Expression of Biologically Active Human Antithrombin III by Recombinant Baculovirus in Spodoptera frugiperda Cells*

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(Received for publication, August 29, 1990)

Antithrombin III (ATIII) is a plasma-borne serine protease inhibitor that plays a pivotal role in the regulation of hemostasis. The cDNA for ATIII has been available, but genetic studies on the functional domains of ATIII have not progressed because of the absence of an expression system that will yield sufficient quantities of biologically active protein for biochemical analyses. In the present studies the cDNA of the human antithrombin III gene was inserted into the vector pVL 1393, which is suitable for cotransfection of Spodoptera frugiperda (Sf9) insect cells with Baculovirus wild-type DNA. Recombinant virus particles were selected by the presence of occlusion-negative plaques. Upon infection with purified recombinant virus, Sf9 cells secreted 1.0–35 μg of ATIII/1 x 10⁶ cells. Southern analysis of DNA from infected cells demonstrated incorporation of the full-length cDNA into the Baculovirus recombinant, and RNase protection experiments verified the presence of full-length transcript. This recombinant ATIII protein was immunologically reactive with antisera raised against native human ATIII, formed stable complexes with thrombin, and was heparin-accelerated at the same concentration as native human ATIII. In addition, the recombinant ATIII retained specificity for the same molecular species of heparin that activates authentic human ATIII. This is the first successful production of active, recombinant ATIII in quantities that will allow purification on the milligram scale and permit a biochemical analysis of genetically engineered variants.

Antithrombin III (ATIII) is a plasma-borne serine protease inhibitor that acts as a key regulatory element in the blood clotting cascade (1–3). This regulation is principally due to the inhibition of thrombin (4), although ATIII inhibits several other proteases in the cascade less efficiently, including factors IXa and Xa (5), Xa (6), and XIla (7). Although the mechanism underlying the inhibition of thrombin by ATIII is not completely understood, it is agreed that a small fragment of ATIII is cleaved and released during the process (8–10). Thrombin and ATIII remain in tight association in a 1:1 stoichiometry in complexes that are stable to boiling in SDS and electrophoresis in the presence of βME (1). Although the precise nature of the ATIII-thrombin association has not been clearly demonstrated, it is assumed that it represents the covalent acylation intermediate that arises during serine protease hydrolysis.

The anti-protease activity of ATIII, as well as several other plasma-borne serine protease inhibitors, is dramatically activated by the glycosaminoglycan heparin (11). This activation results in a several thousandfold acceleration in complex formation between ATIII and thrombin and is initiated by the binding of heparin to ATIII (1). Since the binding of heparin to ATIII appears to be a prerequisite for acceleration, elucidation of the structure and location of the heparin-binding site in ATIII is fundamental to the mechanistic understanding of its activation. To date, there have been several studies indicating that the first third of the molecule contains the heparin-binding domain. H nuclear magnetic resonance studies demonstrate that a conformational change occurs in the first third of the molecule upon heparin addition (11), and analysis of CNBr fragments of ATIII show heparin binding to this region (12). Studies employing chemical modification of lysine residues 107, 125, 133, and 136 (13–15), which alter the heparin binding capacity, also support the premise that the first third of the molecule contains the heparin-binding site. More recently, the heparin-binding site has been narrowed to the region of the molecule containing residues 124–145 (16, 17). Because the heparin-binding domain has been narrowed to such a short region of the molecule, the next logical step in finding the exact amino acids involved in heparin binding is to employ genetic manipulation of the protein sequence.

In the present studies we have taken the first step toward this goal by generating a recombinant Baculovirus containing the human ATIII cDNA in place of the major coding region of the polyhedrin gene (18). Sf9 insect cells infected with the recombinant virus produce and secrete ATIII which is biologically active, heparin-activated, and strongly reactive with antisera raised against authentic human ATIII purified from human plasma. These studies represent the first successful expression of biologically active recombinant human ATIII in quantities sufficient for biochemical analysis.

