Secretion of Active Kringle-2–Serine Protease in *Escherichia coli*

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ABSTRACT: Active human tissue plasminogen activator variant kringle-2–serine protease (K_2 + SP domains; referred to as MB1004) was synthesized as a secreted protein in *Escherichia coli*, isolated, and characterized. MB1004 is a relatively large and complex protein, approximately 38 kDa in size and containing nine disulfide bonds. MB1004 without a pro region was secreted into the periplasm of *E. coli* by fusing the protein to the PhoA leader peptide expressed from the *tac* promoter. Approximately 1% (20 μg/L broth) of the secreted MB1004 was purified from *E. coli* homogenates as a soluble, active enzyme by using a combination of lysine and *Erythrina* inhibitor affinity chromatography. Purified MB1004 was monomeric and single-chain, and the N-terminus was identical with the predicted amino acid sequence. The specific activity of purified MB1004 from *E. coli* was compared against the equivalent recombinant material purified from mammalian cells that was naturally glycosylated (MB1004G) or deglycosylated after treatment with *N*-glycanase (MB1004N). Results from four different in vitro assays showed that MB1004 and MB1004N had similar activities. Both exhibited 4–12-fold higher specific activity than MB1004G in plasminogen activation assays. These results suggest that an inaccurate picture of specific activity can be obtained if the effects of glycosylation are not considered. By utilization of secretion in *E. coli*, nonglycosylated MB1004 was purified without in vitro refolding and was shown to be suitable for structure–function studies.

Human tissue-type plasminogen activator (tPA)[1] is a key component in fibrinolysis, converting plasminogen to plasmin (Collen, 1980, 1988; Vehar et al., 1986; Astrup, 1978; Harris, 1987). tPA is a glycosylated serine protease containing multiple structural domains. Regions homologous to the finger repeats in fibronectin, mouse epidermal growth factor, and the triple disulfide-bonded structures termed "kringles" are present in addition to the serine protease domain (Pennica et al., 1983; Banyai et al., 1983).

Various efforts have been made to correlate tPA structure with function (Harris, 1987; Krause & Tanswell, 1989). These have identified the finger and second kringle (K_2) domains as important for fibrin binding and fibrin stimulation of activity (van Zonneveld et al., 1986a; Verheijen et al., 1986). Although the nature of the fibrin binding site in the finger domain remains obscure, it appears to interact with native fibrin, while the K_2 domain has a binding site for lysine-like ligands (van Zonneveld et al., 1986b; Cleary et al., 1989). This lysine binding site is hypothesized to be important in binding to fibrin which has undergone limited digestion with plasmin (van Zonneveld et al., 1986b), a process which generates primarily C-terminal lysine residues (Castellino, 1981). A tPA variant comprised of only K_2 and the serine protease domain (K_2–SP) might therefore be expected to show some, but not all, of the fibrin-stimulatory properties of native tPA. Work by other laboratories has shown this to be true (van Zonneveld et al., 1986a,b; Verheijen et al., 1986; Burck et al., 1990).

Recombinant tPA (Vehar et al., 1986) and tPA variants (Lau et al., 1987) have potential as therapeutic agents by specifically mediating thrombolysis. Synthesis of tPA or tPA variants as secreted proteins by mammalian cells has typically been the method of choice because properly folded, glycosylated material can be recovered directly from the growth medium. Synthesis of tPA has also been documented in *Saccharomyces cerevisiae* (Lemontt et al., 1985), *Aspergillus nidulans* (Upshall et al., 1987), and also with transgenic animals (Gordon et al., 1987). However, yields are typically relatively low, and recent results have shown that differences in glycosylation may affect activity (Wittwer et al., 1989). As an alternative, intracellular synthesis of tPA in *Escherichia coli* followed by in vitro refolding has been described to obtain nonglycosylated protein (Pennica et al., 1983; Sarmientos et al., 1989). Due to the complexity of the tPA molecule (527 amino acids, 14 disulfide bonds), efficient in vitro refolding remains a major hurdle.

This report describes secretion of the tPA variant kringle-2–serine protease (K_2 + SP domains; herein referred to as MB1004) into the periplasm of *E. coli* using the PhoA leader peptide with expression being controlled by the *tac* promoter. MB1004 is approximately 38 kDa in size and contains nine disulfide bonds. Typically, such relatively complex proteins are inactive when expressed intracellularly in *E. coli*. Ex-

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[1] Abbreviations: Ap^R, ampicillin resistance; bp, base pair(s); EI, *Erythrina caffra* tPA inhibitor; g10L, phage T7 gene 10 leader sequence; kb, kilobase(s); kDa, kilodalton(s); K_2–SP, tPA variant consisting of the second kringle (K_2) and serine protease (SP) domains; MB1004, K_2–SP produced by *Escherichia coli*; MB1004G, glycosylated K_2–SP produced by mammalian cells; MB1004N, MB1004G treated with *N*-glycanase to remove oligosaccharides; RBS, ribosome binding site; Sp^R, spectinomycin resistance; tPA, tissue-type plasminogen activator.

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pression, N-terminal sequencing, purification, and specific activity data are presented which show that soluble, fully active MB1004 could be recovered as a secreted protein in *E. coli*. Nonglycosylated MB1004 purified from *E. coli* has a significantly higher specific activity than its mammalian-derived glycosylated counterpart. The effect of glycosylation on MB1004 specific activity is the largest observed with any enzyme to date. By utilization of secretion in *E. coli*, sufficient levels of MB1004 were purified without in vitro refolding. MB1004 from *E. coli* is suitable for structure–function studies which are not complicated by the effects of glycosylation.

Experimental Procedures

Bacterial Strains, Growth Media, and Antibiotics. The following *E. coli* expression strains were used in this study: (1) JM101 F′ *traD*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15/Δ(*proAB-lac*) *supE thi*-1 (Messing, 1979); (2) RB791 F$^+$ *lac*^{q} L8 IN-(*rrnD-rrnE*)1 λ$^-$ (referred to as W3110 *lacI*^{q} L8; Brent & Ptashne, 1981).

Cell cultures were brown in L broth (LB) or M9 minimal medium supplemented with thiamin (Maniatis et al., 1982). Bench level inductions were performed by growing the cultures in M9 medium supplemented with casamino acids, thiamin, and trace minerals (Obukowicz et al., 1988).

Antibiotics and concentrations were as follows: ampicillin (Ap), 200 μg/mL (agar plates) or 100 μg/mL (liquid); spectinomycin (Sp), 50 μg/mL.

Shake Flask Expression Analysis. Cells were grown overnight at 30 or 37 °C in 2 mL of LB plus Sp. The overnight cultures were diluted to 20–25 Klett units (Klett–Summerson photoelectric colorimeter with a green 54 filter) in 10 mL of M9 induction medium or LB and grown with vigorous shaking (approximately 300 rpm). Expression from the *tac* promoter was induced by adding IPTG to a final concentration of 1 mM at approximately 150 Klett units. One-milliliter aliquot samples were taken for SDS–polyacrylamide gel analysis just prior to the addition of IPTG (preinduction) and at hourly intervals postinduction.

SDS–Polyacrylamide Gel Electrophoresis and Western Blot Analysis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with 15% or 8–16% gradient polyacrylamide gels followed by staining with Coomassie blue or silver (Laemmli, 1970; Morrissey, 1981). Western blot analyses were performed by using diazotized paper according to Renart et al. (1979). tPA was identified by using iodinated protein A (Amersham) which had bound to the anti-tPA polyclonal antibodies. Whole cell samples were prepared by pelleting 1 mL of culture and solubilizing the cell pellet in SDS sample buffer (Laemmli, 1970) to a final concentration of 1 Klett unit equiv/mL.

