Characterization of $^{125}\text{I}$-Tissue Plasminogen Activator Binding to Cerebellar Granule Neurons

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**Abstract.** Mouse cerebellar cells in culture secrete tissue plasminogen activator (tPA) into the culture medium. Fibrin overlays have shown tPA to be associated with granule neurons in these cultures. This cell associated tPA can be displaced by extensive washing of the cells or by a brief lowering of the pH (<4), which leads to a loss of fibrinolytic activity by the cells. Incubation of these fibrinolytically inactive cells with exogenously added murine tPA leads to the restoration of the fibrinolytic activity, indicating the presence of tPA binding sites on these granule neurons. Using $^{125}\text{I}$-tPA, the binding to cerebellar granule neurons is rapid, saturable, specific, high affinity ($K_d = 50 \text{ pM}$) and reversible. Both murine and human tPA compete with $^{125}\text{I}$-tPA for binding, while both murine and human urokinase (uPA) as well as human thrombin and plasminogen fail to compete. Neither the catalytic site nor the carbohydrate moiety of tPA appear to be involved in the binding, since both diisopropylfluorophosphate-treated tPA and endoglycosidase-H-treated tPA compete with $^{125}\text{I}$-tPA for binding. Furthermore, epidermal growth factor does not compete well with tPA for binding even at a 10:1 molar excess, suggesting that the epidermal growth factor–like (EGF) domain of tPA may not be involved in the binding mechanism. Autoradiography and antibody immunofluorescence show the specific tPA binding is to granule neurons in these cultures. Thus, granule neurons possess tPA receptors on their surface, where this protease binds retaining its functional activity and may play a role in cell and axon migration.

**Materials and Methods**

**Cell Culture**

Cerebella were removed from postnatal day eight mice and the finely diced tissue was dissociated enzymatically in 0.17% trypsin in saline (0.138 M NaCl, 5.4 mM KCl, 1.1 mM Na$_2$HPO$_4$, 1.1 mM KH$_2$PO$_4$) containing 0.4% glucose, 0.01% CaCl$_2$, and 3 μg/ml DNase (United States Biochemical Corp., Cleveland, OH) and incubated at 37°C with constant rotation for 12 rain. The dissociation medium was replaced by basal Eagle’s medium (BEM) containing 10% heat inactivated fetal bovine serum (FCS), and the tissue was dispersed by gentle pipetting and passed through a nylon screen to collect single cells. Cells were collected by centrifugation and enriched for granule cells by adsorption of nonneuronal cells on glass beads during an hour incubation in BEM with 10% FCS. The nonattached cells were aspirated from the medium surrounding the beads and collected by centrifugation. The cells were resuspended in BEM with 10% acid-treated (20) FCS and plated at 200,000 cells/cm$^2$ on poly-lysine coated rectangular plastic coverslips (Miles Scientific, Naperville, IL). Rous sarcoma virus transformed vole (Microtus agrestis) fibroblasts, clone IT (9), were grown in DME containing 5% FCS. Conditioned medium was obtained by rinsing confluent cultures three times with saline 1 and incubating them in serum-free DME for 24 h.

**tPA and Other Proteases**

Murine tPA was isolated from serum-free conditioned medium from Rous sarcoma virus-transformed vole fibroblasts by adsorption on zinc chelate-agarose (Pharmacia Fine Chemicals, Piscataway, NJ) as described by Rijken and Colisen (36), followed by immunoadfinity chromatography using Affigel-linked (Bio-Rad Laboratories, Cambridge, MA) anti-mouse tPA IgG.
The monospecific anti-mouse-tPA was developed in rabbits and the IgG isolated from antiserum on protein A-agarose (Pierce Chemical Co., Rockford, IL) (48). Briefly, pooled tPA fractions from the zinc chelate-agarose column were applied to the immunoaffinity column (0.9 × 3.3 cm) equilibrated with 0.2 M ammonium bicarbonate (pH 7.5) at a flow rate of 0.5 ml/h. The column was extensively washed with the same buffer, the tPA eluted with 0.1 M ammonium formate (pH 3). Fractions containing tPA were neutralized with 17% NH_{4}OH, pooled and lyophilized. A single protein species of approximate M, 70,000 was observed in silver-stained (30) polyacrylamide gels under reducing (not shown) and nonreducing conditions (Fig. 1 a), suggesting the secretion of single-chain tPA molecules into the culture medium by the vole IT fibroblasts.

