Quantitative Immunocytochemical Staining for Recombinant Tissue-Type Plasminogen Activator in Transfected Chinese Hamster Ovary Cells (43294)

DORIS E. GENNARO,* SYLVIA T. HOFFSTEIN,* GARY MARKS,* LUCIANO RAMOS,† MELVIN S. OKA,† MITCHELL E. REFF,‡,[1] TIMOTHY K. HART, AND PETER J. BUGELSKI*,[2]

Departments of Experimental Pathology, Biological Process Sciences,† and Molecular Genetics,‡ SmithKline Beecham Pharmaceuticals, Philadelphia, Pennsylvania 19406*

Abstract. **Tissue-type plasminogen activator (tPA) is a serine protease which cleaves plasminogen to its active form, plasmin. tPA plays a physiologic role in hemostasis, wound healing, and embryogenesis. Therapeutically, recombinant human tPA is used as a thrombolytic in myocardial infarction. Although production of therapeutic quantities of tPA in Chinese hamster ovary (CHO) cells transfected with the human gene for tPA is practical, production costs remain high. One important factor which determines the ultimate cost of tPA (or any other recombinant protein expressed in mammalian cells) is its production level on a per cell basis. We have used postembedding immunocytochemical staining with colloidal gold to study the subcellular localization of tPA in CHO cells expressing recombinant tPA (rCHO) in an effort to understand the factor(s) which might limit secretion. Staining for tPA was evaluated visually and by morphometric analysis and was specific and reproducible. Serially passaged rCHO showed no significant change in staining density over 31 serial passages. Staining density was greatest over dilated cisternae of the rough endoplasmic reticulum and nuclear envelope. Golgi stacks and large acid phosphatase-positive vacuoles (probably lysosomes) were also heavily stained. Staining of lysosomal vacuoles suggested that rCHO might be degrading nascent tPA. Incubation of rCHO with ^{125}I-tPA showed that the cells were not internalizing tPA from the media. These results suggest that rCHO fail to secrete a portion of the tPA they synthesize and that it is degraded in lysosomes. This observation may have important implications on the choice of expression systems for efficient production of large quantities of recombinant proteins.** [P.S.E.B.M. 1991, Vol 198]

Human proteins produced by recombinant DNA techniques hold great promise as therapeutic agents in many disease settings. Although some recombinant proteins can be expressed in non-mammalian cells, many proteins that are dependent on intra-chain disulfide bonding, proper glycosylation, or other posttranslational modifications for functional activity must be expressed in mammalian cells (1). However, mammalian expression systems typically produce recombinant proteins at lower levels than bacterial, yeast, or insect expression systems. Production levels of tens of milligrams per liter per day (or pg/cell/day) are typical for an optimized mammalian expression system (2). This low production level impacts adversely on the ultimate availability and cost of therapeutic proteins. For example, streptokinase is a thrombolytic that is a natural product of bacteria and is used to treat acute myocardial infarction. The cost of a therapeutic dose of streptokinase in 1991 is approximately 200 U.S. dollars. Recombinant tissue-type plasminogen activator (tPA) is also a thrombolytic used in acute myocardial infarction but costs approximately $2000, 10 times the cost of streptokinase. tPA is produced in Chinese hamster ovary (CHO) cells (3), and higher costs of production are responsible, in large part, for this substantial

[1] Present address: IDEC Pharmaceutical Corporation, La Jolla, CA 92037.

[2] To whom correspondence and requests for reprints should be addressed at Department of Experimental Pathology, L-60, SmithKline Beecham Pharmaceuticals, Box 1539, King of Prussia, PA 19406.

Received March 1, 1991. [P.S.E.B.M. 1991, Vol 198]
Accepted May 9, 1991.

0037-9727/91/1981-0591$3.00/0

difference in price. While this is admittedly an extreme case, high production costs and difficulty in producing large quantities of recombinant proteins in mammalian cells remain significant issues in exploiting these proteins therapeutically.

tPA is a serine protease, with an approximate mol wt of 65,000, that has three glycosylation sites and elaborate intra-chain disulfide bonding (4). The protein is folded into five domains, which are named on the basis of homology with other proteins. From the N terminus, these are: finger, a domain homologous to a domain of fibronectin; growth factor like, homologous to epidermal growth factor; two kringles, homologous to plasmin; and protease, homologous with the active site domain of many other serine proteases (5). The proteolytic activity of tPA is dependent on proper folding of the peptide chain. The principal physiologic function of tPA is to proteolytically cleave plasminogen to its active form, plasmin. This function is exploited clinically in thrombolytic therapy of myocardial infarction (6).

tPA is produced constitutively in a number of tissues, most notably by endothelial cells (7–9). Some tumor cells also produce tPA (10). For production of recombinant tPA, the human gene has been transfected into a variety of continuous cell lines (11–13). The current therapeutically approved tPA is produced in transfected Chinese hamster ovary cells (3). Production of large amounts of functional tPA in bacterial or yeast expression systems has not been reported, presumably because of improper folding of the molecule. Although there are many factors that contribute to the final cost of recombinant proteins produced in mammalian cells (14), production on a per cell basis is one of the more important. In the experiments described herein, we have used postembedding immunocytochemical staining with colloidal gold to study the subcellular localization of tPA in CHO cells expressing recombinant tPA (rCHO). In an effort to better understand the factor(s) which might limit secretion of tPA, production on a per cell basis and subcellular localization were correlated in stablely transfected cells for 31 serial passages. We found that there was no significant change in the staining density with serial passage, but found evidence that CHO cells may fail to secrete a portion of nascent tPA.