Cell extract samples were obtained by sonicating 1-mL culture samples (4 × 20 s on ice) and then subjecting the samples to centrifugation. Insoluble proteins were present in the pellet fraction following centrifugation. Soluble proteins remaining in the supernatant were precipitated with trichloroacetic acid (0.1 volume of 50% trichloroacetic acid containing 2 mg/mL sodium deoxycholate). Soluble and insoluble sonicate fractions were solubilized in SDS sample buffer to a final concentration of 1 Klett unit equiv/mL.

Quantitative estimations of the relative amount of MB1004 were determined from Western blots by performing linear scans with a Joyce-Loebl Chromscan III densitometer (0.3-mm slit width, 530-nm green filter).

Construction of the K_2–SP tPA Variant. A synthetic gene encoding human tPA (Bell et al., 1988) was used to construct the tPA variant, K_2–SP (MB1004). The synthetic tPA gene

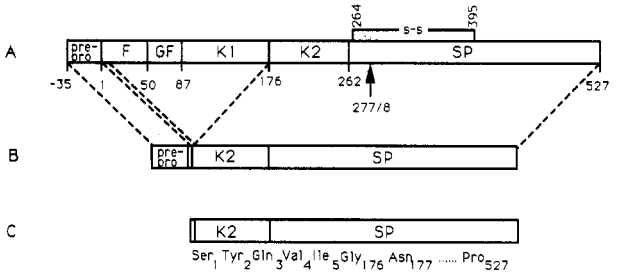

FIGURE 1: Schematic showing construction of K_2–SP (MB1004). (A) Full-length tPA, including the prepro region. The numbers beneath the block schematic designate amino acid intron positions which separate the different domains (F, finger; GF, growth factor; K1, kringle-1; K2, kringle-2; SP, serine protease) (Ny et al., 1984). A disulfide bridge between Cys(264) and Cys(395) links the K_2 and SP domains following cleavage by plasmin between amino acids 277/8 (vertical arrow). The molecule is referred to as single-chain if intact or two-chain if cleaved by plasmin between amino acids 277/8. (B) K_2–SP tPA variant, including the prepro region. K_2–SP fused in-frame with the prepro region was constructed to direct secretion of the molecule in mammalian cells (see Experimental Procedures for details). The molecule includes the 35 amino acids of the prepro region and the first 5 amino acids of the F domain [Ser(1), Tyr(2), Gln(3), Val(4), Ile(5)] fused to Gly(176) of the K_2 domain. The molecule contains the intact K_2 and SP domains and terminates at Pro(527), the last amino acid in the SP domain. (C) K_2–SP variant described in (B), excluding the prepro region. This molecule is referred to as MB1004 and was used in the construction of the *E. coli* secretion and intracellular expression vectors (see Experimental Procedures for details).

cloned in pML2 (Bell et al., 1988) was digested with *Nde*I and *Kpn*I to delete the F, GF, and K_1 domains (Figure 1A). The resulting linear DNA fragment was recircularized by ligation with two complementary oligonucleotides with the sequences:

5′ TAGGTAACTCTGACTGTTACTTCGGTAACGGTTCTGCTTACAGAGGTAC 3′

3′ CCATTGAGACTGACAATGAAGCCATTGCCAAGACGAATGTCTC 5′

These oligonucleotides reconstruct the amino-terminal portion of the K_2 domain, resulting in the fusion of the first five residues of mature tPA [Ser(1), Tyr(2), Gln(3), Val(4), Ile(5)] to the first residue of the K_2 domain [Gly(176)] (Figure 1). The first five residues of mature tPA were retained in the K_2–SP variant in order to limit possible amino acid context effects on efficient processing of the prepro fragment in mammalian cells.

The tPA variant MB1004 was expressed with the bovine papilloma virus vector pMON1123. This vector is based on the complete viral genome and utilizes the mouse metallothionein I promoter and SV40 late poly(A) addition site to regulate the expression of foreign genes (Keck et al., 1989). The MB1004 gene was excised from pML2 by digestion with *Bam*HI and was ligated into the unique *Bam*HI site of pMON1123 to yield the vector pMON1393 (Figure 2A). pMON1393 served as the source of the MB1004 gene for construction of the *E. coli* expression vectors.

Mammalian Expression of MB1004. Mouse C127 cells (ATCC CRL 1616) were grown and cotransfected with pMON1393 and pSV2neo as previously described (Ramabhadran et al., 1984). Neomycin-resistant transfectants were selected with G418 (Southern & Berg, 1982). Resistant colonies were picked and assayed for expression of MB1004 by ELISA (American Diagnostica). Cell lines were established and then expanded for production of the variant protein. Cells were seeded into 6000 cm^2 Nunc cell factories and allowed to proliferate until confluent. The cells were washed with phosphate-buffered saline and then maintained on serum-free Dulbecco's modified Eagle's medium supplemented with 2× penicillin/streptomycin, 50 KIU/mL aprotinin, and 0.3%

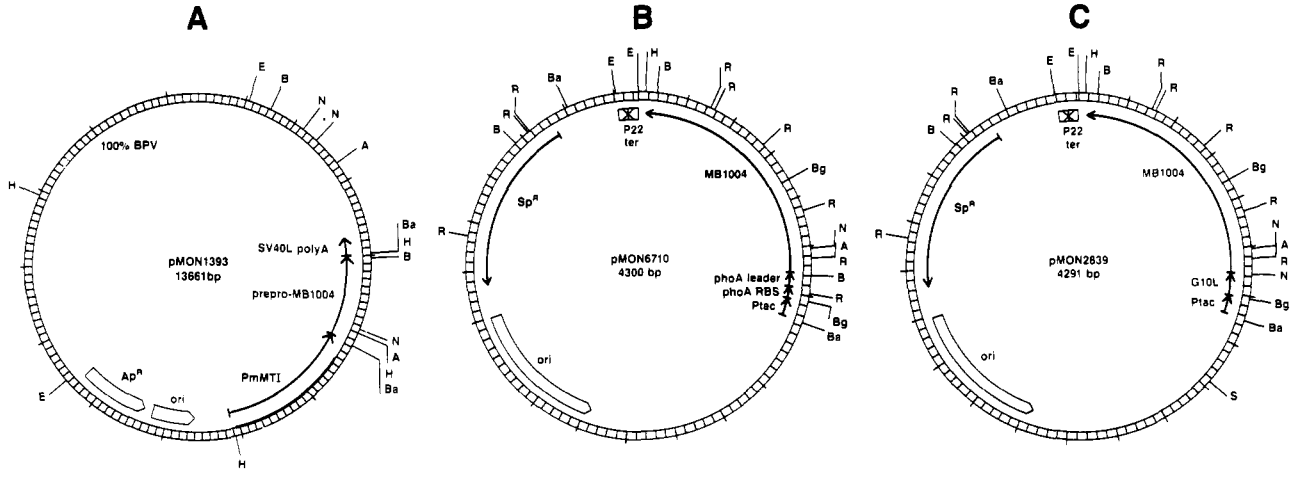

FIGURE 2: Expression plasmids. See Experimental Procedures for a detailed description of the construction of each plasmid. With each plasmid, the gene encoding the K_2-SP variant enzyme is referred to as MB1004. Restriction enzyme abbreviations: A, *Asp*718; B, *Bst*EII; Ba, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; N, *Nco*I; R, *Rsa*I; S, *Sal*I. Other abbreviations: Ap^R, ampicillin resistance; G10L, phage T7 gene 10 leader sequence; *ori*, origin of replication; P22 *ter*, P22 transcription terminator; PmMTI, metallothionein promoter; Ptac, *tac* promoter; Sp^R, spectinomycin resistance.

lactalbumin hydrolysate. Conditioned medium was harvested every 3 days and used for protein purification.