The tPA was radiolabeled with ^{125}I-Na (Amersham International, Amersham, UK) using Iodo-Beads (Pierce Chemical Co.) for 15 min at 20°C. The free iodine was removed by passing the labeled protein over an exocellose GF-S column (Pierce Chemical Co.), precolumns with 0.1 M Tris-HCl (pH 7.4) containing 1 mg/ml BSA. Only a single radiolabeled species comigrating with murine tPA was seen (Fig. 1 b). DFP-tPA was prepared by treating the tPA with 10 mM diisopropyl-fluorophosphate for 2 h at 20°C; the inactivation was > 97%. Carbohydrate depleted tPA was prepared as described by Little et al. (25).

Mouse urokinase (uPA) was prepared from mouse urine by affinity chromatography on p-aminobenzamidine-agarose (Pierce Chemical Co.) as described by Holmberg et al. (19), followed by passage over an anti-tPA-Affigel column to remove residual tPA.

The amino-terminal 30 kD finger-domain fragment of human fibronectin (the generous gift of Dr. P. McGuire, University of Colorado Health Science Center) was prepared by mild trypsinization, and isolated following its exclusion from gelatin-Sepharose and its specific elution from heparin-Sepharose chromatography according to the procedure of Sekiguchi et al. (40).

Human (melanoma) tPA, reference standard, was obtained from the National Institute for Biological Standards and Control, Hertfordshire, UK. Recombinant tPA (human) was from Genentech Inc. (San Francisco, CA). Human uPA was purchased from Calbiochem-Behring Corp. (San Diego, CA); receptor grade epidermal growth factor was from Collaborative Research, Inc. (Waltham, MA); and human plasminogen was from Kabo (Helena Laboratories, Beaumont, TX). Human zymogen-thrombin was a gift from Dr. J. Fenson (Albany Medical College). Heparin (clinical grade; Upjohn Corp., Kalamazoo, MI) was isolated by precipitation with 3 vol of 95% ethanol, centrifuged, boiled for 10 min, and then placed in ice at 0.25 mg/ml.

**Assays for Plasminogen Activator**

tPA activity was quantified by a chromogenic assay of Gilboa et al. (14), essentially as described by Hawkins and Seeds (17). tPA samples were incubated with plasminogen for 2 h followed by the addition of the chromogenic plasmin substrate Kabl S-2251 for another 2 h.

The fibrin overlayer procedure of Todd (45) was used as modified by Krystosiek and Seeds (22). The cultures were washed three times with saline 1, coated with a drop of thrombin (50 U/ml), followed by a drop of crude fibronectin (75% clottable; ICN Lisle, IL), and drained. The thin fibrin clot was incubated in a moist chamber for 90 min, fixed and stained with Coomasie brilliant blue and mounted for microscopic observation and counting of the fibrinolytic zones.

**^{125}I-tPA Binding Assay**

The binding experiments were performed on 5-6-d cerebellar cultures grown on coverslips. Endogenous cell-bound PA was dissociated by rinsing the coverslips 3 times in saline 1, 0.1 mM CaCl_{2}, 1 mM MgCl_{2}, and placing the coverslips in 50 mM glycine-HCl (pH 3), 0.1 M NaCl for 5 min at 20°C, then neutralizing rapidly with 0.5 M Hepes (pH 7.5), and rinsing 5 times in saline 1 (Ca, Mg). In some studies, the endogenous tPA was displaced by repetitive washings of the cultures in serum-free BEM over several hours. The rinsed coverslips were incubated for 30 min at 20°C with ^{125}I-tPA in binding buffer (BEM containing 15 mM Hepes, 10 Kallikrein inhibitor units/ml aprotinin (Sigma Chemical Co., St. Louis, MO) and 1 mg/ml BSA), maintaining a final volume of 0.1 ml. The cells were rapidly washed 5 times with saline 1 (Ca, Mg) and the cell-associated radioactivity was determined.

