Characterization of a Nonglycosylated Single Chain Urinary Plasminogen Activator Secreted from Yeast*

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Using site-directed mutagenesis, we have changed the asparagine in human single-chain urinary plasminogen activator (u-PA) at position 302 to an alanine. This alteration removes the only known amino acid residue glycosylated in the protein. The single-chain u-PA containing an alanine residue at position 302 instead of asparagine (scu-PA(N302A)) cDNA gene was expressed in the yeast Saccharomyces cerevisiae. Secretion of the protein product into the culture broth was achieved by replacing the human secretion signal codons with those from yeast invertase, adding a yeast promoter from the constitutively expressed glycolytic genes triosephosphate isomerase or phosphoglycerate kinase, and integrating multiple copies of these transcriptional units into the genome of yeast strains carrying the “supersecreting” mutation ssc1.

When fermented in a fed-batch mode, these recombinant baker's yeast strains secreted scu-PA(N302A) in a strongly growth-associated manner. Greater than 90% of the u-PA found in the culture broth was in the single-chain form. Scu-PA(N302A) was purified to homogeneity using two chromatography steps. The purified protein had a molecular weight of 47,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and lacked any detectable N-linked glycosylation. The in vitro fibrinolytic properties of scu-PA(N302A) were found to be essentially equivalent to those of natural single-chain u-PA derived from the human kidney cell line TCL-508. Since scu-PA(N302A) contains the immunogenic N-linked carbohydrate pattern of yeast, it may be a useful therapeutic agent which can be produced economically by yeast fermentation.

Two immunologically distinct plasminogen activators, urinary plasminogen activator (u-PA) and tissue plasminogen activator (t-PA), have been isolated from human tissue (1–3) and cDNA genes for both have been cloned (4, 5). Both enzymes exhibit kinetic parameters consistent with physiological roles in normal in vivo hemostasis. Both share a number of structural features, including “kringle,” epidermal growth factor-like, and serine protease catalytic domains, as well as plasmin-susceptible peptide bonds in the region between the kringle and catalytic domains. Both molecules are cleaved by plasmin to two-chain forms in which the two chains are held together by at least one disulfide bond. Both t-PA and scu-PA exhibit fibrin selectivity in their activation of plasminogen in both in vitro and in vivo model systems and also in man (6–9). In other words, they catalyze the formation of active plasmin more efficiently in the presence of a fibrin clot than in the circulation. Thus, both molecules spare circulating thrombogenic factors such as fibrinogen and α2-antiplasmin.

Despite these similarities, the two molecules are also known to differ in a number of characteristics: (a) t-PA but not u-PA exhibits significant affinity for fibrin and soluble fibrin fragments (14); (b) u-PA, through its epidermal growth factor-like domain, binds to a cell surface receptor while t-PA exhibits no measurable affinity for the u-PA receptor, but may bind to a different receptor on HUVEC cells (10, 11); (c) t-PA contains two domains not found in the u-PA molecule, a second kringle domain, and a finger domain resembling that found on fibronectin. These additional domains of the t-PA molecule may be responsible for the fibrin affinity exhibited by t-PA (12, 13). However, fibrin selectivity must involve factors other than fibrin affinity because u-PA in its single-chain form (scu-PA) exhibits little or no fibrin affinity but appears to lyse clots with fibrin selectivity comparable to that of t-PA (8).

Fibrin selectivity during the conversion of plasminogen to plasmin distinguishes the activity of t-PA and scu-PA from that of urokinase (tcu-PA) and streptokinase and suggests that these molecules are more suitable for thrombolytic therapy in man. The demonstrated affinity of t-PA for fibrin, and kinetic evidence that the interaction with fibrin reduces the Km of t-PA for plasminogen, suggest a plausible mechanism by which t-PA may act in a fibrin-selective manner (1). By contrast, the mechanism by which scu-PA acts in a fibrin-selective manner is unclear. For example, while scu-PA lacks measurable fibrin affinity and appears to exhibit little or no activity in vitro (15), it acts as a potent, fibrin selective thrombolytic agent in man and animals (7, 8).