Materials and Methods

Cell Culture. CHO cells were transfected with the human gene for tPA in an expression vector similar to that described previously for bovine endothelial cells (12) and selected in methotrexate for high level tPA secretion. The recombinant CHO cells were maintained as suspension cultures (5% CO_2, 95% air, 37°C) in spinner flasks in minimal essential medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum and 200 n*M* methotrexate. Recombinant DNA and transfected cells were handled in compliance with the NIH guidelines for physical and biological containment. The cells were passaged by dilution on a twice-weekly basis. Passage numbers 25, 30, 43, and 56 (from a 15-week period) were immunostained for tPA.

Immunocytochemistry. Aliquots of approximately 1×10^6 cells were centrifuged and fixed with 0.5% paraformaldehyde and 0.05% glutaraldehyde in 0.1 *M* cacodylate buffer (pH 7.3) for 2 hr at 4°C. The cells were washed in 0.2 *M* cacodylate buffer and partially dehydrated in ethanol (90%). The cells were then incubated in 90% ethanol and L.R. White embedding resin (1/2), followed by 100% L.R. White resin at room temperature for 9 h (Polysciences Inc., Warrington, PA). The cells were infiltrated with L.R. White resin (without catalyst) overnight at 4°C and embedded in gelatin capsules in L.R. White resin (with catalyst) at −20°C (15). The resultant blocks were sectioned at approximately 60 nm and the sections were collected on Formvar-coated 200-mesh nickel grids. The grids were stored at −20°C until immunostained.

Cells were stained for tPA using goat antihuman uterine tPA antibodies. This antisera was obtained commercially (American Diagnostica, New York, NY) and has been characterized previously (16).

Grids were stained by sequential immersion in 100-μl reagent drops on Parafilm at room temperature according to the following protocol: (i) 1% cold water fish gelatin in phosphate-buffered saline (CWFG-PBS; 17) (Sigma Chemical Co., St. Louis, MO) for 60 min; (ii) goat antihuman tPA 1/500 in CWFG-PBS for 90 min; (iii) wash in CWFG-PBS 6 times, 10 min each time; (iv) colloidal gold-conjugated rabbit antigoat Ig (15 nm) (E-Y Laboratories, San Mateo, CA) 1/5 in CWFG-PBS for 90 min; and (v) wash in distilled water (20 immersions).

The grids were then post-stained with osmium, uranyl acetate, and lead citrate. Controls for immunostaining included omission of primary antibody tPA, immunostaining of a sample of rCHO cells mixed with amphibian erythrocytes, and immunostaining of CHO cells transfected with the gene for an unrelated human protein (control-CHO).

Morphology and Acid Phosphatase Cytochemistry. To allow detailed examination of organelles, rCHO and control-CHO were fixed in 2% glutaraldehyde in 0.1 *M* cacodylate, postfixed in OsO_4 stained *en bloc* with uranyl acetate, dehydrated, and embedded in Spurr's resin. Sections were cut at approximately 60 nm and stained with lead citrate. rCHO were also stained cytochemically for acid phosphatase activity using modified Gomori medium, as described previously (18). All grids were examined with a JEOL 100CX or 1200EX transmission electron microscope operating at an accelerating voltage of 60–80 kV.

Morphometry. Ten to 17 consecutive cell profiles from each immunogold-stained sample were photographed at ×10,000 magnification. To ensure that data would be collected from comparable regions, only cells that were sectioned through the nucleus were included. Negatives were printed as ×2.5 enlargements. The number of colloidal gold particles overlaying the nucleus, cytoplasm, and extracellular space was counted and the areas of these regions were measured digitally with a SigmaScan semiautomatic image analysis system (Jandel Scientific, Corte Madera, CA). These data were used to calculate staining density (number of gold particles per μm^2) for each region.

Statistical analysis of the gold unit area was performed using the general linear models procedure and the Tukey studentized range test (SAS Institute, Cary, NC). *P*-values less than or equal to 0.05 were accepted as significant.

