989): Basic fibroblast growth factor binds to subendothelial extracellular matrix and is released by heparitinase and heparin-like molecules. Biochemistry 28:1737–1743.

Bissel MJ, Hall HG, Parry G (1982): How does the extracellular matrix direct gene expression? J Theor Biol 99: 31–68.

Brantly M, Nukina T, Crystal RG (1988): Molecular basis of alpha-1-antitrypsin deficiency. Am J Med 84 (Suppl. 6A): 13–31.

Cambell A, Wicha MS, Lang M (1985): Extracellular matrix promotes the growth and differentiation of murine hematopoietic cells in vitro. J Clin Invest 75:2085–2090.

Cawston TE, Barrett AJ (1979): A rapid and reproducible assay for collagenase using [1-^{14}C] acetylated collagen. Anal Biochem 99:340–345.

Declerck PJ, DeMol M, Alessi M-C, Bauder S, Paques EP, Preissner KT, Muller-Berghaus G, Collen D (1988): Purification and characterization of a plasminogen activator inhibitor binding protein from human plasma. J Biol Chem 263:15454–15461.

Geboes K, Ray MB, Rutgeerts P, Callea F, Desmet VJ, Vantrappen G (1982): Morphological identification of alpha-1-antitrypsin in the human small intestine. Histopathology 6:55–60.

Highsmith RF (1981): Isolation and properties of a plasminogen activator derived from canine vascular tissue. J Biol Chem 256:6788–6795.

Huber AR, Weiss SJ (1989): Disruption of the subendothelial basement membrane during neutrophil diapedesis in an in vitro construct of a blood vessel wall. J Clin Invest 83:1122–1136.

Humphries MJ, Olden K, Yamada KM (1986): A synthetic peptide from fibronection inhibits experimental metastasis of murine melanoma cells. Science 233:467–470.

Johnson-Witt B (1980): A quantitative collagen film collagenase assay for large numbers of samples. Anal Biochem 104:175–181.

Kay EP, Smith RE, Nimni ME (1985): Type I collagen synthesis by corneal endothelial cells modulated by polymorphonuclear leukocytes. J Biol Chem 260:5139–5146.

Kittas C, Aroni K, Kotsis L, Papadimitriou CS (1982a): Distribution of lysozyme, α1-antichymotrypsin and α1-antitrypsin in adenocarcinomas of the stomach and large intestine. Virchows Arch [A] 398:139–147.

Kittas C, Aroni K, Matani A, Papadimitriou C (1982b): Immunocytochemical demonstration of α1-antitrypsin and α1-antichymotrypsin in human gastrointestinal tract. Hepatogastroenterology 29:275–277.

Kleinman HK, McGarvey ML, Hassell JR, Star VL, Cannon FB, Laurie GW, Martin GR (1986): Basement membrane complexes with biological activity. Biochemistry 25:312–318.

Knudsen BS, Silverstein RL, Leung LLK, Harpel PC, Nachman RL (1986): Binding of plasminogen to extracellular matrix. J Biol Chem 261:10765–10771.

Kushner I (1988): The acute phase response: An overview. Methods Enzymol 163:373–383.

LeBaron RG, Hook A, Esko JD, Gay S, Hook M (1989): Binding of heparan sulfate to type V collagen. A mechanism of cell-substrate adhesion. J Biol Chem 264:7950–7956.

Madri JA, Williams SK (1983): Capillary endothelial cell cultures: Phenotypic modulation by matrix components. J Cell Biol 97:153–165.

Majack RA, Cook CA, Bornsetin P (1985): Platelet-derived growth factor and heparin-like glycosaminoglycans regulate thrombospondin synthesis and deposition in the matrix by smooth muscle cells. J Cell Biol 101:1059–1070.

Miller EJ, Rhodes RK (1982): Preparation and characterization of the different types of collagen. Methods Enzymol 82A:33–64.

Mimuro J, Loskutoff DJ (1989): Purification of a protein from bovine plasma that binds to type I plasminogen activator inhibitor and prevents its interaction with extracellular matrix. J Biol Chem 264:936–939.

Molhuizen HOF, Alkemede HAC, Zeeuwen PLJM, deJongh GJ, Wieringa B, Schalkwijk J (1993): SKALP/Elafin: An elastase inhibitor from cultured keratinocytes. J Biol Chem 268:12028–12032.

Nielsen K (1984): Coeliac disease: Alpha-1-antitrypsin content in jejunal mucosa before and after gluten-free diet. Histopathology 8:759–764.