EXPERIMENTAL PROCEDURES

Materials—The ATIII cDNA (pBATII-113) cloned into pBR322 was a generous gift from Dr. Savio Woo (Howard Hughes Medical Institute, Houston, TX). ATIII was purified from human plasma as previously described (19). This preparation is referred to as authentic ATIII throughout the paper. Thrombin was a generous gift from Dr. John Fenton. Anti-human ATIII antisera and radiolnuclide enzyme immunoassay grade bovine serum albumin were from Sigma. Na⁺¹¹⁰Iodide and Hydroxylamine were purchased from Amersham Corp. Electrophoresis reagents were purchased from Bio-Rad. Protein G-Sepharose was from Pharmacia LKB Biotechnology Inc. Endoglycosidase F and porcine...
moval washes were performed using 0.1 M 3996 NaCl, pH 7.7) for 1 h at 30 °C. The reaction was stopped by the addition of 30 μl of 10% SDS and 50 μl of proteinase K. The reactions were incubated for an additional 30 min at 37 °C. The protected RNA was extracted with phenol/chloroform and precipitated with isopropl alcohol. The RNA was redissolved in 30 μl of water and 3 μl of NaCl, pH 7.5, 1% SDS, and 1 unit(s) of endoglycosidase F (Behring Diagnostics), respectively. The digested proteins were precipitated with ice cold 10% trichloroacetic acid, pelleted by centrifugation for 10 min at 10,000 × g in a microcentrifuge, rinsed with ice-cold acetone, repelleted, and air-dried. The protein pellets were resuspended in 25 μl of Excel1 medium) were digested for approximately 20 h with 3 μl of 10% SDS and 50 μg of proteinase K. The reactions were terminated by the addition of 25 μl of SDS sample buffer containing 2 mM βME and boiled for 5 min. Thirty μl of each sample was separated by SDS-PAGE on 10% polyacrylamide gels (20). The gels were dried and exposed to x-ray film (XAR-5) overnight at -70 °C in the presence of a Cronex Lightning plus enhancement screen. 

Immunoprecipitation—ATIII-thrombin complexes were formed as described above, with the exception that the reactions were not terminated with SDS sample buffer or boiled. The complexes were immunoprecipitated at 37 °C for 30 min, using anti-ATIII antibodies at a final dilution of 1:100. The antibody complexes were precipitated with a 30-min incubation with 100 μl of Protein G-Sepharose (a 1:5 slurry in phosphate-buffered saline), and pelleted by centrifugation in a microcentrifuge for 30 s at 10,000 × g. The pellets were washed twice with 2 ml of HES, pH 7.5 (10 mM HEPES, pH 7.5, 0.15 M NaCl, 0.5% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml ovalbumin, and centrifuged again. The samples were resuspended in 50 μl of SDS sample buffer containing 2 mM βME and analyzed by SDS-PAGE on 10% polyacrylamide gels followed by autoradiography as described above.

Immunoblotting—Twenty-five μl of each medium sample and 50 ng of ATIII were separated on 10% polyacrylamide gels and transferred to nitrocellulose for 1 h at 70 V using a semidry blotting apparatus. The blots were blocked overnight at room temperature with 3% radioimmuno assay grade bovine serum albumin in Tris-buffered saline, pH 7.5, and processed for autoradiography as described above.

Baculovirus Recombinant ATIII

Construction and Transformation—The ATIII cDNA was cut out of pBR322 with PstI and SalI and subcloned into Bluescript (Stratagene) at the PstI and SalI sites. The gene was then digested out of Bluescript with PstI and XbaI, and inserted into vector pVL 1393 at those sites in the polylinker. The resulting pVL 1393-ATIII vector was purified by cesium chloride centrifugation (18). Two × 10^7 Sf9 cells were cotransfected with 2 μg of the purified vector and 1 μg of wild-type viral DNA as described (18). Recombinant virus particles were purified by plaque assay (18), stained with Coomassie brilliant blue, and analyzed by SDS-PAGE. 

RNA extraction Mammalian heparin were purchased from Behring Diagnostics. Autoradiography supplies including x-ray film, developers, and fixers were from Kodak. Sf9 cells were purchased from American Type Culture Collection, Rockville, MD. Wild-type Baculovirus DNA and the pVL 1393 vector were a generous gift from Max D. Summers, Texas A&M, College Station, TX. Tissue culture media and reagents were from J. R. Soriano. All labeled amino acids were from Sigma, Cal-Biochem, and Bethesda Research Laboratories.

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reactions were placed on ice, and 50 μl of a 1 mM solution of Chromozyme-TH substrate was added to each well. The reactions were brought to room temperature and incubated for an additional 10 min. The reactions were terminated by the addition of 100 μl of 5 M acetic acid and quantitated by absorbance at 405 nm.