Escherichia coli Plasmid Expression Vectors. Standard cloning procedures (Maniatis et al., 1982) were used for construction of expression plasmids containing the MB1004 gene from pMON1393 (Figure 2A). Two MB1004 expression plasmids were constructed; pMON6710 (Figure 2B) was utilized to secrete MB1004 into the periplasm, while pMON2839 (Figure 2C) was utilized for cytoplasmic expression of MB1004. Plasmid constructs were verified by restriction fragment analysis. If synthetic DNA linker fragments were used in plasmid assembly, the expected DNA sequence was verified by restriction fragment analysis as well as plasmid DNA sequencing of the entire synthetic fragment and junction regions.

pMON6710 Secretion Vector. The pBR327-derived expression plasmid pMON5842 containing the *tac* promoter (de Boer et al., 1983), the phage T7 g10L ribosome binding site (Olins et al., 1988), the synthetic double terminator based on the *ant* gene terminator (Berget et al., 1983) of phage P22, and the gene encoding spectinomycin resistance (Sp^R) was used as the parental vector. Plasmid pMON6710 (Figure 2B) containing the *tac* promoter controlling expression of MB1004 fused in-frame to the native PhoA leader peptide was constructed as follows. A three-fragment ligation was performed using (1) the 3.2-kb *Bgl*II–*Hin*dIII vector fragment containing the *tac* promoter, (2) the 1.0-kb *Asp*718–*Hin*dIII fragment encoding MB1004, except for the first 20 amino acids at the N-terminus, and (3) a 172 bp *Bgl*II–*Asp*718 synthetic linker fragment encoding the indigenous *phoA* ribosome binding site and leader peptide (Shuttleworth et al., 1986) and 20 amino acids of the N-terminus of MB1004.

The 172 bp *Bgl*II–*Asp*718 synthetic linker fragment was assembled as three complementary synthetic fragments (60-mer + 60-mer + 52-mer). To facilitate future cloning, a *Bst*EII site was introduced just upstream of the signal peptidase cleavage site in the *phoA* leader sequence (Figure 2B). The introduction of the *Bst*EII site did not alter the amino acid sequence of the PhoA leader peptide.

pMON2839 Intracellular Expression Vector. Plasmid pMON2839 (Figure 2C), derived from pMON5842 (described above), was constructed for the intracellular synthesis of MB1004. pMON2839 contains the *tac* promoter, the phage T7 g10L ribosome binding site, the MB1004 structural gene, and the synthetic *ant* gene terminator from phage P22. pMON2839 was constructed by performing a three-fragment ligation using (1) the 3.3-kb *Nco*I–*Hin*dIII vector fragment containing the *tac* promoter and g10L ribosome binding site, (2) the 65 bp *Nco*I–*Asp*718 synthetic linker fragment encoding the N-terminus of MB1004, and (3) the 1.0-kb *Asp*718–*Hin*dIII fragment encoding MB1004, except for 20 amino acids at the N-terminus.

Immunoassay Quantification of MB1004. Samples were quantified by particle concentration fluorescence immunoassay (PCFIA). The polystyrene beads and tracer antibody were prepared according to procedures suggested by Pandex Laboratories. The polystyrene beads were coated with tPA antibody available commercially from American Diagnostica. The tracer antibody was prepared with affinity-purified rabbit antibody. Aliquots of samples in 50 μL of phosphate-buffered saline (0.1 M sodium phosphate/0.15 M NaCl, pH 7.4) containing 1% BSA and 0.5% Tween 20 were added to a 96-well plate. To this was added 15 μL of a suspension of polystyrene particle-bound polyclonal antibody (50 μg/mL). The FITC-labeled antibody (30 μL of a 5 mg/mL stock) was added after a 10-min incubation. The plates were allowed to incubate an additional 20 min at room temperature after which they were vacuumed, washed, and then assayed for fluorescence. A calibration curve was calculated by linear regression of the data obtained from the standards and used to determine the concentration of samples.

Fibrin Plate Activity Assay. A fibrin plate activity assay was used as an initial screen to identify (1) soluble, active MB1004 synthesized in *E. coli* either intracellularly or as a secreted protein or (2) insoluble MB1004 which first had to be solubilized with guanidine hydrochloride before generation of active enzyme.

Thin-layer fibrin assay plates (20-cm diameter) for the detection of active MB1004 in *E. coli* extracts were prepared according to a modified method of Goldberg (1974), except that bovine fibrin was substituted for casein to provide specificity (Rijken & Collen, 1981). Soluble cell extract from sonicated samples or whole cell samples solubilized in guanidine hydrochloride followed by dialysis (Pennica et al., 1983) were spotted directly onto the surface of the fibrin assay plate. Five-microliter aliquots from 1 mL of cell culture samples, corresponding to approximately 5 μg of total soluble cell protein, were spotted directly onto the fibrin assay plate. Total

soluble cell protein in each sample was quantified by using the Bradford microassay (Bio-Rad Laboratories). Human tPA or MB1004 derived from mammalian cell culture served as a positive control. Cell samples from *E. coli* strains lacking the MB1004 expression plasmid served as negative controls. The fibrin plates were incubated at 37 °C and monitored hourly for the presence of fibrin clearing. Active MB1004 from *E. coli* extracts could be easily detected after a 2-h incubation.

Protein Sequence Analysis. Automated Edman degradation chemistry was used to determine the N-terminal protein sequence of MB1004. Samples were prepared either by blotting onto PVDF paper from SDS–PAGE profiles of total *E. coli* solubilized protein pellets (Matsudaira, 1987), or, subsequently, as directly applied after purification. In both cases, an Applied Biosystems, Inc., Model 470A gas-phase sequencer (Foster City, CA) was employed for the degradations (Hunkapiller et al., 1983). The respective PTH-aa derivatives were identified by RP–HPLC analysis in an on-line fashion using an Applied Biosystems, Inc., Model 120A PTH analyzer fitted with a Brownlee 2.1-mm i.d. PTH-C18 column.

Activity Gel Analysis. Enzymatic assays of MB1004 in SDS-containing gels were performed essentially as described by Ryan et al. (1984). SDS–polyacrylamide gradient gels (8–16%) were copolymerized with 0.1% gelatin and 0.12 mg/mL (0.012%) Lys-plasminogen. Control gels lacking plasminogen were used to correct for plasminogen-independent proteolysis in *E. coli* cell extracts. Proteolysis was detected by clear bands against a dark, Coomassie blue stained background. Optimal results were obtained by using 0.75-mm-thick slab gels.

High Cell Density Fermentation. Ten-liter fermentations were run in a 15-L LSL Biolafitte fermenter using M9 induction medium which contained 2% casamino acids (Obukowicz et al., 1988). Glucose was added to maintain a final concentration of approximately 2.5 g/L. The fermentation temperature was maintained at 30 °C, the pH was controlled at 7.0 with ammonium hydroxide, and the airflow rate was fixed at 15 L/min. Dissolved oxygen was controlled at 30% by increasing the agitation speed from 500 to 1000 rpm as needed. Culture growth was monitored by measuring the optical density at 550 nm with a Gilson Stasar II spectrophotometer. IPTG was added to a final concentration of 1 mM at late-log (OD_{550} = 55) phase to induce the *tac* promoter.

Purification of MB1004 from E. coli Cells. (A) Extraction. Frozen cell paste (558 g, equivalent to 7.5 L of broth) was suspended to 4.2 L in 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA and 0.1% Tween 80. The cell suspension was passed through a Gaulin 15M homogenizer 4 times at 8000 psi, cooling to 4–8 °C between passes. The homogenate was clarified by passage through a 0.2-μm Durapore membrane, after which it was concentrated by using a 10-kDa cutoff spiral cartridge and then filtered through a 0.22-μm Millipak-60 unit.