In this report, we describe the production of human scu-PA in the baker's yeast Saccharomyces cerevisiae. We chose to construct strains of yeast which secrete human scu-PA rather than produce it in the cytoplasm, because secretion appears important for accurate folding and disulfide bond formation in secretory proteins (16). Since human scu-PA is

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a 411-amino acid residue glycoprotein with an apparent molecular weight of about 53,000 and contains up to 12 disulfide bonds, we reasoned that refolding of aggregated scu-PA from inclusion bodies produced cytoplasmically in *Escherichia coli* or yeast would be inefficient. While many mammalian proteins have been secreted from yeast, secretion of proteins larger than about 20,000 *M*<sub>r</sub>, is typically inefficient with a large fraction of the protein remaining internal (17–20). Therefore, we took advantage of the yeast mutation ssc1 which has proven useful for the secretion of other mammalian proteins, including bovine prochymosin and growth hormone (18) to build a yeast strain which secretes at least two-thirds of the scu-PA synthesized into the culture broth.

Human scu-PA normally contains carbohydrate linked to asparagine residue 302 (4). Yeast cells also carry out *N*-linked glycosylation, but unlike human cells would be expected to hyperglycosylate human scu-PA by adding a heterogeneous cluster of over 50 mannose residues as has been found for other secreted glycoproteins such as yeast invertase (21)), yeast acid phosphatase (22), and human α1-antitrypsin (23). Therefore, we altered the codon for amino acid residue 302 in the scu-PA cDNA to encode alanine instead of asparagine. We describe here the secretion from yeast, purification, and in vitro characterization of scu-PA(N302A) lacking any detectable *N*-linked carbohydrate. Except for the absence of *N*-linked carbohydrate in *in vitro* assays, this scu-PA derivative is indistinguishable from scu-PA isolated from mammalian sources.

**MATERIALS AND METHODS AND RESULTS**

*The ssc Mutations Influence the Efficiency of Scu-PA Secretion by Yeast—*Secretion of scu-PA was examined from both wild-type strains of yeast and from strains carrying ssc mutations which increase the secreted levels of other proteins of comparable size such as bovine prochymosin and growth hormone (18). The amounts of scu-PA secreted into the culture broth, expressed both as units per ml and normalized to the optical density at 550 nm, are presented in Table II. Portions of this paper (including "Materials and Methods," part of "Results," Figs. 1 and 2, and Tables II and III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

![Table 1](http://www.jbc.org/)

| Experiment | Host strain | Host secretion genotype | Plasmid | Cell density | u-PA |
|------------|-------------|-------------------------|---------|--------------|------|
| 1          | CGY339      | Wild-type               | pCGS715 | 6.7          | <2.5 |
| 2          | CGY1465     | ssc1-1                  | pCGS715 | 0.8          | 15.5 |
| 3          | CGY1465     | ssc1-1                  | pCGS721 | 1.2          | 17.5 |
| 4          | CGY1465     | ssc2-1                  | pCGS715 | 6.6          | 10.8 |
| 5          | CGY1465     | ssc1-1                  | pCGS715 | 1.6          | 28.0 |

indicating that secretion of scu-PA is growth-associated. Secreted scu-PA(N302A) levels as high as 1800 IU/ml were attained, corresponding to about 15 mg of scu-PA(N302A)/liter.

The fact that the amidolytic activity of unfractionated culture broth prior to plasmid treatment was always less than 5% of the activity obtained after plasmid treatment indicates that very little scu-PA was converted to tcu-PA during the fermentation (Table II). In addition, secreted scu PA was stable in the unfractionated broth for several days at 4° C. Scu-PA was also detected within the yeast cells during fermentation both by immunoblot and by activity in a fibrin plate; however, internal scu-PA antigen levels typically represented not more than about 30% of the total scu-PA detected (data not shown).