Specific binding was defined as the difference between cell-bound radioactivity in the absence and presence of a 50-fold molar excess of nonlabeled tPA. The binding data expressed in molar terms are based on the conversion of IU of tPA to its weight in grams as compared with the specific activity of the second international standard for tPA, 80/6670. Cerebellar cell number was determined on the basis of DNA content (6) of random cultures, using a value of 5 picogram DNA/cell.

**Results**

Plasminogen activator secreted by mouse cerebellar cells in culture is primarily tPA (>90%) with a small amount of uPA (10%). PA activity in the culture medium is highest at day 3 and declines, while zones of fibrinolytic activity increase 4-fold from day 3 to a high at day 6 (50). This observation coupled with the finding that antibody to tPA binds to the surface of granule cell neurons (39), suggested that the fibrinolytic zones may reflect cell bound tPA. This possibility was explored by attempting to displace cell bound tPA by a brief lowering of the pH; a procedure used successfully for uPA bound to fibroblasts (2). Fig. 2 shows that the fibrinolytic activity of the cultures (a) is lost after a brief exposure to low pH (b). The low pH treatment does not inactivate the tPA.
Figure 2. Fibrinolysis by cerebellar cell-bound tPA. Cerebella from 8-d mice were dissociated and cultured for 6 d as described in Materials and Methods. The cells were rinsed and incubated for 5 min at 20°C with 50 mM phosphate buffer (pH 7.4), 0.1 M NaCl (a), or 50 mM glycine-HCl (pH 3), 0.1 M NaCl (b and c). The cells were rinsed in saline 1 and overlaid with a fibrin clot (a and b), or incubated 30 min with 250 mU RSV-vole cell-derived tPA in 0.25 ml Hepes (15 mM) buffered BEM containing 1 mg/ml BSA, and then rinsed and overlaid with a fibrin clot (c). Bar, 50 μm.

but releases tPA into the culture medium where it can be identified by zymography (50). Furthermore, incubation of these low pH-treated cerebellar cells with exogenous murine tPA for 30 min followed by several rinses in BEM shows (c) the restoration of plasminogen-dependent fibrinolytic activity by the culture; indicating cell binding sites for tPA where tPA retains its proteolytic activity.

A more direct assessment of tPA binding to cerebellar cells is shown in Fig. 3 where 125I-tPA displays rapid and specific binding, being readily competed with a 50-fold molar excess of unlabeled murine tPA. The $K_{d}$ is $0.152$ min$^{-1}$ for this binding to cerebellar neurons. The binding is saturable with increasing tPA concentration as seen in Fig. 4 A. Scatchard analysis of these data (Fig. 4 B) shows $^{125}$I-tPA binding to cerebellar cells is of high affinity with a $K_{d}$ of interaction on the order of $5 \times 10^{-11}$ M and $\sim 30,000$ binding sites/cell. The $^{125}$I-tPA binding is reversible with $>75\%$ of the specifically bound $^{125}$I-tPA released by 15 min incubation in tPA-free medium.

 Autoradiography of the cell bound $^{125}$I-tPA shows that the specific binding is localized to small (6 μm) granule neurons that tend to cluster in these cultures, although radiolabeled singlets and doublets are seen (Fig. 5 a). In the presence of a 50-fold molar excess of unlabeled tPA, nonspecific binding of the radioligand is seen (Fig. 5 b) over the coverslip and the larger and more extended glial and epithelial cells, and is not concentrated over the granule neuron clusters as seen in Fig. 5 a. The identity of the radiolabeled small round cells as granule neurons was further substantiated by double label experiments, where an antibody Cbi-1, specific for mouse neurons (37), was included in the binding assay and shown to bind to those same cells that showed high amounts of $^{125}$I-tPA binding (Fig. 6, a and b). Similar results were found with tetanus toxin binding (data not shown). Although the granule neurons often cluster on top of astrocytes, the glial fibrillary acidic protein reactive glia do not bind the $^{125}$I-tPA to their surface (Fig. 6, c and d). Furthermore, the possibility that the tPA was binding not to the cells but to fibronectin in the matrix was ruled out by antibodies to fibronectin, whose binding does not coincide with the binding of $^{125}$I-tPA (Fig. 6, e and f). Thus, tPA binding is a property of granule neurons in these cultures.