(B) Lysine–Sepharose Affinity Chromatography. A column of lysine–Sepharose (Pharmacia), 8.3 × 4.4 cm in size, was equilibrated with 50 mM Tris-HCl, 5 mM EDTA, and 0.1% Tween 80, pH 7.4. The clarified concentrated extract (1.7 L) was applied to the column at a flow rate of 6.2 cm/h. After being loaded, the column was washed with 6 column volumes of equilibration buffer, followed by 5 column volumes of equilibration buffer containing 1.5 M NaCl, and finally with equilibration buffer + 1.5 M NaCl + 0.2 M L-lysine.

(C) Erythrina Inhibitor–Sepharose Affinity Chromatography. Erythrina inhibitor–Sepharose was prepared by coupling *Erythrina caffra* tPA inhibitor (EI) to cyanogen bromide activated Sepharose 4B by the method described by Heussen et al. (1984). A 7.5 × 1.6 cm column of the prepared gel was equilibrated with 0.5 M NH_4HCO_3 containing 1% Triton X-100. The 1.5 M NaCl + 0.2 M lysine eluate (214 mL) from the lysine–Sepharose column was loaded onto the *Erythrina* inhibitor column at a flow rate of 5 cm/h. After the column was loaded, it was washed with 5 column volumes of 0.5 M NH_4HCO_3 containing 1% Triton X-100, followed by 15 column volumes of 0.05 M NH_4HCO_3. Elution was achieved with 1.6 M KSCN in 0.05 M NaH_2PO_4, pH 7.3. Nine-milliliter fractions were collected. The fraction eluted from the lysine–Sepharose column by 1.5 M NaCl alone was also passed over the *Erythrina* inhibitor–Sepharose column.

All fractions containing plasminogen activation activity (based on gelatin clearing gel activity) were pooled and concentrated in an Amicon 8050 stirred cell using a YM5 membrane. The original volume of 86 mL was concentrated to 20 mL, diluted to 40 mL with 0.15 M arginine/0.05 M citric acid, pH 6.0, and then concentrated to 10 mL. The batch diafiltration was repeated until the theoretical KSCN concentration was less than 1 mM and the solution was concentrated to 3 mL. The retentate was removed, and the cell was washed with 3 mL of arginine/citrate buffer. A total of approximately 100 μg of active material was recovered (approximately 20 μg/L broth).

Specific Activity Measurements of MB1004. Direct hydrolysis of S-2322 (H-D-Val-Gly-Arg-*p*-nitroanilide, KabiVitrum) and activation of plasminogen in the presence or absence of stimulator (human fibrinogen fragments) were measured by the direct amidolytic and indirect amidolytic assays previously described (Wittwer et al., 1989). Reactions were performed in 96-well microtitration plates, and reaction mixtures (100-μL volume) were overlaid with 50 μL of mineral oil to prevent evaporation during extended incubations. Because of the known or possible activity differences between single- and two-chain tPA (Figure 1A) in these assays (Ranby et al., 1982; Ranby, 1982; Boose et al., 1989), samples were converted to the two-chain form prior to assay by treating with plasmin (Wittwer et al., 1989). A fibrin clot lysis assay (Wittwer et al., 1989) was also performed as previously described.

The results from each assay were reported in international units per microgram of tPA antigen (IU/μg), using human melanoma tPA (American Diagnostica, Inc.) as both an activity and an antigen standard. In separate studies, the specific activity of this melanoma tPA preparation was 534 IU/μg in the stimulated indirect amidolytic assay when compared to the World Health Organization tPA standard (Gaffney & Curtis, 1985), while protein content was verified by amino acid analysis. Antigen concentration of purified MB1004 proteins was determined by ELISA, using reagents from American Diagnostica, Inc. Since glycosylation does not appear to affect tPA antigen determinations by ELISA (Wittwer et al., 1989; Wittwer & Howard, 1990), equivalent amounts of MB1004 proteins could be compared.

N-Glycanase Treatment of MB1004. Deglycosylation of MB1004 derived from mammalian cell culture was carried out by incubating purified MB1004 (0.2 mg/mL in 0.5 M NH_4HCO_3 1‰ Triton X-100) with one-tenth volume of *N*-glycanase (Genzyme product, 250 units/mL in 50% glycerol/2.5 mM EDTA) at room temperature for 24 h.

Results

MB1004 Expression in E. coli. By constructing secretion (pMON6710, Figure 2B) and intracellular (pMON2839,

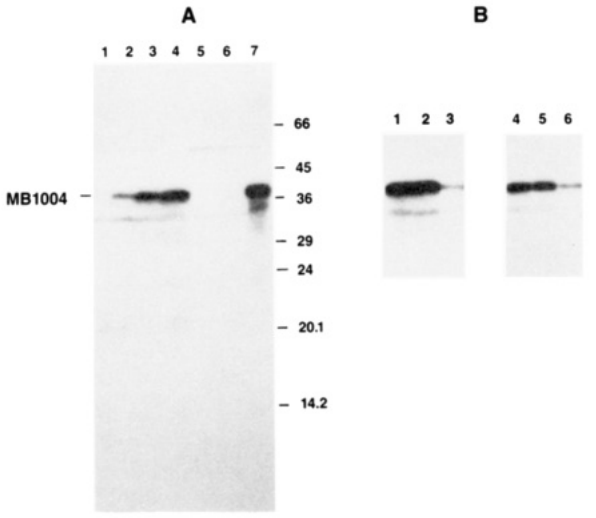

FIGURE 3: Western blot analyses assessing accumulation (A) and solubility (B) of MB1004 synthesized in *E. coli*. (A) Accumulation levels of MB1004. Secretion of MB1004 was assessed in JM101 [pMON6710] (lanes 1–4). Cytoplasmic synthesis of MB1004 was assessed in W3110 *lacI*^{q} L8 [pMON2839] (lane 7). Samples from strain JM101 without any expression plasmid were also included as negative controls (lanes 5 and 6). Both JM101 and W3110 *lacI*^{q} L8 encode an endogenous immunogenic protein that is slightly smaller in size than MB1004, running just beneath the 36-kDa marker (see also Figure 3B). Ten Klett unit equivalents of each sample was loaded per lane, except for W3110 *lacI*^{q} L8 [pMON2839] where 3 Klett unit equiv was loaded (lane 7). Molecular weight markers are indicated ($\times 10^{-3}$). (Lanes 1–4) JM101 [pMON6710]: lane 1, preinduction (I_0); lane 2, 2-h postinduction (I_2); lane 3, 4-h postinduction (I_4); lane 4, 6-h postinduction (I_6); lanes 5 and 6, JM101 host strain without pMON6710; lane 5, preinduction (I_0); lane 6, 4-h postinduction (I_4); lane 7, W3110 *lacI*^{q} L8 [pMON2839], 2-h postinduction (I_2). (B) Relative solubility of MB1004. One-milliliter culture samples taken 6 h after initiation of MB1004 expression were sonicated (4 × 20 s on ice) and then centrifuged. Soluble proteins remaining in the supernatant were precipitated with trichloroacetic acid. Insoluble proteins were pelleted during centrifugation. In addition to culture sonicate samples, whole cell extract samples (soluble + insoluble proteins) were also included to ensure that most of the MB1004 in the culture sonicate samples was recovered and not lost by proteolysis. Ten Klett unit equivalents per lane was loaded for each sample. The lower immunogenic band is an endogenous *E. coli* protein (see also Figure 3A). (Lanes 1–3) MB1004 secreted in JM101 [pMON6710]: lane 1, whole cell extract; lane 2, sonicate pellet (insoluble protein); lane 3, sonicate supernatant (soluble protein). (Lanes 4–6) MB1004 synthesized cytoplasmically in W3110 *lacI*^{q} L8 [pMON2839]: lane 4, whole cell extract; lane 5, sonicate pellet (insoluble protein); lane 6, sonicate supernatant (soluble protein).