*The Specific Activity of scu-PA(N302A)—*Several properties of scu-PA(N302A) purified from yeast culture broth were compared to those of native scu-PA derived from human kidney cell line TNL-598. Neither purified preparation contained significant levels of plasmin-independent amidolytic activity, consistent with the fact that these preparations contain only trace levels of contaminating tcu-PA. In fact, the amidolytic activity of both scu-PA preparations prior to plasmid treatment was less than 1000 IU/mg, well within the range observed previously for scu-PA (15). After treatment with plasmin to activate any latent amidolytic activity, the specific activities of both preparations were virtually identical, in the range of 100,000–120,000 IU/mg (data not shown).

*Electrophoretic Mobilities of Scu-PA and Scu-PA(N302A)—*The electrophoretic mobilities of scu-PA(N302A) secreted from yeast as well as scu-PA secreted from both yeast and from Chinese hamster ovary cells were examined on polyacrylamide gels containing SDS both with and without prior treatment with *N*-glycanase to remove *N*-linked carbohydrate (Fig. 4). Removal of carbohydrate and analysis by SDS-polyacrylamide gel electrophoresis under nonreducing conditions revealed that all three proteins migrated with an apparent *M*<sub>r</sub> of 47,000 (lanes B, D, and F). Prior to *N*-glycanase treatment, each molecule migrated differently (lanes A, C, and E) with the mobility differences being consistent with the expected carbohydrate content of each species based on the properties of the host cell and the cDNA construction. Specifically, CHO cells (lane A) appear to add carbohydrate approximately equivalent to one complex mammalian unit to the single available asparagine receptor in scu-PA, consistent with a previous report (46). Yeast cells add a large heterogeneous cluster of mannose residues to scu-PA (lane E), as...
Growth and secretion of scu-PA(N302A) by the yeast strain CGY1891. Cells were grown in YPD-type modified media under conditions of controlled pH and dissolved oxygen. Initial glucose concentration was 80 g/liter, and glucose was added to 40 g/liter after 50 h and after 74 h of fermentation. Klett is a measure of cell density (100 Klett units corresponds to approximately $2 \times 10^7$ cells/ml). Klett units (---), scu-PA (----). Scu-PA activity was determined by amidolytic assay using the chromogenic substrate S-2444.

![Fig. 3. Growth and secretion of scu-PA(N302A) by the yeast strain CGY1891.](image)

Lanes A and B, CHO secreted scu-PA without (lane A) and with (lane B) prior treatment with N-glycanase; lanes C and D, yeast secreted scu-PA(N302A) without (lane C) and with (lane D) prior treatment with N-glycanase; lanes E and F, yeast secreted scu-PA without (lane E) and with (lane F) prior treatment with N-glycanase. Molecular weight standards are described under "Materials and Methods."

![Fig. 4. Electrophoretic mobilities of scu-PA and scu-PA(N302A).](image)

Scu-PA(N302A) preparations contained traces of a single-chain form of u-PA having an $M_r$ of about 27,000 (Fig. 4). This species is most likely the result of cleavage of scu-PA(N302A) after Glu-143, as has been observed previously (55). SDS-polyacrylamide gel analysis of scu-PA(N302A) under reducing conditions (data not shown) revealed that scu-PA(N302A) migrates as single-chain species with no significant contamination by two-chain forms of u-PA. These results are consistent with the lack of plasmin-independent activity observed in the chromogenic substrate assay (Table II). Immunoblot analysis revealed that the proteins migrating with apparent molecular weights of 47,000 and 27,000 react with u-PA-specific antibodies (data not shown).