RESULTS

Generation and Characterization of Recombinant Baculovirus—Two μg of the Baculovirus vector pVL 1393 containing the full-length cDNA for ATIII (Fig. 1A) was incubated with Sf9 cells in the presence of 1 μg of wild-type Baculovirus DNA. Eight days later the supernatants were harvested and screened in a plaque assay over a 1000-fold dilution series. Two well-separated, polyhedrin-minus (occlusion body negative) plaques from plates containing approximately 200 plaques were isolated. Rased on the number of polyhedrin-minus plaques observed, the recombination efficiency was estimated to be approximately 0.1% (data not shown). Upon second-round plaque purification, both of the original recombinants gave rise to only polyhedrin-minus plaques. These second-round plaque purification, both of the original recombinants from plates containing approximately 200 plaques were digested and probed with a 32P-phATIII-113 (Fig. 1B). The autoradiogram shows that labeled phATIII probe specifically hybridized to high molecular weight DNA from cells infected with both BrATIII-1 and BrATIII-2. In contrast, the DNA from wild-type virus-infected cells and uninfected cells did not show specific hybridization. When the DNA samples were double-digested with PstI and BamHI, the specific hybridization was localized to a 1.4-kilobase band that migrated identical to the authentic phATIII-113 cDNA that was originally cloned into the pVL 1393 vector using PstI and XbaI sites. These data demonstrate that both BrATIII recombinants which were plaque purified from the primary transfection contain the full-length ATIII cDNA insert.

Production of Biologically Active ATIII by Sf9 Cells Infected with BrATIII Recombinants— Cultures of Sf9 cells were infected with BrATIII-1 and BrATIII-2 or with wild-type virus as described above. Seventy-two h after infection, the culture supernatants were harvested and assayed for the presence of biologically active ATIII using the thrombin linkage assay (see “Experimental Procedures”). Briefly, 20-μl aliquots of the media were incubated with 125I-thrombin and analyzed for the presence of high molecular weight complexes by SDS-PAGE and autoradiography. When 125I-thrombin was incubated with medium from cells infected with BrATIII-1 or BrATIII-2, complexes of approximately 80 kDa were formed (Fig. 2, lanes 1 and 2). In contrast, complexes were absent when 125I-thrombin was incubated with medium harvested from uninfected cultures or cultures infected with wild-type virus (Fig. 2, lanes 3 and 4). These data show that the production of a protein capable of forming complexes with thrombin is dependent on infection with the recombinant viruses.

Fig. 1. A, map of vector pVL 1393. The cDNA of ATIII was inserted into the Baculovirus vector pVL 1393 at the PstI and Xbal sites within the polyhedrin region of the vector. This vector utilizes the translation initiation signals of the inserted gene, resulting in a non-fused protein. B, Southern analysis of total cellular DNA from uninfected cells and cells infected with wild-type or recombinant Baculovirus. Total cellular DNA from BrATIII-1- and BrATIII-2-infected, wild-type-infected, and uninfected Sf9 cells was isolated according to the Baculovirus manual (see “Experimental Procedures”). Two μg of each DNA preparation were digested with PstI and BamHI, separated on 1% agarose gels, and capillary transferred to Hybond N paper. The blots were probed with 32P-phATIII-113 at 55 °C and visualized by autoradiography after a 36-h exposure. Lanes 1 and 2, uninfected; lanes 3 and 4, wild-type; lanes 6 and 7, BrATIII-1; lanes 8 and 9, BrATIII-2; lanes 10 and 11, pVL 1393 containing ATIII. Digested (+); undigested (−). Lanes 5 and 12 contain standards.