Cellular Internalization of ^{125}I-tPA. To determine the role of reuptake of secreted tPA in determining the subcellular localization of tPA, rCHO, control-CHO, and freshly isolated rat hepatocytes (a positive control cell) were incubated with ^{125}I-tPA, as described previously (19). Briefly, ^{125}I-tPA was prepared by incubating tPA with [^{125}I]sodium iodide in an Iodogen coated tube (Pierce, Rockford, IL). Free iodine was separated from ^{125}I-tPA with a tPA affinity gel (American Diagnostica). The ^{125}I-tPA was greater than 90% precipitable by trichloroacetic acid and retained its proteolytic activity. Isolated hepatocytes were prepared by perfusing rat livers with collagenase. The resultant single cell suspensions were greater than 90% hepatic parenchymal cells. Viability of hepatocytes and suspension cultured CHO cells was greater than 90%, as determined by trypan blue exclusion. Cells (1×10^6/ml) were incubated in Kreb's-Heinseleit buffer containing 10 m*M* glucose, 2% bovine serum albumin, and a trace amount (final concentration 10 p*M*) of ^{125}I-tPA in 50-ml Erlenmeyer flasks. The flasks were incubated at 37°C in a shaking bath under 95% O_2 and 5% CO_2. At selected times, triplicate 0.5-ml aliquots were removed and washed, and cell-associated ^{125}I-tPA was measured by gamma counting. Nonspecific uptake of ^{125}I-tPA was determined by incubating cells in the presence of excess (200 m*M*) nonlabeled tPA. Data were expressed as cell-associated tPA by correcting cell-associated counts for the specific activity of ^{125}I-tPA.

Results

Specificity and Reproducibility of Staining. Immunocytochemical staining of tPA in rCHO cells was specific and reproducible. Table I presents data on tPA immunogold staining of the same rCHO cells over 31 passages. Background staining on extracellular spaces were consistently low. No specific staining of control-CHO and amphibian red blood cells was seen and omission of the anti-tPA antibodies from the staining protocol resulted in the complete loss of specific staining, indicating that the staining was specific for tPA.

Specific immunostaining for tPA was observed in rCHO cells examined over 31 passages by morphometric analysis. There were some differences in specific staining density between the passages, but these differences were not statistically significant. Grids from rCHO Passage 43 were stained on five separate occasions. Staining specific for tPA was observed each time these samples were processed, indicating that the tPA immunogold-staining procedure was highly reproducible.

Subcellular Localization of tPA in rCHO. The tPA immunogold-staining procedure was used to study the subcellular distribution of tPA in rCHO cells. The ultrastructure of rCHO was examined following conventional processing procedures, because the fixation for immunostaining did not provide fine ultrastructural preservation.

Profiles of rCHO were generally round (Fig. 1A). Nuclei were eccentric and usually round or bean-shaped. The Golgi region was well-defined and multiple profiles of Golgi were occasionally seen in the same cell. Mitochondria were frequently bracketed by cisternae of rough endoplasmic reticulum (RER) (Fig. 1A). The RER was often dilated and uniformly filled with a moderately electron-dense material. Often the nuclear envelope was dilated and filled with a similar electron-dense proteinaceous material (Fig. 1B). Large vacuoles were prominent in some cells. Some of these vacuoles contained proteinaceous material and/or myelin figures (residual bodies) (Fig. 1C), suggesting that they were secondary lysosomes. Their lysosomal association was also suggested by cytochemical staining for acid phosphatase activity (Fig. 1D).

Representative immunostaining of rCHO is shown in Figure 2. There were deposits of gold beads over dilated cisternae of the RER (Fig. 2A) and nuclear envelope (Fig. 2B). These dilations were similar to those seen in Figures 1A and B. Many cells had a different pattern of staining. Instead of gold beads concentrated over the RER, dilated nuclear envelope, or vacuoles, they were evenly dispersed over the Golgi region (Fig. 2C). Lysosome-like vacuoles, when present, were always intensely stained (Fig. 2D). A qualitative assessment of the distribution of gold particles over the various cellular compartments is summarized in Table II.

Staining of rCHO was heterogeneous on a cell-to-cell basis; some cells showed intense staining and others hardly any. [This is also reflected in the large SE for the morphometry data in Table I.] In cells showing moderate to heavy staining, the pattern of staining was generally consistent for each of the four passages evaluated. RER showed the most intense staining. Staining

Table I. Specificity and Reproducibility of Immunogold Staining for tPA

Cell type	Staining density on cytoplasm[a] (no. gold/μm^2)	Staining density on extracellular space (no. gold/μm^2)	Specific staining density (no. gold/μm^2)
rCHO (Passage 25)	110 ± 40	23 ± 2	87 ± 40
rCHO (Passage 30)	90 ± 27	5 ± 1	85 ± 27
rCHO (Passage 43)	121 ± 20	18 ± 2	103 ± 20
rCHO (Passage 56)	67 ± 9	14 ± 3	53 ± 9
control-CHO	12 ± 2	13 ± 3	−1 ± 3[b]
Amphibian red blood cells	33 ± 8	28 ± 3	5 ± 8[b]

[a] Data are presented as the mean ± SE.
[b] Not significant compared to extracellular space.

of the nuclear envelope and Golgi areas was more variable and, in some cells, the large vacuoles (lysosomes?) were the only structures to be intensely stained.