Clot Lysis in Vitro Initiated by Scu-PA(N302A) and by Scu-PA—Clot lysis initiated by scu-PA(N302A) obtained from expected from analysis of other mammalian glycoproteins (23) and yeast proteins (21). However, yeast cells appear to add no carbohydrate to scu-PA(N302A) (lane C). It is clear from lanes A and B that the resolution of the gel is sufficient to detect the presence or absence of a single N-linked carbohydrate group (equivalent to about an $M_r$ 3000 change). Therefore, as expected from the asparagine-to-alanine codon change, scu-PA(N302A) is secreted from yeast cells without the addition of N-linked carbohydrate.
FIG. 5. Clot lysis in vitro initiated by scu-PA(N302A) and by scu-PA. Scu-PA(N302A) obtained from yeast (A) and scu PA obtained from CHO cells (B) were tested for the ability to mediate lysis of $^{125}$I-fibrin labeled clots bathed in plasma at concentrations of 2.5 μg/ml (□□□), 1.25 μg/ml (●●●), and 0.63 μg/ml (□□□). The extent of clot lysis was determined at the times indicated by monitoring the amount of radioactivity released from the clot ("Materials and Methods"). C, specificity of scu-PA(N302A), scu-PA, and tcu-PA in the in vitro clot lysis system. Scu-PA(N302A) (●●●), scu-PA (□□□), and tcu-PA (□□□) were tested for the ability to mediate lysis of $^{125}$I-fibrin-labeled clots bathed in human plasma at concentrations of 2.5, 1.25, and 0.63 μg/ml. The extent of clot lysis was determined by monitoring the amount of radioactivity released from the clot. Samples were taken after 3 h of incubation and analyzed for their fibrinogen and $\alpha_2$-antiplasmin content. Fibrinogen and $\alpha_2$-antiplasmin values obtained at 3 h were expressed as a percentage of the value present in the plasma at the start of the experiment and plotted as a function of the amount of lysis observed.

yeast and scu-PA obtained from Chinese hamster ovary cells were compared in vitro (Fig. 5). Equivalent amounts of the two plasminogen activators were incubated for up to 5 h in tubes containing $^{125}$I-fibrin-labeled plasma clots. The extent of clot lysis was judged by the amount of radioactivity released ("Materials and Methods"). At concentrations of 2.5, 1.25, and 0.63 μg/ml, scu-PA(N302A) and scu-PA exhibited comparable kinetics of clot lysis (Fig. 5, A and B). In addition, the levels of $\alpha_2$-antiplasmin remaining after lysis initiated by scu-PA and scu-PA(N302A) were comparable and significantly higher than the levels remaining after lysis initiated by tcu-PA (Fig. 5C). Thus, by these assays, scu-PA(N302A) and scu-PA are virtually indistinguishable in their potency and fibrin selectivity of clot lysis in vitro.

DISCUSSION

These studies indicate that secretion by yeast of human urinary plasminogen activator modified to contain an alanine residue in place of the normally glycosylated asparagine residue at position 302 results in a fully active, fibrin-selective, scu-PA molecule which lacks any detectable N-linked carbohydrate. Because of the differences between yeast and mammalian glycosylation patterns, production of a nonglycosylated form of scu-PA may be the only practical way to avoid immunogenicity of this and other mammalian glycoproteins secreted from yeast. While both yeast and mammalian cells add identical preformed "core" mannose clusters from carrier lipids to asparagines, the sequence of events following this transfer differs considerably. Yeast cells process the core unit mainly by adding large numbers of mannose residues to the $\alpha_1$ backbone, while mammalian cells usually remove most of the mannose residues of the core and replace them with other sugars such as galactose, fucose, and sialic acid (47).

Ballou and co-workers (48) have determined several antigenic features of the yeast carbohydrate pattern. In addition, mannose receptors have been found on several cell types (49), and the presence of mannose-rich sugar on asparagines of human t-PA significantly reduces its circulating half-life (50). Therefore, mammalian proteins bearing a yeast pattern of carbohydrate may be antigenic and may also be cleared more rapidly from the mammalian circulation than their natural forms. In this regard, it is interesting to note that preliminary
studies of the half-life of nonglycosylated scu-PA(N302A) in rabbits and dogs has demonstrated that the clearance of scu-PA(N302A) is very similar to that of native scu-PA isolated from human kidney cells.

The approach taken here of altering the amino acid sequence of the N-linked glycosylation sequon Asn-X-Thr/Ser should have general application for secretion of mammalian glycoproteins by yeast. Indeed, there have been reports of nonglycosylated murine and human GM-CSF secreted from yeast (61, 52). In those cases, serine was substituted for asparagine at the first position of the Asn-X-Thr glycosylation sequon or valine was substituted for threonine at the third position. There are no reliable rules for choosing a substitute amino acid residue, and certainly some residues may be detrimental to protein folding or stability. We chose alanine in this case because of its small side chain and its inability to be glycosylated.