Fig. 2. Biological activity of recombinant ATIII. Twenty-five μl of media from infected and uninfected Sf9 cells were used to test for the presence of 125I-thrombin-complexing proteins. The media samples were incubated at 37 °C in the presence of 16 ng of 125I-thrombin for 30 min. After termination, complexes were separated from free thrombin by SDS-PAGE on 10% polyacrylamide gels and visualized by autoradiography. Lane 1, BrATIII-1; lane 2, BrATIII-2; lane 3, uninfected; lane 4, wild-type virus-infected.
Thrombin-inhibitor complexes were allowed to form in samples examined. To confirm the identity of the protein involved in the formation of complexes as ATIII, we examined the ability of anti-ATIII antisera to recognize the protein involved in the formation of complexes as ATIII, we examined the ability of anti-ATIII antisera to recognize the protein involved in the formation of complexes. The samples were then solubilized in SDS sample buffer and resolved by SDS-PAGE on 10% polyacrylamide gels (Fig. 3). Complexes formed by the addition of 125I-thrombin to medium harvested from BrATIII-1- and BrATIII-2-infected Sf9 cells were specifically recognized by the anti-ATIII antisera (Fig. 3, lanes 1 and 4), confirming the identity of the inhibitor in these complexes as human ATIII. These complexes were not immunoprecipitated by control antisera raised against a related serine protease inhibitor, protease nexin-I (Fig. 3, lanes 2 and 5), or in the absence of antisera (Fig. 3, lanes 3 and 6). Immunoprecipitable complexes were absent when 125I-thrombin was incubated with medium harvested from wild-type virus-infected cells (Fig. 3, lanes 7–9), further demonstrating that the inhibitor was not produced in the absence of recombinant virus. It should be noted, however, that the apparent mass of the complexes is smaller than those formed with authentic human ATIII (Fig. 3, lane 10).

Immunoblot Analysis and Endoglycosidase Digestion—Since the immunoprecipitation data of 125I-thrombin-inhibitor complex suggested a difference in the molecular masses of authentic and recombinant ATIII, we examined this difference more carefully. Although this difference appeared to be small, even a relatively large difference could be obscured in the high molecular mass range on 10% polyacrylamide gels. To more accurately quantitate this difference, samples of medium harvested from BrATIII-1- and BrATIII-2-infected cells, and authentic ATIII, were subjected to electrophoresis and immunoblot analysis. In this gel system, the authentic ATIII migrated with an apparent molecular mass of 58 kDa (Fig. 4, lane 1), in precise agreement with previously published values (24). The recombinant ATIII expressed by both BrATIII-1- and BrATIII-2-infected cells (Fig. 4, lanes 3 and 7, 2 and 6 respectively), had an apparent molecular mass of 50 kDa, 8 kDa less than authentic ATIII purified from plasma. The Sf9 insect cells have been shown to glycosylate at the universal consensus sites, but the precise nature of the carbohydrate groups added can differ substantially from those added to a protein in its native expression system (24). To determine if the apparent difference in molecular mass might be due to glycosylation differences, the immunoblot experiment shown in Fig. 4 was repeated with and without endoglycosidase F digestion, to remove N-linked carbohydrate. Both the recombinant and authentic ATIII showed an approximate 4 kDa loss in mass after treatment with endoglycosidase F (data not shown). This apparent difference in molecular mass is addressed further in experiments discussed below.

Recombinant ATIII Is Heparin Activated—Since the original goal of these studies was to develop an ATIII expression system to examine the mechanism of heparin activation by genetic manipulation, we next examined the ability of the recombinant ATIII to be activated by heparin and to recognize the same molecular species of heparin that selectively activates authentic human ATIII. To determine if the recombinant ATIII retains specificity for the same species of heparin that activates authentic ATIII, two different types of heparin were compared in an activation assay. These two different preparations of heparin, termed “high” and “low” affinity, were prepared by the repeated passage of commercial porcine mucosal heparin over an affinity column of authentic human ATIII covalently coupled to Sepharose. On each cycle, the retained heparin was eluted with 1.0 M NaCl and is referred to as “high affinity” heparin. The flow-through heparin is referred to as “low affinity” heparin. Both preparations were quantitatively standardized according to uronic acid content.

![FIG. 3. Immunological reactivity of recombinant ATIII-thrombin complexes with anti-ATIII antisera.](image1)

![FIG. 4. Immunoblot comparison of recombinant ATIII and authentic ATIII.](image2)
the addition of SDS sample buffer. Free thrombin and complexes proceed for an additional 30 s. The reactions were rapidly terminated from BrATIII-1-infected Sf9 cells were preincubated with the indicated concentrations of heparin at 37 °C for 30 min. Sixteen ng of 125I-thrombin (125I-T) were added, and the reactions were allowed to proceed for an additional 30 s. The reactions were rapidly terminated by the addition of SDS sample buffer. Free thrombin and complexes were separated by SDS-PAGE on 10% polyacrylamide gels and visualized by autoradiography. Bands corresponding to the position of high molecular weight 125I-thrombin-ATIII complexes were excised and quantitated by gamma counting. The high affinity heparin displayed a sharp dose-response curve and was maximally active in accelerating complex formation at a concentration of 100 ng/ml.