Figure 2C) expression plasmids, it was possible to compare accumulation levels and activity of MB1004 secreted or synthesized cytoplasmically in *E. coli*.

Induction of the *tac* promoter on pMON6710 (Figure 2B) by addition of IPTG to JM101 cells at late-log phase resulted in secretion of MB1004. Western blot results showed that only completely processed MB1004 accumulated steadily with time from 2–6-h postinduction (Figure 3A, lanes 1–4). N-Terminal protein sequencing results confirmed that the PhoA leader peptide was properly processed from MB1004.

The cytoplasmic form of MB1004 was synthesized in W3110 *lacI*^{q} L8 [pMON2839] by inducing the *tac* promoter at late-log phase (Figure 3A, lane 7). The accumulation level of MB1004 reached approximately 5% of total cell protein as judged by a Coomassie blue stained protein gel (data not shown).

MB1004 Solubility. The aim of this study was to obtain soluble, active MB1004 from *E. coli* extracts without performing an in vitro refolding step. The relative solubility of MB1004 was thus measured in cell extracts where MB1004 was secreted or synthesized cytoplasmically. Cell extracts were sonicated, and the soluble and insoluble fractions were separated by centrifugation. Both fractions were subjected to Western blot analysis. Approximately 70% of the MB1004 secreted in JM101 [pMON6710] and synthesized cytoplasmically in W3110 *lacI*^{q} L8 [pMON2839] was insoluble (Figure 3B).

Fibrin Clearing Assay. Both soluble cell extracts and whole cells solubilized with guanidine hydrochloride were used in the fibrin plate clearing assay to determine if the MB1004 was active. When soluble sonicate samples of JM101 [pMON6710] were spotted, relatively fast (1 h) and significant clearing occurred (Figure 4). This suggested that a portion of soluble MB1004 (Figure 3B, lane 3) was properly folded in the periplasm. In contrast, soluble sonicate samples from the intracellular MB1004 construct, W3110 *lacI*^{q} L8 [pMON2839], did not cause any clearing on fibrin plates (Figure 4), even though comparable levels of soluble MB1004 accumulated compared to JM101 [pMON6710] (compare Figure 3B, lanes 3 and 6). This result implied that MB1004 must be secreted in order to obtain proper folding and accompanying activity.

MB1004 activity in whole cell extracts was also determined by solubilizing the cell pellet with guanidine hydrochloride followed by dialysis. In all cases, clearing was obtained (Figure 4). The fibrin plate clearing was due to MB1004 activity because the parental control strains (JM101 and W3110 *lacI*^{q} L8) lacking the corresponding expression plasmid did not cause any clearing (Figure 4) and also did not synthesize any immunogenic protein related to MB1004 (Figure 3A, lanes 5 and 6). In addition, preinduction samples of JM101 [pMON6710] and W3110 *lacI*^{q} L8 [pMON2389] did not cause any clearing when cell pellets were solubilized with guanidine hydrochloride and then dialyzed or when soluble cell sonicate samples were spotted directly onto the fibrin assay plate (Figure 4).

Experiments were performed to determine whether active MB1004 could be extracted from the insoluble material secreted in JM101 [pMON6710] (Figure 3B, lane 2). Results showed that only very low levels of activity could be recovered using standard solubilization procedures based on extraction by various chaotrophic agents or pH (data not shown). Since the already soluble, active MB1004 could be readily extracted and purified, no further effort was made in quantifying the amount of insoluble MB1004 present.

Purification of MB1004 from JM101 [pMON6710]. In order to purify and further characterize MB1004, a 10-L fermentation was performed using JM101 [pMON6710]. The temperature was maintained at 30 °C, and IPTG was added to a final concentration of 1 mM at $OD_{550} = 55$ to induce expression of MB1004. Results showed that accumulation levels of soluble, active MB1004 were similar to the levels obtained in shake flasks (data not shown). PCFIA immunoassays showed that no MB1004 was detectable preinduction, whereas levels steady increased to approximately 20 ng mL^{-1} OD^{-1} by 4-h postinduction.

The unique characteristics of the kringle-2 (K_2) and serine protease (SP) domains of MB1004 were exploited in the purification scheme by using two sequential affinity chromatographic steps, lysine–Sepharose and *Erythrina* inhibitor–Sepharose. Lysine affinity chromatography would take advantage of the presence of lysine binding sites on the K_2 portion of the molecule, while the *Erythrina* inhibitor (EI) would bind fairly specifically to the substrate binding pocket of a properly folded SP domain.

Approximately 16 g of total protein from 1.7 L of a soluble cell extract of JM101 [pMON6710] was loaded onto the lysine–Sepharose column. The bulk of the protein isolated

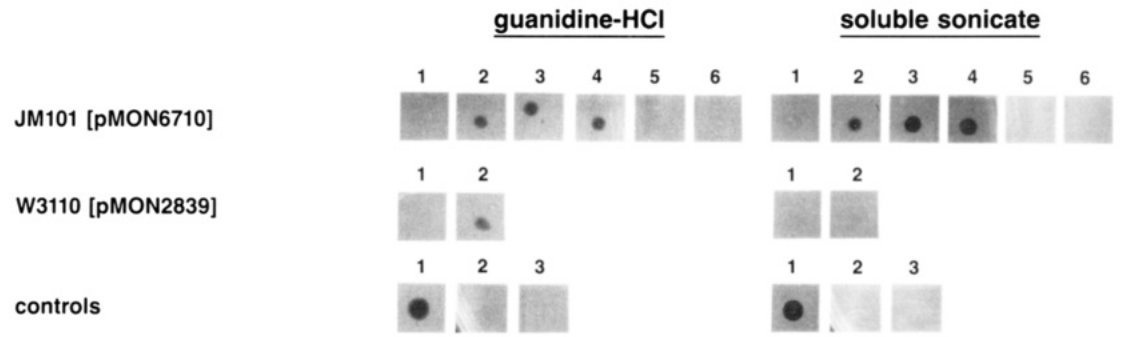

FIGURE 4: Fibrin clearing by MB1004. Whole *E. coli* cell extracts from JM101 [pMON6710] (secreted MB1004) or W3110 *lacI*^{q} [pMON2839] (cytoplasmic MB1004) were assayed for fibrin clearing ability. Samples were taken at preinduction and at several time points postinduction and prepared for assay two different ways: (1) Following sonication and centrifugation, soluble cell extracts were spotted directly onto the surface of a fibrin assay plate (right-hand side). (2) Following centrifugation, pelleted cells were first solubilized in guanidine hydrochloride, after which the extract was dialyzed and then spotted directly onto the surface of a fibrin assay plate (left-hand side). See Experimental Procedures for a detailed description of sample preparation. Cell samples from *E. coli* strains lacking the MB1004 expression plasmid served as negative controls. JM101 [pMON6710]: sample 1, preinduction (I_0); sample 2, 2-h postinduction (I_2); sample 3, 4-h postinduction (I_4); sample 4, 6-h postinduction (I_6); samples 5 and 6, JM101 control; sample 5, preinduction (I_0); sample 6, 4-h postinduction (I_4). W3110 *lacI*^{q} L8 [pMON2839]: sample 1, preinduction (I_0); sample 2, 2-h postinduction (I_2). Controls: sample 1, 0.5 ng of tPA standard in Tris–phosphate-buffered saline (TPBS) (positive control); sample 2, TPBS; sample 3, guanidine solubilization buffer (guanidine hydrochloride samples) or M9 + CAA induction medium (soluble sonicate samples).

from the lysine–Sepharose column was immunoreactive to anti-tPA antisera (data not shown). Of the 15 mg of immunogenic protein eluted from the lysine–Sepharose column, only approximately 0.1 mg, or <1% of the immunoreactive material that bound to the column, also bound and was eluted from the *Erythrina* inhibitor–Sepharose column (20 μg of MB1004/L of broth recovered, representing 1% of total immunogenic protein). The *Erythrina* inhibitor interaction is apparently very specific for the proper SP domain configuration, as the only material that bound to the *Erythrina* inhibitor–Sepharose column was the material which showed plasminogen activation activity on gelatin clearing gels (data not shown).