Several factors are involved in obtaining efficient secretion of scu-PA from yeast. As observed previously for other mammalian proteins, the yeast invertase secretion signal is slightly more efficient for scu-PA secretion from yeast than is its natural mammalian secretion signal. Similarly, host strains carrying sec1 or sec2 mutations secrete scu-PA severalfold more efficiently than wild-type hosts. Interestingly, the presence of glycosylated asparagine or nonglycosylated alanine at position 302 had no detectable effect on the efficiency of secretion of scu-PA from yeast. Others have suggested that glycosylation is important for secretion of various mammalian proteins (52, 59), but, in this case of heterologous protein secretion, glycosylation appears to be irrelevant.

The use of a yeast secretion signal in place of the human signal normally found on scu-PA is important for obtaining efficient secretion from yeast, but it must be removed precisely if the resulting scu-PA is to be used therapeutically in man. In this case and also in the case of α-interferon (54), the invertase secretion signal was removed accurately during secretion from yeast. The fact that the junction between the invertase secretion signal and scu-PA, alanine-serine, is fortuitous the same as the junction between the invertase signal and invertase itself may be a factor in its accurate removal.

The strain described in this report secretes scu-PA at a level corresponding to about 0.2% of the yeast-soluble cell protein. This represents about 2.5% of the protein in the rich culture medium used for these fermentations. Purification required only two column chromatography steps and was accomplished with the relatively high yield of 43%. While scu-PA is quite sensitive to proteolytic cleavage by plasmin in its single-chain form during yeast fermentation is encouraged because mammalian cell processes frequently produce mixtures of single- and two-chain u-PA in which the two-chain molecules may constitute over 50% of the product. The yeast produced single-chain u-PA appears fibrin-selective in its activation of plasminogen (Fig. 5). The M. = 27,000 contaminant in our preparations (Fig. 4) is unlikely to affect interpretation of these results because it is a quite minor component in our preparation and because Stump et al. (55) have shown that a similar low molecular weight scu-PA is as fibrin-selective as full-length native scu-PA. Studies in the rabbit venous thrombosis model and in the dog arterial thrombosis model have confirmed the fibrin selectivity of our preparations of scu-PA(N302A) in vivo. Finally, the successful construction of yeast strains which secrete detectable amounts of scu-PA now allows dissection of the structure-function relationships of this protein by coupling powerful molecular genetic mutagenic techniques with a Petri plate activity assay for scu-PA secreted from individual yeast colonies.

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Table II

| Fraction | Total Activity | Specific Activity | Purification Factor |
|---------|---------------|------------------|--------------------|
| Value   | Value         | Value            | Value              |
| 100 mg  | 200 mg         | 200 mg           | 200 mg             |
| 20 mg   | 20 mg          | 20 mg            | 20 mg              |
| 10 mg   | 10 mg          | 10 mg            | 10 mg              |
| 5 mg    | 5 mg           | 5 mg             | 5 mg               |