Finally, it was of interest to examine the kinetics of high affinity heparin activation, comparing recombinant and authentic plasma ATIII in parallel. While the experiment shown in Fig. 5 demonstrates that the specificity for high affinity heparin is retained by recombinant ATIII, a different type of experiment was required to gain the resolution needed to examine the kinetics. To accomplish this, the linkage assay shown in Fig. 5 was repeated with authentic ATIII and recombinant ATIII in parallel, under conditions where the concentration of ATIII was approximately equal to or less than the concentration of 125I-thrombin. Using this approach we were able to more accurately define the relationship between heparin concentration and activation (Fig. 6). The activation of both authentic and recombinant ATIII was sharply dependent on the concentration of heparin, and nearly identical activation curves were seen for each. The difference in the amount of high affinity heparin required to achieve half-maximal activation of each was less than 0.3 ng. These data are of particular importance, since they demonstrate that the kinetic interactions of high affinity heparin with authentic ATIII and recombinant ATIII are functionally identical.

\[ \text{BrATIII-1 and BrATIII-2 Produce Full-length ATIII Tr}\]
FIG. 7. RNA nuclease protection of full-length ATIII antisense RNA by RNA extracted from BrATIII-1-infected Sf9 cells. Total RNA from both uninfected cells and BrATIII-1-infected cells, (5 and 15 μg from each) was cocrupipitated with 4 × 10^6 cpm of ^32P-antisense ATIII RNA. After denaturation, the RNA was annealed for 4 h at 42 °C, and the unprotected RNA was digested with 100 ng of RNase A and 100 units of RNase T1 (lanes 1–4). The reaction was stopped by the addition of SDS, followed by proteinase K digestion (see “Experimental Procedures”). Protected RNA was separated by 5% denaturing PAGE and visualized by autoradiography. Lanes 1 and 2, RNA from BrATIII-1-infected cells, 15 and 5 μg, respectively; lanes 3 and 4, RNA from uninfected cells, 15 and 5 μg, respectively; lane 5, ^32P-antisense ATIII RNA; lane 6, standards, given in number of bases.

FIG. 8. Partial purification of recombinant ATIII by ion-exchange chromatography on DEAE-Sephacel. An aliquot of 150 μl of the peak fraction of thrombin inhibitory activity obtained by the fractionation of medium harvested from BrATIII-1-infected Sf9 cells on DEAE-cellulose (see text) was precipitated in 10% trichloroacetic acid. The precipitated proteins were divided into two equal aliquots and separated by SDS-PAGE on 10% polyacrylamide gels. The lanes were transferred to polyvinylidene difluoride paper; one lane was stained with Coomassie Brilliant Blue R (lane 1), the other lane was subjected to immunodetection with anti-ATIII antisera (lane 2). The positions of molecular weight standards are indicated. The band indicated by the arrow that comigrates with the immunoreactive band was excised and subjected to amino acid sequence analysis (see Table I).

### Table I

**NH2-terminal amino acid sequence of recombinant ATIII**

| Cycle Number | Residue | Yield (μg) | hATIII |
|--------------|---------|------------|--------|
| 1            | Gly     | 5.2        | His    |
| 2            | Gly     | 3.93       | Gly    |
| 3            | Ser     | 1.3        | Ser    |
| 4            | Pro     | 2.9        | Pro    |
| 5            | Val     | 1.7        | Val    |
| 6            | Asp     | 2.3        | Asp    |
| 7            | Ile     | 2.0        | Ile    |

Partially purified recombinant ATIII was separated by SDS-PAGE and transferred to polyvinylidene difluoride. The band corresponding to ATIII, determined by parallel immunoblotting was visualized by Coomassie staining, excised, and subjected to automated Edman degradation. Shown in the table are the cycle numbers, residues identified, and yields in picomoles. Shown for comparison in the last column is the NH2-terminal sequence of authentic human ATIII.