On the basis of clearing gel results, a small amount of MB1004 activity may have eluted through the lysine–Sepharose column without binding. Approximately 10% of the total pooled activity from the lysine–Sepharose column was derived from the 1.5 M NaCl elution fraction. The total amount of protein eluted from the lysine–Sepharose column with just 1.5 M NaCl was significant (1.26 g), suggesting that a good deal of the capacity of the column involved nonspecific ion-exchange interactions with impurities.

Since plasminogen activation on gelatin clearing gels gave the most specific MB1004 activity, it was the primary analytical technique used to follow the purification. Demonstration of plasminogen-dependent proteolytic activity at the appropriate molecular weight was the key criterion for MB1004 identification. Western blots of crude cell extracts and processing intermediates showed multiple immunoreactive bands of both higher and lower molecular weight relative to MB1004 with nonreducing gels. The band representing the active MB1004 was a very minor component of the total immunoreactive profile and was only distinguishable as a distinct band after the lysine–Sepharose chromatography step. The major immunoreactive band in the lysine–Sepharose column product migrated at a molecular weight several kilodaltons higher than the active material on a nonreducing gel. The identity of this material was not established. Several immunoreactive bands of lower molecular weight than MB1004 were also also in the lysine–Sepharose elution product. Since the crude cell extract also contained these lower molecular weight bands, it is unlikely that these lower molecular weight bands resulted from proteolytic degradation during purification. However, since no protease inhibitors were included in the processing steps, this possibility exists. Intracellular degradation of intact proenzyme, premature termination, or incorrect translation initiation was also possible.

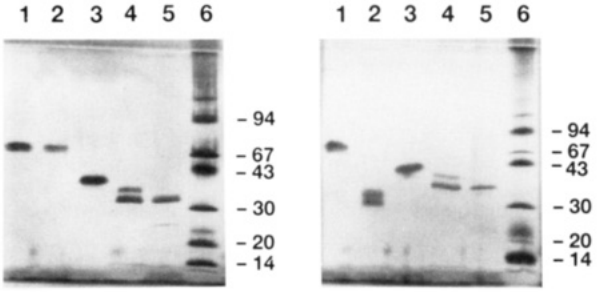

FIGURE 5: SDS–PAGE of native tPA and K_2–SP variant preparations under nonreduced (left) and reduced (right) conditions. Lane 1, native single-chain recombinant C127 tPA. Lane 2, native two-chain recombinant C127 tPA. Lane 3, mammalian cell-derived MB1004 variant. Lane 4, mammalian cell-derived MB1004 variant treated with *N*-glycanase. Lane 5, *E. coli* derived MB1004 variant. Lane 6, molecular weight markers ($\times 10^{-3}$). Approximately 70 ng of sample was loaded per lane. Electrophoresis was performed using a PhastSystem unit and 10–15% gradient gels from Pharmacia. Gels were stained with silver following the procedure of Morrissey (1981).

Quantification of MB1004 in crude cell extracts and during purification was attempted by using a quantitative fluorescence immunoassay (PCFIA) in conjunction with an extended, direct amidolytic assay using the synthetic substrate S-2288 (KabiVitrum). Both "quantitative" techniques showed 2–3 mg/L of immunogenic or amidolytic activity. Both assays suffered from a severe lack of specificity for the active, monomeric MB1004 protein. The multitude of immunoreactive species seen by Western blot analysis minimized the value of the PCFIA immunoassay as a means of specifically quantifying proteolytic activity observed with S-2288.

No attempt was made to increase activity by refolding. Significant quantities of proteins that were immunoreactive with anti-tPA antisera were produced during the fermentation. Other than the material isolated through the lysine–Sepharose/*Erythrina* inhibitor–Sepharose columns, none of this material showed plasminogen activation on a gelatin clearing gel.

Molecular Weight and Purity of MB1004. Gradient SDS–PAGE under both reduced and nonreduced conditions showed that MB1004 purified by both lysine and *Erythrina* inhibitor affinity chromatography migrated between the 30K and 43K molecular weight markers, consistent with a predicted

Table I: Specific Activities of the K_2–SP tPA Variant Produced in *E. coli* or Mammalian Cells, with or without *N*-Glycanase Treatment[a]

	assay						
			indirect amidolytic				
	direct amidolytic		unstimulated		stimulated		
tPA species[b]	mean	SD	mean	SD	mean	SD	clot lysis
MB1004G	760	67	216	58	34	2	32
MB1004N	623	57	453	76	159	30	112
MB1004	791	81	915	111	396	23	182

[a] Activities are expressed in international units per microgram using human melanoma tPA as both activity and antigen standard. See Experimental Procedures for details. Mean and standard deviation (SD) are given for 3 separate assays with 4 replicates per assay (12 total determinations). For the clot lysis assay, activities are from a single assay with four dilutions for each sample. [b] Abbreviations: MB1004G, glycosylated K_2–SP variant produced in mammalian cells; MB1004N, mammalian K_2–SP variant treated with *N*-glycanase; MB1004, K_2–SP variant produced in *E. coli*.

molecular weight of 38K (Figure 5). For comparison, gradient SDS–PAGE was also performed on MB1004 produced in recombinant mammalian cells before and after treatment with *N*-glycanase to remove N-linked oligosaccharides. Mammalian cell MB1004 migrated at a higher molecular weight, nearer to the 43K marker (Figure 5). *N*-Glycanase treatment produced two lower molecular weight species, the smaller of which migrated with *E. coli* produced MB1004 (Figure 5). This suggested that the higher molecular weight of mammalian cell-produced MB1004 was due to glycosylation. The tPA variant MB1004 has two potential N-linked glycosylation sites. The band present in the *N*-glycanase-treated protein which migrated at a position intermediate between mammalian MB1004 and *E. coli* MB1004 may represent a species with only one of the two potential oligosaccharide chains removed (Figure 5). Compared to native two-chain tPA (Figure 1), the absence of a shift to a lower molecular weight upon reduction with 2-mercaptoethanol suggested that the MB1004 samples were in a single-chain form (Figure 5). Only low levels of contaminants were present in the *E. coli* MB1004 enzyme. Amino acid analysis through 25 positions confirmed that the correct sequence was present in the purified MB1004. Secondary signals accounted for <20% of the output signal relative to the MB1004. The similar migration of the major band from this preparation and the lower molecular weight band from the *N*-glycanase-treated mammalian MB1004 enzyme provided additional evidence that the *E. coli* product had the expected size for a K_2–SP variant.