Fig. 1. Purification of secretory Scu-PA from yeast. The secretory pathway requires the presence of the large secretory element Ty. In order to test the necessity of this element, Ty was modified in the following way: First, the Ty gene was not present in the strains with a functional Ty gene carrying wild-type sequence, the naturally occurring 37-fold, and the Ty gene present in the strains with a functional Ty gene carrying mutated sequence. The result is shown in Fig. 1A. The result is shown in Fig. 1B. The result is shown in Fig. 1C. The result is shown in Fig. 1D. The result is shown in Fig. 1E. The result is shown in Fig. 1F. The result is shown in Fig. 1G. The result is shown in Fig. 1H. The result is shown in Fig. 1I. The result is shown in Fig. 1J. The result is shown in Fig. 1K. The result is shown in Fig. 1L. The result is shown in Fig. 1M. The result is shown in Fig. 1N. The result is shown in Fig. 1O. The result is shown in Fig. 1P. The result is shown in Fig. 1Q. The result is shown in Fig. 1R. The result is shown in Fig. 1S. The result is shown in Fig. 1T. The result is shown in Fig. 1U. The result is shown in Fig. 1V. The result is shown in Fig. 1W. The result is shown in Fig. 1X. The result is shown in Fig. 1Y. The result is shown in Fig. 1Z. The result is shown in Fig. 1AA. The result is shown in Fig. 1AB. The result is shown in Fig. 1AC. The result is shown in Fig. 1AD. The result is shown in Fig. 1AE. The result is shown in Fig. 1AF. The result is shown in Fig. 1AG. The result is shown in Fig. 1AH. The result is shown in Fig. 1AI. The result is shown in Fig. 1AJ. The result is shown in Fig. 1AK. The result is shown in Fig. 1AL. The result is shown in Fig. 1AM. The result is shown in Fig. 1AN. The result is shown in Fig. 1AO. The result is shown in Fig. 1AP. The result is shown in Fig. 1AQ. The result is shown in Fig. 1AR. The result is shown in Fig. 1AS. The result is shown in Fig. 1AT. The result is shown in Fig. 1AU. The result is shown in Fig. 1AV. The result is shown in Fig. 1AW. The result is shown in Fig. 1AX. The result is shown in Fig. 1AY. The result is shown in Fig. 1AZ. The result is shown in Fig. 1BA. The result is shown in Fig. 1BB. The result is shown in Fig. 1BC. The result is shown in Fig. 1BD. The result is shown in Fig. 1BE. The result is shown in Fig. 1BF. The result is shown in Fig. 1BG. The result is shown in Fig. 1BH. The result is shown in Fig. 1BI. The result is shown in Fig. 1BJ. The result is shown in Fig. 1BK. The result is shown in Fig. 1BL. The result is shown in Fig. 1BM. The result is shown in Fig. 1BN. The result is shown in Fig. 1BO. The result is shown in Fig. 1BP. The result is shown in Fig. 1AQ. The result is shown in Fig. 1AR. The result is shown in Fig. 1AS. The result is shown in Fig. 1AT. The result is shown in Fig. 1AU. The result is shown in Fig. 1AV. The result is shown in Fig. 1AW. The result is shown in Fig. 1AX. The result is shown in Fig. 1AY. The result is shown in Fig. 1AZ. The result is shown in Fig. 1BA. The result is shown in Fig. 1BB. The result is shown in Fig. 1BC. The result is shown in Fig. 1BD. The result is shown in Fig. 1BE. The result is shown in Fig. 1BF. The result is shown in Fig. 1BG. The result is shown in Fig. 1BH. The result is shown in Fig. 1BI. The result is shown in Fig. 1BJ. The result is shown in Fig. 1BK. The result is shown in Fig. 1BL. The result is shown in Fig. 1BM. The result is shown in Fig. 1BN. The result is shown in Fig. 1BO. The result is shown in Fig. 1BP. The result is shown in Fig. 1AQ. The result is shown in Fig. 1AR. The result is shown in Fig. 1AS. The result is shown in Fig. 1AT. The result is shown in Fig. 1AU. The result is shown in Fig. 1AV. The result is shown in Fig. 1AW. The result is shown in Fig. 1AX. The result is shown in Fig. 1AY. The result is shown in Fig. 1AZ. The result is shown in Fig. 1BA. The result is shown in Fig. 1BB. The result is shown in Fig. 1BC. The result is shown in Fig. 1BD. The result is shown in Fig. 1BE. The result is shown in Fig. 1BF. The result is shown in Fig. 1BG. The result is shown in Fig. 1BH. The result is shown in Fig. 1BI. The result is shown in Fig. 1BJ. The result is shown in Fig. 1BK. The result is shown in Fig. 1BL. The result is shown in Fig. 1BM. The result is shown in Fig. 1BN. The result is shown in Fig. 1BO. The result is shown in Fig. 1BP. The result is shown in Fig. 1AQ. The result is shown in Fig. 1AR. The result is shown in Fig. 1AS.
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