The binding of $^{125}$I-tPA to granule neurons is specific for tPA (Table I); neither mouse nor human uPA compete for this tPA binding site. Furthermore, two other serine proteases, thrombin and plasminogen that have been reported to bind to cell surfaces (16, 27), do not effectively compete with tPA binding. As expected from the fibrinolytic results (Fig. 1), tPA binding to the granule cells does not involve the catalytic
site, since DFP-tPA competes with \(^{125}\text{I}\)-tPA for binding. Although tPA with a blocked catalytic site can bind, tPA which loses activity upon standing, by repetitive freeze-thaws or heat denaturation does not compete for binding to these sites. About 7% of tPAs mass is carbohydrate; however, removal of the mannose-rich oligosaccharides with Endo-H has no effect on tPAs binding activity. Furthermore, epidermal growth factor, a molecule that shares structural and sequence homology with tPA (3) shows very little competition for binding even at a 10-fold molar excess. Heparin, which is known to bind to tPA and enhance its activity (1), has also been implicated in the cell binding of several proteins (26); however, heparin has little apparent effect on tPA binding to granule neurons. The amino-terminal fibronectin-like finger domain of tPA has been implicated in tPAs interaction with fibrin (3, 46). Therefore, the finger domain of human fibronectin was tested for its ability to compete with human \(^{125}\text{I}\)-recombinant tPA (whose binding properties are similar to that of murine tPA). A 100-fold molar excess of this fibronectin finger region showed <20% decrease in the specific binding of tPA (data not shown).

Unless bound ligand is displaced by lowering the pH, very
Figure 6. Neuronal cell surface labeling and $^{125}$I-tPA binding. Cell identity and $^{125}$I-tPA binding were monitored simultaneously by immunofluorescent antibody (a, c, and e) and bright field autoradiography (b, d, and f) of the same fields. Rabbit anti-Cbl-1 (a), a neuron-specific cell surface marker, was used to visualize cerebellar neurons in these cultures, and this antigen colocalized with $^{125}$I-tPA binding (b). tPA binding (d) was not associated with astrocytes (c, glial fibrillary acidic protein). Very little fibronectin is seen in cerebellar cultures as visualized with rabbit anti-fibronectin (e) and fibronectin did not colocalize with the $^{125}$I-tPA binding (f). Bar, 15 μm.

little $^{125}$I-tPA binds to 6-d cerebellar cell cultures; suggesting that the receptors are saturated under these conditions. However, endogenously bound tPA should be freely reversible and subject to dilution of the free-tPA concentration. Repetitive media changes bring about a reduction in the free-tPA concentration, such that bound tPA is released into the medium and $^{125}$I-tPA binding to these cells increases (data not shown). Similarly, Fig. 7 shows the effect of diluting the free-tPA concentration on release of cell-bound tPA. Released tPA is seen as an increase in tPA in the medium as assessed by the amidolytic assay, while there is a parallel loss of cell-bound tPA activity as indicated by the decrease in the relative number of fibrinolytic zones associated with the cells. The dissociation rate constant ($k_d$) is 0.10 min$^{-1}$ for the reversibility of endogenously bound tPA, when combined with the association constant $k_a = 7 \times 10^8$ M$^{-1}$ min$^{-1}$ calculated from $K_a$ (Fig. 3), the $K_d$ (70 pM) obtained from these kinetic data agrees well with the $K_d$ obtained from the Scatchard plot (Fig. 4).

Discussion

These studies have demonstrated the specific binding of tPA to the surface of developing granule neurons. This binding is rapid, saturable, reversible, and of high affinity; furthermore, tPA interacts with the cell surface in such a manner that tPA retains its full proteolytic activity. This localization of tPA to the cell surface provides an optimal site to facilitate cell movement.