Enzymatic Activity. To determine the enzymatic activity of the purified MB1004 (soluble, active, monomeric; Figure 5), four different in vitro assay conditions were employed (Table I). The direct amidolytic assay was used to measure the active-site functionality of the SP domain. The other assays employed, unstimulated and stimulated indirect amidolytic and clot lysis, are more physiologically relevant since they are coupled to plasminogen activation in the absence (unstimulated) or presence (stimulated) of fibrinogen fragments or fibrin (clot lysis). The activities were compared to those of a purified mammalian cell-derived MB1004 preparation (MB1004G). *N*-Glycanase-treated mammalian cell MB1004 (MB1004N) was included because of the negative effect glycosylation might have on the in vitro assay results (Wittwer et al., 1989). From results of these assays (Table I), it can be seen that the relative activities depended on the assay employed. The three preparations were all similar in activity when the hydrolysis of a chromogenic peptide substrate was measured in the direct amidolytic assay. These results indicated that the SP domains were equally functional and that glycosylation had no effect on this activity. In the other assays, however, the *E. coli* MB1004 had about twice the activity of the *N*-glycanase-treated mammalian cell MB1004 and substantially greater activity (4–12-fold) than untreated, glycosylated MB1004, suggesting a dramatic effect of glycosylation on plasminogen and fibrin binding. The *E. coli* MB1004 had similarly high specific activities in the direct and unstimulated indirect amidolytic assays, suggesting that proper interactions with plasminogen were mediated by the SP domain. The significantly lower specific activities in the fibrinogen fragment stimulated indirect and fibrin clot lysis assays may reflect the absence of the fibrin binding finger domain in the variant K_2–SP enzyme.

It should be noted that activity of the native melanoma tPA standard employed for these assays was 534 IU/μg. A proper comparison with native tPA, however, was not possible because the antigen response of MB1004 in the tPA ELISA assay relative to native tPA is not known. Others have found up to a 2-fold difference for tPA variants where the finger or both the finger and EGF domains were deleted (Larsen et al., 1988).

Discussion

Recent results with trypsin (Graf et al., 1989) provided the precedent that a tPA variant could also be secreted into the periplasm of *E. coli* in a soluble, active form since trypsin also has numerous disulfide bonds and shares 60% identity with the serine protease domain of tPA (Strassburger et al., 1983). The tPA variant K_2–SP (MB1004) was initially chosen because it is relative small, resembles trypsin, and contains an even number of cysteines (18), all of which pair to form 9 disulfide bonds. In addition, secretion of the K_2 domain in *E. coli* has been described (Cleary et al., 1989).

This is the first report of a tPA variant synthesized as a secreted, active protein in *E. coli* having a significantly higher specific activity than its mammalian-derived counterpart. Expression, N-terminal protein sequencing, and specific activity data have shown that a small percentage of soluble, active MB1004 (approximately 20 μg/L of broth recovered, representing 1% of total immunogenic protein) was secreted into the periplasm of *E. coli* using the PhoA leader peptide and *tac* promoter. In contrast, no soluble activity was detected when MB1004 was synthesized cytoplasmically, even though comparable accumulation levels were obtained (Figures 3 and 4). An added advantage with *E. coli* expression was that the MB1004 variant was not glycosylated. Specific activity comparisons showed that the *E. coli* derived MB1004 had similar activity to mammalian cell-derived deglycosylated MB1004, but, depending on the assay, was 4–12-fold higher than mammalian cell-derived glycosylated MB1004 (Table I). This increase in specific activity due to the lack of glycosylation was much greater than anticipated. Previous results with full-length tPA showed that a 2-fold increase in fibrinogen fragment stimulated plasminogen activation was observed with nonglycosylated full-length tPA (Wittwer et al., 1989). In the present study using nonglycosylated MB1004, a 12-fold in-

crease in specific activity was observed. The results with MB1004 demonstrate the biggest effect of glycosylation on activity of any enzyme observed to date. These results suggest that the presence of carbohydrate substantially inhibits full activity and that removal is essential before a proper comparison with the mammalian cell produced enzyme can be made (Table I).

The specific activity measurements presented here suggest that the *E. coli* produced enzyme has the enzymatic properties which might be expected of a K_2–SP tPA variant. Similar activity in assays employing a low molecular weight peptide or plasminogen as substrate (direct and unstimulated indirect assays, respectively, Table I) suggested proper functioning of the SP domain. On the other hand, a significantly lower activity in the fibrin clot lysis assay is consistent with the absence of the finger domain of native tPA. Activity in the fibrinogen fragment stimulated indirect assay was twice that seen for the clot lysis assay, reflecting the interaction of these fragments with the K_2 domain. Others who have examined the properties of K_2–SP variants have also found stimulation of activity by fibrinogen fragments and defects in fibrin binding or fibrin-stimulated activity (van Zonneveld et al., 1986a,b; Verheijen et al., 1986; Burck et al., 1990).

Removal of oligosaccharide from mammalian cell-produced MB1004 resulted in a dramatic increase in activity in assays employing plasminogen as substrate. While the MB1004 enzyme treated with *N*-glycanase was more comparable in activity to the nonglycosylated *E. coli* MB1004 enzyme, it was still somewhat less active. This may be at least partially due to the incomplete removal of oligosaccharides by *N*-glycanase (Figure 5). The different activity assays used in this study (Table I) provide insight into the reasons behind these effects of carbohydrate on activity. The direct amidolytic assay measures hydrolysis of a low molecular weight peptide, while the other three assays employ plasminogen as substrate. The enzymes were found to have similar direct amidolytic activities, while the fully glycosylated enzyme had much lower activity in the plasminogen substrate assays. The K_2–SP variant has a potential glycosylation site on each domain. The presence of oligosaccharide on the SP domain might hinder the binding of the large (90 kDa) plasminogen molecule, but not affect hydrolysis of a peptide substrate. In addition, oligosaccharide on the K_2 domain may decrease the interaction of the K_2 domain with fibrin or fibrinogen fragments, explaining why the activity of fully glycosylated enzyme is further depressed in assays where these stimulators are present. Studies with native tPA from melanoma and fibroblast sources also suggested that glycosylation at the site on K_2 depressed fibrinogen fragment stimulated activity, while glycosyulation of the SP domain affected interactions with both plasminogen and fibrin (Wittwer et al., 1989).

Although expression of the K_2–SP variant in *E. coli* resulted in a higher activity enzyme when compared to expression in a mammalian cell line, several factors should be considered before this nonglycosylated enzyme could be used for therapeutic use. First, the practicality of large-scale bacterial production would need to be demonstrated. Second, since plasminogen activation both in the presence and in the absence of stimulator showed significant increases (Table I), the desirability of nonglycosylation remains to be shown. The effect of the increased activity seen in this study on clot-specific compared to systemic plasminogen activation should be determined in appropriate in vitro and in vivo systems. Third, the effect of glycosylation on clearance from circulation, stability, and immunogenicity would have to be determined. With mammalian cell-derived tPA, the high-mannose oligosaccharide at position 117 in the K_1 domain is in part responsible for the short circulation half-life (3–5 min). Deglycosylation resulted in a 3–5-fold longer circulating half-life (Lau et al., 1987; Hotchkiss et al., 1988).

The majority (approximately 70%) of secreted MB1004 was insoluble in the periplasm when synthesized in JM101 [pMON6710] (Figure 3B). Even the soluble MB1004 fraction from JM101 [pMON6710] was not liberated from the periplasm using routine extraction methods such as osmotic shock, lysozyme–EDTA treatment, or treatment with various chaotrophs and ionic and nonionic detergents (unpublished data). A similar problem was encountered with BPTI (Marks et al., 1986), a related protein to tPA in that it is basic and could bind to anionic periplasmic components. As with IGF-1 (Obukowicz et al., 1988), this problem of insolubility is a major hindrance in using secretion for the synthesis of other heterologous proteins where intracellular expression is not well-suited. MB1004 serves as an ideal model protein to address solubility either genetically by strain/expression plasmid manipulation or by designing MB1004 variants having full activity, but altered solubility properties.