Cellular Uptake of ^{125}I-tPA by Transfected Cells. To determine if reuptake tPA could account for the putative lysosomal localization of tPA in rCHO, we examined uptake of ^{125}I-tPA by these cells. Freshly isolated hepatocytes were included in the experiment to serve as a positive control for uptake of tPA. Rat hepatocytes have been shown to rapidly internalize tPA by receptor-mediated endocytosis (19). As shown in Figure 3, isolated rat hepatocytes progressively took up radiolabel over the 30-min incubation. In contrast, rCHO cells took up very little radioactivity. Extending the incubation time to 2 h did not increase uptake by rCHO cells. To eliminate the possibility that rCHO cells may have autosaturated a putative tPA receptor, we also examined CHO cells transfected with the gene for human CD4. Again, little uptake of ^{125}I-tPA was seen. When rCHO cells were incubated with ^{125}I-tPA in the presence of a large excess of nonlabeled tPA, the same amount of cell-associated radioactivity was found, suggesting that all uptake of ^{125}I-tPA by rCHO cells is due to nonspecific interactions and that it is not a receptor-mediated process.

Discussion

In this report, we describe a technique for postembedding immunogold staining of tPA. We demonstrate that the staining is specific and reproducible and have applied it to study the subcellular localization of tPA in CHO cells transfected with the human gene for tPA.

Plasminogen activators are believed to play a role in many physiologic functions, e.g., thrombolysis, embryogenesis, and metastases (7–9), and numerous investigators have studied their distribution by immunohistochemical staining (20–26). With the exception of a study by Paul *et al.* (27) of the plasminogen activator produced by pig kidney cells, we believe that ours is the first report of an ultrastructural localization of tissue-type plasminogen activator. And, although our studies have been limited to transfected cells, the technique may be sufficiently sensitive to detect indigenously expressed tPA *in vivo*, e.g., in endothelial cells (7–9). Since all necessary reagents are commercially available, application of the staining technique described in this report can be carried out by other laboratories.

rCHO cells were examined for over 30 continuous passages as suspension cultures. Although there were variations in the subcellular localization of tPA from pass to pass, e.g., staining of the nuclear envelope, the average density of staining was relatively stable. This stability in staining correlates with stable levels of secretion of tPA (4–6 pg/cell/day) by rCHO. A notable finding of our study was the variation in staining density for tPA from cell to cell in a given passage population. This was reflected in the large SE for tPA-specific staining density (Table I). Hammel *et al.* (28) have used quantitative immunocytochemistry to evaluate production of peptide hormones and have found similar cell-to-cell variation in morphometrically similar populations of pituitary melanotrophs and cells in the neurohypophysis. They suggest that staining density reflects physiologic differences between the cells. The observed variations in staining density of rCHO cells, thus, may correlate with the fluctuations in production of tPA by individual cells. CHO cells transfected with foreign proteins consistently showed dilated cisternae of the endoplasmic reticulum and nuclear envelope that were filled with proteinaceous deposits. Our results show that these deposits contained tPA-derived material. Nontransfected CHO cells rarely had regions of dilated endoplasmic reticulum. In addition to immunocytochemical localization in organelles of the secretory pathway, tPA was also found in vacuoles containing densely staining material. These structures were similar in size, shape, density, and location to structures identified as lysosomes by acid phosphatase cytochemistry. To elucidate whether the tPA in these structures was a result of shunting out of the secretory pathway or reuptake of secreted tPA from the medium, we assessed the ability of rCHO to endocytose ^{125}I-tPA. Several cell types, including endothelial cells (29), fibroblasts (30),