Niemann MA, Narkates AJ, Miller EJ (1992): Isolation and serine protease inhibitory activity of the 44-residue C-terminal fragment of α1-antitrypsin from human placenta. Matrix 12:233–241

Niemann MA, Baggott JE, Miller EJ (1997): Inhibition of human serine proteases by SPAAT, the C-terminal 44-residue peptide from α1-antitrypsin. Biochim Biophys Acta, in press.

Orkin RW, Gehron P, McGoodwin EB, Martin GR, Valentine T, Swarm R (1977): A murine tumor producing a matrix of basement membrane. J Exp Med 145:204–220.

Pei D, Majmudar G, Weiss SJ (1994): Hydrolytic inactivation of a breast carcinoma cell-derived serpin by human stromeylsin-3. J Biol Chem 269:25849–25855.

Rao CN, Liu YY, Peavey CL, Woodley DT (1995a): Novel extracellular matrix-associated serine proteinase inhibitors from human skin fibroblasts. Arch Biochem Biophys 317:311–314.

Rao CN, Gomez DE, Woodley DT, Thorgeirsson UP (1995b): Partial characterization of novel serine proteinase inhibitors from human umbilical vein endothelial cells. Arch Biochem Biophys 319:55–62.

Ray MB, Desmet VJ, Gepts W (1977): Alpha-1-antitrypsin immunoreactivity in islet cells of adult human pancreas. Cell Tissue Res 185:63–68.

Ray MB, Geboes K, Callea F, Desmet VJ (1982): Alpha-1-antitrypsin immunoreactivity in gastric carcinoid. Histopathology 6:289–297.

Reich E (1978): Activation of plasminogen: A widespread mechanism for generating localized extracellular proteolysis. In Ruddon RW (ed): "Biological Markers of Neoplasia: Basic and Applied Aspects." Amsterdam: Elsevier/North Holland, pp 491–498.

Rice WG, Weiss SJ (1990): Regulation of proteolysis at the neutrophil-substrate interface by secretory leukoproteinase inhibitor. Science 249:178–181.

Rinehart AR, Mallya S, Simon SR (1993): Human α1-proteinase inhibitor binds to extracellular matrix in vitro. Am J Respir Cell Mol Biol 9:666–679.

Schreiber G (1987): Synthesis, processing and secretion of plasma proteins by the liver and other organs and their regulation. In Putnam FW (ed): "The Plasma Proteins," 2nd ed, vol 5. New York: Academic, pp 292–363.

Sheela S, Barrett JC (1982): In vitro degradation of radiolabelled, intact basement membrane mediated by cellular plasminogen activator. Carcinogenesis (London) 3:363–369.

Siegal GP, Wang M-H, Rinehart Jr CA, Kennedy JW, Goodly LJ, Miller Y, Kaufman DG, Singh RK (1993): Development of a novel human extracellular matrix for quantitation of the invasiveness of human cells. Cancer Lett 69:123–132.

Tahara E, Ito H, Taniyama K, Yokozaki H, Haita J (1984): Alpha1-antitrypsin, alpha1-antichymotrypsin, and alpha2-macroglobulin in human gastric carcinomas. Hum Pathol 15:957–964.

Timpl R (1989): Structure and biological activity of basement membrane proteins. Eur J Biochem 180:487–502.

Vlodavsky I, Lui GM, Gospodarowicz D (1980): Morphological appearance, growth behavior and migratory activity of human tumor cells maintained on extracellular matrix versus plastic. Cell 19:607–616.

Vlodavsky I, Fuks Z, Ishai-Michaeli R, Bashkin P, Levi E, Korner G, Bar-Shavit R, Klagsbrum M (1991): Extracellular matrix-resistant basic fibroblast growth factor: Implication for the control of angiogenesis. J Cell Biochem 45:167–176.

Wagner DD, Urban-Pickering M, Marden VJ (1984): von Willerbrand protein binds to extracellular matrices independently of collagen. Proc Natl Acad Sci USA 81:471–475.

Wiedow O, Schroder J-M, Gregory H, Young JA, Christophers E (1990): Elafin: An elastase-specific inhibitor of human skin. J Biol Chem 265:14791–14795.

Wittekind CH, Wachiner R, Henke W, Kleist S (1986): Localization of CEA, HCG, lysozyme, alpha-1-antitrypsin and alpha-1-antichymotrypsin in gastric cancer and prognosis. Virchows Arch [A] 409:715–724.

Yurchenco PD, Schittny (1990): Molecular architecture of basement membranes. FASEB J 4:1577–1590.