The success with MB1004 secretion using the *phoA* leader sequence in combination with the *phoA* ribosome binding site and *tac* promoter illustrates the unpredictability of whether secretion will occur. Previous attempts to secrete MB1004 or just the serine protease domain of tPA using the *lamB* or *ompF* leader sequence in combination with the phage T7 g10L ribosome binding site and *recA* promoter failed. The failure in secretion of full-length tPA using the *ompA* or native tPA leader sequence has also been reported (Sarmientos et al., 1989). A systematic analysis of the importance of the promoter, ribosome binding site, and leader sequence in mediating secretion of a given heterologous protein thus appears to be necessary.

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REFERENCES

Astrup, T. (1978) *Prog. Fibrinolysis 3*, 1–57.

Banyai, L., Varadi, A., & Patthy, L. (1983) *FEBS Lett. 163*, 37–41.

Bell, L. D., Smith, J. C., Derbyshire, R., Finlay, M., Johnson, I., Gilbert, R., Slocombe, P., Cook, E., Richards, H., Clissold, P., Meredith, D., Powell-Jones, C. H., Dawson, K. M., Carter, B. L., & McCullagh, K. G. (1988) *Gene 63*, 155–163.

Berget, P. B., Poteete, A. R., & Sauer, R. T. (1983) *J. Mol. Biol. 164*, 561–572.

Boose, J. A., Kuismanen, E., Gerard, R., Sambrook, J., & Gething, M.-J. (1989) *Biochemistry 28*, 635–643.

Brent, R., & Ptashne, M. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4204–4208.

Burck, P. J., Berg, D. H., Warrick, M. W., Berg, D. T., Wallis, J. D., Jaskunas, S. R., Crisel, R. M., Weigel, B., Vlahos, C. J., McClure, D. B., & Grinnell, B. W., (1990) *J. Biol. Chem. 365*, 5170–5177.

Castellino, F. J. (1981) *Chem. Rev. 81*, 431–446.

Cleary, S., Mulkerrin, M. G., & Kelley, R. F. (1989) *Biochemistry 28*, 1884–1891.

Collen, D. (1988) *Thromb. Res* (*Suppl. VIII*), 3–14.

Collen, D. (1980) *Thromb. Haemostasis 43*, 77–89.
de Boer, H. A., Comstock, L. J., & Vasser, M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 21–25.
Gaffney, P. J., & Curtis, A. D. (1985) *Thromb. Haemostasis 53*, 134–136.
Goldberg, A. R. (1974) *Cell 3*, 95–102.
Gordon, K., Lee, E., Vitale, J. A., Smith, A. E., Westphal, H., & Henninghausen, L. (1987) *Bio/Technology 5*, 1183–1187.
Graf, L., Craik, C. S., Patthy, A., Roczniak, S., Fletterick, R., & Rutter, W. J. (1987) *Biochemistry 26*, 2616–2623.
Harris, T. J. R. (1987) *Protein Eng. 1*, 449–458.
Heussen, C., Joubert, F., & Dowdle, E. B. (1984) *J. Biol. Chem. 259*, 11635–11638.
Hotchkiss, A., Refino, C. J., Leonard, C. K., O'Connor, J. V., Crowley, C., McCabe, J., Tate, K., Nakamura, G., Powers, D., Levinson, A., Mohler, M., & Spellman, M. W. (1988) *Thromb. Haemostasis 60*, 255–261.
Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J., & Hood, L. E. (1983) *Methods Enzymol. 91*, 399–413.
Keck, P. J., Hauser, S. D., Krivi, G., Sanzo, K., Warren, T., Feder, J., & Connolly, D. T. (1989) *Science 246*, 1309–1312.
Krause, J., & Tanswell, T. (1989) *Drug Res. 39*, 632–637.
Laemmli, U. K. (1970) *Nature 227*, 680–688.
Larsen, G. R., Henson, K., & Blue, Y. (1988) *J. Biol. Chem. 263*, 1023–1029.
Lau, D., Kuzma, G., Wei, C.-M., Livingson, D. J., & Hsiung, N. (1987) *Bio/Technology 5*, 953–958.
Lemontt, J. F., Wei, C. M., & Dackowski, W. R. (1985) *DNA 4*, 419–428.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Marks, C.-B., Vasser, M., Ng, P., Henzel, W., & Anderson, S. (1986) *J. Biol. Chem. 261*, 7115–7118.
Matsudaira, P. (1987) *J. Biol. Chem. 263*, 10035–10038.
Messing, J. (1979) *Recomb. DNA Tech. Bull. 2*, 43–48.
Morrissey, J. H. (1981) *Anal. Biochem. 117*, 307–310.
Ny, T., Elgh, F., & Lund, B. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5335–5339.
Obukowicz, M. G., Turner, M. A., Wong, E. Y., & Tacon, W. C. (1988) *Mol. Gen. Genet. 215*, 19–25.
Olins, P. O., Devine, C. S., Rangwala, S. H., & Kavka, K. S. (1988) *Gene 73*, 227–235.
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeberg, P. H., Heyneker, H. L., & Goeddel, D. V. (1983) *Nature 301*, 214–220.
Ramabhadran, T. V., Reitz, B. A., & Tiemeier, D. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6701–6705.
Ranby, M. (1982) *Biochim. Biophys. Acta 704*, 461–689.
Ranby, M., Bergsdorf, N., & Nilsson, T. (1982) *FEBS Lett. 146*, 289–293.
Renart, J., Reiser, J., & Stark, G. R. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3116–3120.
Rijken, D. C., & Collen, D. (1981) *J. Biol. Chem. 256*, 7035–7041.
Ryan, T. J., Seeger, J. I., Kumar, S. A., & Dickerman, H. W. (1984) *J. Biol. Chem. 259*, 14324–14327.
Sarmientos, P., Duchesne, M., Denefle, P., Boiziau, J., Fromage, N., Delporte, N., Parder, F., Lelievre, Y., Mayaux, J.-F., & Cartwright, T. (1989) *Bio/Technology 7*, 495–501.
Southern, E. M., & Berg, P. (1982) *J. Mol. Appl. Genet. 1*, 327–341.
Strassburger, W., Wollmer, A., Pitts, J. E., Glover, I. D., Tickle, I. J., Blundell, T. L., Steffens, G. J., Gunzler, W. A., Otting, F., & Flohe, L. (1983) *FEBS Lett. 157*, 219–223.
Upshall, A., Kumar, A. A., Bailey, M. C., Parker, M. D., Favreau, M. A., Lewison, K. P., Joseph, M. L., Maraganore, J. M., & McKnight, G. L. (1987) *Bio/Technology 5*, 1301–1304.
van Zonneveld, A.-J., Veerman, H., & Pannekoek, H. (1986a) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4670–4674.
van Zonneveld, A.-J., Veerman, H., & Pannekoek, H. (1986b) *J. Biol. Chem. 261*, 14214–14218.
Vehar, G. A., Spellman, M. W., Keyt, B. A., Ferguson, C. K., Keck, R. G., Chloupek, R. C., Harris, R., Bennett, W. F., Builder, S. E., & Hancock, W. S. (1986) *Cold Spring Harbor Symp. Quant. Biol. 5*, 551–562.
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., de Munk, G. A. W., Pouwels, P. H., & Enger-Valk, B. E. (1986) *EMBO J. 5*, 3525–3530.
Wittwer, A. J., & Howard, S. C. (1990) *Biochemistry 29*, 4175–4180.
Wittwer, A. J., Howard, S. C., Carr, L. S., Harakas, N. K., Feder, J., Parekh, R. B., Rudd, P. M., Dwek, R. A., & Rademacher, T. W. (1989) *Biochemistry 28*, 7662–7669.