Quantitation of ATIII Production by BrATIII Recombinants—Finally, it was of interest to determine the relative levels of ATIII expression by Sf9 cells infected with BrATIII-1 and BrATIII-2 since one of the goals of this study was to produce recombinant ATIII in quantities sufficient for biochemical analyses. A chromogenic assay that specifically measures thrombin activity was employed. Separate flasks of Sf9 cells (3 × 10^6) were infected with BrATIII-1 and BrATIII-2 obtained from second-round plaque purification, at a multiplicity of infection of 0.2:1.0. Seventy-two h later the medium was harvested and assayed for thrombin inhibitory activity (Fig. 9B). A standard curve of thrombin inhibition was generated in parallel using authentic human ATIII (Fig. 9A). The data are presented as the percent remaining thrombin activity versus μl of conditioned medium added (see “Experimental Procedures” for details). In the standard curve, 65 ng of authentic ATIII were required to achieve 50% thrombin inhibition under these assay conditions (Fig. 9A). While medium harvested from uninfected Sf9 cells showed no thrombin inhibitory activity, medium harvested from BrATIII-1- and BrATIII-2-infected cells were potently inhibitory. Based on the volumes of medium required for 50% inhibition, and the total volume of medium harvested from the cells, we estimate a recombinant ATIII production of 10 and 35 μg from 3 × 10^6 Sf9 cells infected with BrATIII-1 and BrATIII-2, respectively. These experiments have been repeated at a 50-fold higher multiplicity of infection, and production was found not to be significantly increased (data not shown).

**DISCUSSION**

In the present studies we used the Baculovirus-insect cell expression system to produce recombinant human ATIII. The
cotransfection of Sf9 cells with wild-type Baculovirus and pVL 1393 vector carrying a full-length human ATIII cDNA with polyhedrin gene-flanking sequences resulted in the formation of independent recombinant Baculovirus carrying the ATIII cDNA. Both of the recombinants, designated BrATIII-1 and BrATIII-2, were purified by two rounds of plaque purification, are stable, and give rise to the production of recombinant ATIII in Sf9 insect cells.

The recombinant ATIII produced by BrATIII-1 and BrATIII-2 appear to be identical to each other and retain all of the biological properties of authentic ATIII that were tested. Both are immunologically reactive with antisera raised against ATIII purified from human plasma, form complexes with thrombin, and are maximally activated by heparin at the same molar concentration required to maximally activate authentic ATIII. The heparin activation data are of particular importance, since many studies are now directed at determining the mechanism of heparin activation.

The only difference observed between authentic ATIII and the recombinant forms generated in insect cells infected with BrATIII-1 and BrATIII-2 is the apparent molecular mass determined by SDS-PAGE. We initially thought this difference was probably due to differences in glycosylation, but endoglycosidase treatment suggested that both the authentic and recombinant ATIII were glycosylated to the same degree.

While we still have not determined the reason for the apparent mass difference, three lines of evidence suggest that it is not due to a difference in the polypeptide chain per se. First, RNase protection experiments showed that the transcript produced in the insect cells completely protected full-length antisense transcript generated in vitro, demonstrating that the full-length transcript is produced in infected cells. Second, the complexes formed between thrombin and recombinant ATIII showed the same apparent mass difference. Since thrombin cleaves ATIII at a site 38 residues from the carboxyl terminus (Arg393–Ser394), the modification(s) causing this apparent mass difference must be to the amino side of Arg393. Finally, the recombinant ATIII was partially purified and the amino terminus was sequenced. The amino terminus of the recombinant and authentic ATIII were identical for the first 7 residues. Therefore, the recombinant signal sequence is processed properly, and the difference in mass is not due to a proteolytic cleavage at the amino terminus. Thus, we conclude that the apparent mass difference is due either to post-translational modifications of the complete polypeptide chain, which has been shown to occur with other proteins expressed in Baculovirus (24), or is simply an SDS-PAGE artifact. Importantly, this apparent mass difference does not affect any of the biological or biochemical properties of activities of the recombinant ATIII that we studied.

These studies were initiated so that an expression system could be developed for human ATIII for use in site-directed mutagenesis experiments, primarily in the region of heparin binding. Previous attempts to express active ATIII have been largely unsuccessful, with the exception of expression in COS cells (25). Unfortunately, the level of transient expression in that system was so low (1 ng/10^6 cells) that its utility value in site-directed mutagenesis studies is doubtful. Using the Baculovirus expression system, we have obtained expression at a 30,000–60,000-fold higher level than in COS cells. Most importantly, this recombinant ATIII appears to retain functional identity to authentic ATIII. The recombinant ATIII presented in these studies is activated by heparin and retains specificity and the same apparent affinity for the same molecular species of heparin that activates authentic ATIII. ATIII expressed in the Baculovirus expression system should now prove to be a valuable tool in further dissecting the heparin-binding site(s) of ATIII through genetic manipulation.

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