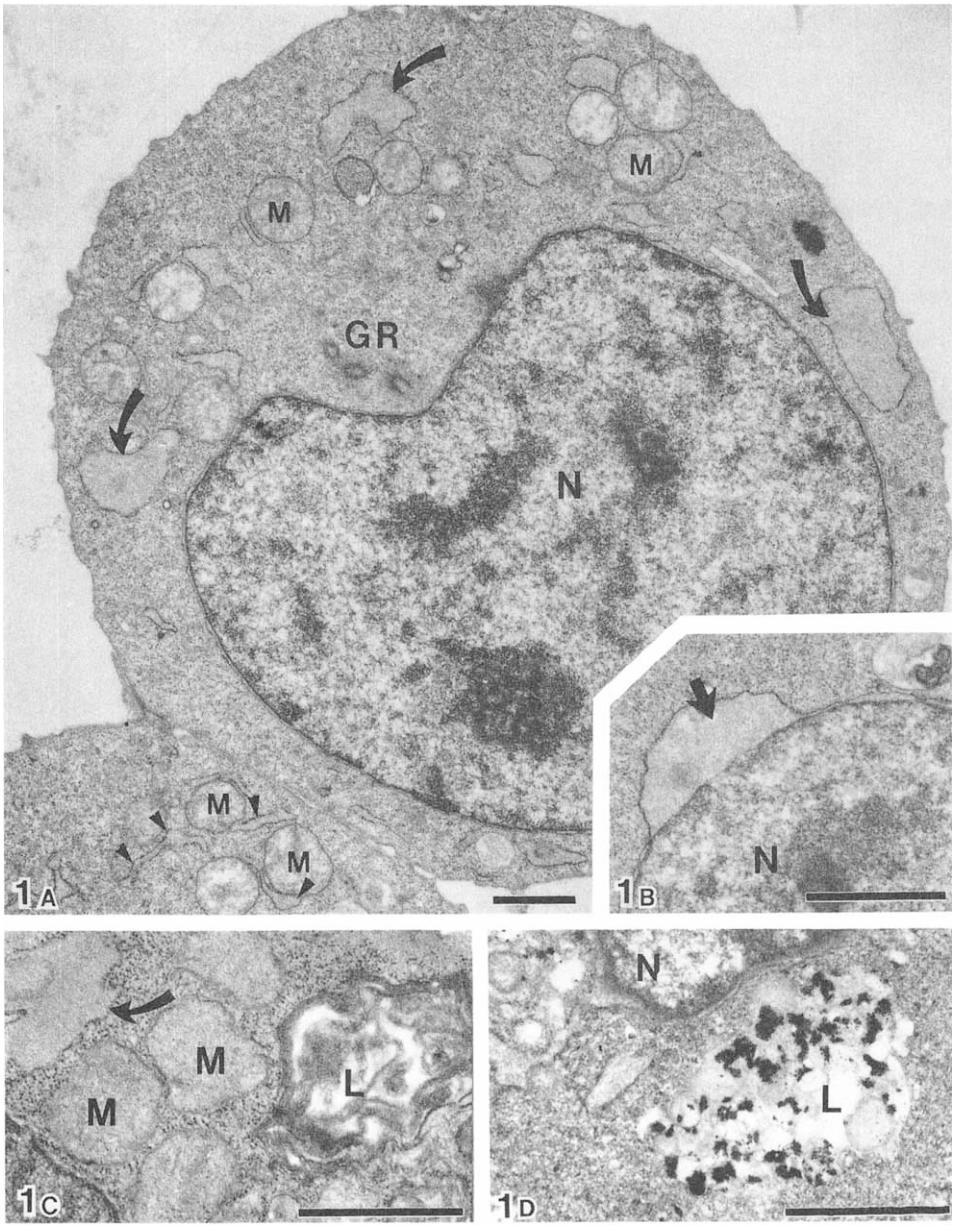

Figure 1. (A) CHO cell transfected with the human gene for tPA (rCHO) fixed in glutaraldehyde and postfixed in osmium. The nucleus (N) is eccentric and is associated with a prominent Golgi region (GR). The cytoplasm has relatively few organelles except for mitochondria (M) and a few dilated cisternae of rough endoplasmic reticulum (arrows). In an adjacent cell, the cisternae of the rough endoplasmic reticulum are only slightly dilated (arrow heads). Lead citrate and uranyl acetate. Bar equals 1 μm. (B) Detail of an rCHO cell process, as described above. The cisternae of the nuclear envelope is dilated (arrow) and contains moderately electron-dense proteinaceous material. Lead citrate and uranyl acetate. Bar equals 1 μm. (C) Detail of an rCHO cell showing the dilated cisternae of the endoplasmic reticulum (arrow) and mitochondria. A cytoplasmic vacuole contains a whorl of electron-dense material, suggesting that it is a secondary lysosome (L). Lead citrate and uranyl acetate. Bar equals 1 μm. (D) Detail of an rCHO cell stained for acid phosphatase activity using a modified Gomori method. A cytoplasmic vacuole (L) similar to that shown in C is positively stained. Lead citrate and uranyl acetate. Bar equals 1 μm.

and especially hepatocytes (19, 31, 32) can internalize tPA. However, the ^{125}I-tPA uptake studies with rCHO cells show that only very low levels of tPA become cell associated. Thus, it is unlikely that reuptake of secreted tPA accounts for the vacuolar localization. Another hypotheses is that tPA is transcribed at a faster rate than the Golgi can process it and that a portion accumulates in the RER and may be routed to lysosomes, rather than being secreted. Recent work on glycoprotein synthesis suggests that the transit time for proteins from the RER to the Golgi is relatively long (33). In addition, improperly folded proteins cannot be transferred to the Golgi for glycosylation (34), but are degraded either lysosomally or in a novel, nonlysosomal compartment (35). A mechanism that may be involved in regulating secretion of proteins is binding to the so-called heavy chain binding protein (BiP). BiP has been shown to bind immunoglobulin heavy chains, preventing transfer