Since DFP-tPA competes for the tPA receptor, the catalytic site is not involved in this binding, contrary to other reports of high affinity binding to endothelial cells where tPA is bound covalently via the active site, presumably to a plasminogen activator inhibitor (4, 5). The most likely portion of the tPA molecule to be involved in cell binding is the amino terminal one-half. This proposal is supported by our observation that only the 65,000-Mr form of tPA is displaced from the cells by low pH, although the 32,000-Mr catalytic carboxyl one-half of the tPA is seen in the culture medium (50). The amino-terminal portion of the tPA molecule contains several domains that are shared by other proteins and proteases (31). The finger domain characteristic of fibronectin (3), the growth factor domain that is similar to epidermal growth factor and is also found in uPA (3), the kringles (Nos. 1 and 2), similar to those in plasminogen and uPA (32, 35), are all candidates for this binding interaction; however, the failure of epidermal growth factor, uPA and plasminogen to compete effectively for binding make the

Table I. Specificity of $^{125}$I-tPA Binding to Cerebellar Neurons

| Nonlabeled protein     | Amount | Molarity | Binding % |
|------------------------|--------|----------|-----------|
| None                   | 0      | 0        | 100       |
| Murine tPA             | 32.5 IU| 11.5 nM  | 0         |
| Human tPA              | 32.5 IU| 11.5     | 0         |
| Murine urokinase       | 32.5 IU| 11       | 106       |
| Human urokinase        | 32.5 IU| 10       | 110       |
| Human thrombin         | 36 ng  | 11.5     | 94        |
| Human plasminogen      | 110 ng | 11.5     | 78        |
| Murine DFP-tPA         | 75 ng  | 11.5     | 5         |
| Murine denatured tPA   | 75 ng  | 11.5     | 100       |
| Deglycosylated tPA     | 32.5 IU| 11.5     | 0         |
| Heparin                | 24 ng  | 11.5     | 93        |
| Epidermal growth factor| 0.15 ng| 0.23     | 102       |
| Epidermal growth factor| 1.5 ng | 2.3      | 86        |

Cerebellar cells were stripped of endogenous PA by mild acid treatment, washed, and incubated for 30 min at 20°C with murine $^{125}$I-tPA (0.65 IU = 1.3 ng = 0.23 nM) in the absence or presence of an excess of nonlabeled competitors, as indicated. Percentage of (specific) binding was calculated relative to inhibition by a 50-fold molar excess of active nonlabeled tPA.
ous localization of fibrinolytic zones to only granule neurons in cerebral cultures. This finding agrees well with our previous reports in its high affinity for tPA, reversibility and retention of enzymatic activity.

The Journal of Cell Biology, Volume 109, 1989

...may represent only those granule neurons actively involved in cell migration from the external to the internal granule cell layer of the cerebellum. This proposal is supported by observations of granule neurons migrating on neural fascicles in culture that bind antibody to tPA (39). If only migrating granule cells have cell bound tPA, this adds an important new level of regulation to the PA-PA inhibitor (15, 21, 22, 28, 29, 33, 34, 42) system; therefore, regulation of tPA receptor synthesis and membrane insertion may be the key steps.

The presence of tPA receptors on granule neurons may be disadvantageous to the cell for several reasons. Cell binding prevents diffusion of tPA away from the granule neuron. The tPA bound to the granule neuron receptor may be protected from inactivation by protease inhibitors present in the extracellular environment, as has been demonstrated for macrophage uPA (8) and monocytes (44). Furthermore, the possibility should not be overlooked that tPA may interact with its receptor in an autocrine system to elicit some second-messenger response within the cell. The tPA receptor provides a mechanism whereby cells can "arm themselves" with functionally active protease. Furthermore, the receptor may permit both a temporal regulation as to when a cell may have this activity, and a spatial regulation to provide for highly localized and concentrated areas of extracellular protease activity at sites of cell-cell and cell-matrix interaction. Thus, the process of cell and axonal migration may be regulated in part by the presence of a membrane tPA receptor and its occupancy by tPA.

The authors would like to express appreciation of Dr. P. G. McGuire's gift of the fibronectin finger domain, and to thank Ms. K. Christensen and Ms. S. Haffke for their excellent technical assistance.

These studies were supported in part by grants from the National Institutes of Health (NS-09818), and the National Science Foundation (BNS-86-07719). Some of these studies have been submitted by S. Verrall in partial fulfillment of the requirements for the Ph.D. degree at the University of Colorado Health Science Center.

Received for publication 22 November 1988 and in revised form 14 March 1989.

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