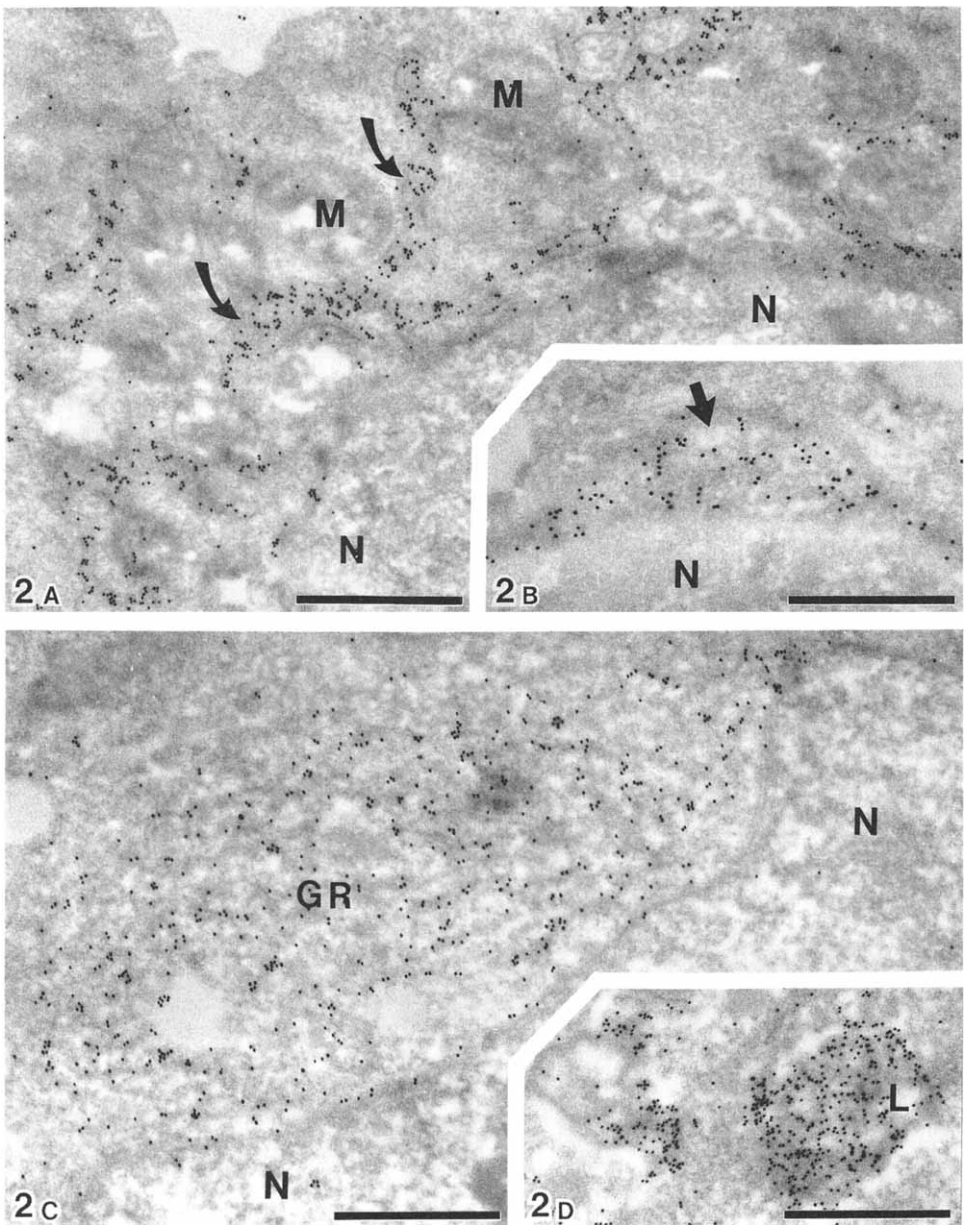

Figure 2. (A) CHO cells transfected with the human gene for tPA (rCHO) and immunostained for tPA, as described in the text. Dilated cisternae of endoplasmic reticulum are heavily stained (arrows), while the mitochondria (M) and nucleus show no specific staining. Bar equals 1 μm. (B) Detail of an rCHO showing immunostaining for tPA in the nuclear envelope. N indicates the nucleus. Bar equals 1 μm. (C) rCHO showing diffuse staining of the Golgi region (GR) delimited on one side by the nucleus. Bar equals 1 μm. (D) Detail of an rCHO showing heavy immunostaining of a lysosome-like cytoplasmic vacuole (L). Bar equals 1 μm.

Table II. Subcellular Localization of tPA in rCHO[a]

Passage number	Nuclei and mitochondria	Nuclear envelope	RER	Golgi regions	Vacuoles
25	—	—	++	+++	+
30	—	+++	++++	+	+++
43	—	++++	++++	+++	+++
56	—	—	++	+	++

[a] Symbols used in table: + = minimal specific staining; ++ = moderate specific staining; +++ = heavy specific staining; and ++++ = very heavy specific staining.

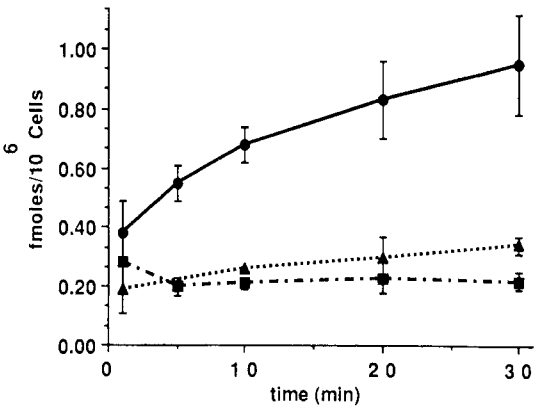

Figure 3. Uptake of ^{125}I-tPA by cultured cells. Freshly isolated hepatocytes (circles), CHO cells transfected with the human gene for tPA (triangles), or CHO cells transfected with the human gene for an unrelated protein (squares) were incubated with ^{125}I-tPA at 37°C for 1 to 30 min. Cell-associated radioactivity was used to calculate fmole equivalents of tPA per 10^6 cells and the results (± SD) were plotted against time. Rat hepatocytes took up ^{125}I-tPA in a time-dependent fashion. CHO cells showed little uptake of ^{125}I-tPA.

to the Golgi prior to their assembly with light chains (36). In CHO cells transfected with the gene for tPA, normally glycosylated tPA shows low levels of association with BiP. However, blockade of glycosylation of tPA by site-directed mutagenesis or treatment of the cells with tunicamycin enhanced association of tPA with BiP and decreased secretion (37). Our results demonstrating that wild-type tPA-derived material is present in dilated RER and lysosome-like vacuoles suggests that slow processing and trapping of tPA occurs in rCHO cells. Direct evidence of involvement of BiP in this process will require further studies.

Taken together, our data suggest that proteins transcribed from amplified genes in transfected CHO cells may not be efficiently secreted. CHO cells are a commonly used expression system for recombinant proteins (1). Given the high cost of production of pharmaceutically relevant recombinant proteins in mammalian systems, understanding this phenomenon and optimizing the culture conditions to improve secretion of recombinant proteins is an important area for further research.

1. Bendig MM. The production of foreign proteins in mammalian cells. Genetic Engineer **7**:91–127, 1988.
2. Marino MH. Expression systems for heterologous protein production. BioPharm **1**:18–33, 1989.
3. Kaufman RJ, Wasley LC, Spiliotes AJ, Gossels SD, Latt SA, Larsen GR, Kay RM. Coamplification and coexpression of human tissue-type plasminogen activator and murine dihydrofolate reductase sequences in Chinese hamster ovary cells. Molec Cell Biol **5**:1750–1759, 1985.
4. Hotchkiss A, Refino CJ, Leonard CK, O'Connor JV, Crowley C, McCabe J, Tate K, Nakamura G, Powers D, Levinson A, Mohler M, Spellman MW. The influence of carbohydrate structure on the clearance of recombinant tissue-type plasminogen activator. Thromb Haem **60**:255–261, 1988.
5. van Zonneveld AJ, Veerman H, MacDonald ME, van Mourik JA, Pannekoek H. Structure and function of human tissue-type plasminogen activator. J Cell Biochem **32**:169–178, 1986.
6. Brower RW, Arnold AER, Lubsen J, Verstraete M. Coronary patency after intravenous infusion of recombinant tissue-type plasminogen activator in acute myocardial infarction. J Am Col Cardiol **11**:681–688, 1988.
7. Bachman F, Kruithof IEKO. Tissue plasminogen activators: Chemical and physiological aspects. Semin Thromb Hemosta **10**:6–17, 1984.
8. Hanss M, Collen D. Secretion of tissue-type plasminogen activator inhibitor by cultured human endothelial cells: Modulation by thrombin, endotoxin and histamine. J Lab Clin Med **109**:97–104, 1987.
9. Wun TC. Plasminogen activator: Biochemistry, physiology and therapeutics. Crit Rev Biotech **8**:131–148, 1988.
10. Rifkin DB, Loeb JN, Moore G, Reich E. Properties of plasminogen activators formed by neoplastic human cell cultures. J Exp Med **139**:1317–1328, 1974.
11. Collen D, Stassen JM, Marafino BJ Jr, Builder S, DeCock F, Ogez J, Tajiri D, Pennica D, Bennett WF, Salwa J, Hoyng CF. Biological properties of human tissue-type plasminogen activator obtained by expression for recombinant DNA in mammalian cells. J Pharm Exp Ther **231**:146–152, 1984.
12. Connors RW, Sweet RW, Noveral JP, Pfarr DS, Trill JJ, Shebuski FJ, Berkowitz BA, Williams D, Franklin S, Reff ME. DHFR coamplification of tPA in DHFR+ bovine endothelial cells: In vitro characterization of the purified serine protease. DNA **7**:651–661, 1988.
13. Kalyan NK, Lee SG, Wilhelm J, Fu KB, Hum WT, Rappaport R, Hartzell RW, Urdane C, Hung PP. Structure-function analysis with tissue-type plasminogen activator. Effect of elimination of NH_2-terminal domains on its biochemical and biological properties. J Biol Chem **263**:3971–3978, 1988.
14. Cartwright T. Isolation and purification of products from animal cells. Trends Biotech **5**:25–30, 1987.
15. Milici AJ, Watrous NE, Stukenbrok H, Palade GE. Transcytosis of albumin in capillary endothelium. J Cell Biol **105**:2603–2612, 1987.
16. Fong K-L, Crysler CS, Mico BA, Boyle KE, Kopia GA, Kopaciewicz L, Lynn RK. Dose-dependent pharmacokinetics of recombinant tissue-type plasminogen activator in anesthetized dogs following intravenous infusion. Drug Met Disp **16**:201–206, 1988.
17. Birrell GB, Hedberg KK, Griffith OH. Pitfalls of immunogold labeling: Analysis by light microscopy, transmission electron microscopy and photoelectron microscopy. J Histochem Cytochem **35**:843–853, 1987.
18. Hoffstein ST, Weissmann G. Mechanisms of lysosomal enzyme release from leukocytes. IV: Interaction of monosodium urate crystals with dogfish and human leukocytes. Arth Rheumat **18**:153–165, 1975.
19. Marks G, Hart TK, Rush GF, Hoffstein ST, Fong K-LL, Bugelski PJ. Internalization of recombinant tissue-type plasminogen activator by isolated hepatocytes is via coated pits. Thromb Haem **63**:251–258, 1990.
20. Larsson Å, Åstedt B. Immunohistochemical localization of tissue plasminogen activator and urokinase in the vessel wall. J Clin Pathol **38**:140–145, 1985.
21. Kristensen P, Nielson JH, Larsson L-I, Danø K. Tissue type plasminogen activators in somatostatin cells of rat pancreas and hypothalamus. Endocrinol **121**:2238–2244, 1987.
22. Tripathi BJ, Geanon JD, Tripathi RC. Distribution of tissue plasminogen activator in human and monkey eyes, an immunohistochemical study. Ophthalmol **94**:1434–1438, 1987.

23. Grøndahl-Hansen J, Lund LR, Ralfkiger E, Ottevanger V, Danø K. Urokinase and tissue-type plasminogen activators in keratinocytes during wound reepithelialization in vivo. J Invest Derm **90**:790–795, 1988.
24. Jeanneau C, Sultan Y. Tissue plasminogen activator in human megakaryocytes and platelets: Immunohistochemical localization, immunoblotting and zymographic analysis. Thromb Haem **59**:529–534, 1988.
25. Jensen PJ, Baird J, Morioka S, Lessin S, Lazarus GS. Epidermal plasminogen activator is abnormal in cutaneous lesions. J Invest Derm **90**:777–782, 1988.
26. Franks AJ, Ellis E. Immunohistochemical localization of tissue plasminogen activator in human brain tumors. Br J Cancer **59**:462–466, 1989.
27. Paul DC, Bobbit JL, Williams DC, Hull RN. Immunocytochemical localization of plasminogen activator on porcine kidney cell strain. LLC-PK_1 (LP_{100}). J Histochem Cytochem **27**:1035–1040, 1979.
28. Hammel I, Elmalek M, Castel M, Kalina M. Variability in gold bead density in cells. Quantitative immunocytochemistry. Histochem **91**:527–530, 1989.
29. Beebe DP. Binding of tissue plasminogen activator to human umbilical vein endothelial cells. Thromb Res **46**:241–254, 1987.
30. Hoal EG, Wilson EL, Dowdle EB. The regulation of tissue plasminogen activator activity by human fibroblasts. Cell **34**:273–279, 1983.
31. Bakhit C, Lewis D, Billings R, Malfroy B. Cellular catabolism of recombinant tissue-type plasminogen activators. J Biol Chem **262**:8716–8720, 1987.
32. Einarsson M, Smedsrød B, Pertoft H. Uptake and degradation of tissue plasminogen activator in rat liver. Thromb Haem **59**:474–479, 1988.
33. Lodish HF. Transport of secretory and membrane glycoproteins from the rough endoplasmic reticulum to the Golgi. A rate-limiting step in protein maturation and secretion. J Biol Chem **263**:2107–2110, 1988.
34. Lingappa VR. Intracellular traffic of newly synthesized proteins. Current understanding and future prospects. J Clin Invest **83**:738–751, 1989.
35. Lippincot-Schwartz J, Bonifiacino JS, Yuan LC, Klausner RD. Degradation from the endoplasmic reticulum: Disposing of newly synthesized proteins. Cell **54**:209–220, 1988.
36. Bole DG, Hendershot LM, Kearney JF. Posttranslational association of immunoglobulin heavy chain binding protein with recent heavy chains in non-secreting and secreting hybridomas. J Cell Biol **102**:1558–1560, 1986.
37. Dorner AJ, Bole DG, Kaufman RJ. The relationship of N-linked glycosylation and heavy chain-binding protein association with the secretion of glycoproteins. J Cell Biol **105**:2665–2674, 1987.