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Spatial Orientation of Tissue-Type Plasminogen Activator Bound at the Melanoma Cell Surface[1]

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Human melanoma cells produce tissue-type plasminogen activator (tPA) which is bound to the cell surface where it effectively mediates generation of plasmin. The present study is focused on analysis of involvement of the tPA domains in binding of the enzyme to the cell surface. The extent of plasminogen activation by tPA of melanoma cells was measured using an immunocapture assay. The activator anchored to solid surface via monoclonal antibodies directed to the individual domains of the activator exhibited variable enzymatic activity. The tPA was the most effective when bound by the antibodies against kringle-1 or kringle-2. Accessibility of the epitopes within cell surface-bound tPA was probed by the same set of monoclonal antibodies. FACS analysis showed that the epitopes within the finger/growth factor domain, one part of the kringle-2 domain and the active site epitope were the most exposed. The kringle-1 domain epitope and the protease region epitope appeared partially exposed. Full-length melanoma-derived tPA and three recombinant domain-deletion variants of tPA were compared for their capacity to bind to the melanoma cells. The estimated IC_{50} value for the melanoma-derived tPA was 2.3 ± 0.25 μM. Comparable IC_{50} values were found for the tPA variant lacking the finger domain (3.6 ± 0.6 μM) as well as for the variants consisting only of the kringle-2 and protease domains (7.5 ± 0.45 μM). In contrast the value found for a tPA variant lacking the kringle-2 domain was >100 μM. The consistent results obtained by the three different experimental approaches provide evidence that tPA binds to melanoma cells via its kringle-2 domain but binding sites within kringle-1 domain and protease domain may support the interaction. The finger domain did not contribute to the binding.

Tissue-type plasminogen activator (tPA) is a serine protease that plays an essential role in the fibrinolytic system (1,2). In addition, we and others have demonstrated previously that tPA secreted by human melanoma cells is bound to the cell surface where it effectively mediates generation of cell-bound plasmin (3,4) which is used for matrix degradation and consequently supports melanoma cell invasion (5). Based on the mentioned results and the recent immunohistological studies where elevated levels of tPA were found in human melanomas in situ (6) this particular histological type of tumor is being designated as "tPA tumor" (7).

Several cellular binding sites for tPA have been described; however, the biochemical nature of these sites remains insufficiently characterized. Related studies have been focused in two directions. The first one is to identify a receptor which is responsible for the rapid clearance of tPA from circulation (8,9). A mechanism involved proposes the interaction of tPA with its primary physiological inhibitor, plasminogen activator inhibitor type-1 (PAI-1) (10). The formed complex or free tPA is endocytosed and degraded in the lysosomes after binding to the low-density lipoprotein receptor-related protein (LRP)/α_2-macroglobulin (α_2M) receptor (11,12). The second series of experiments represent studies designed to identify tPA-binding protein(s) with an analo-

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Abbreviations: tPA, tissue-type plasminogen activator; FG, finger/growth factor domain; K1, K2, kringle-1, -2 domains, P, protease domain; LRP, low density lipoprotein receptor-related protein; α_2M, alpha-2-macroglobulin; RAP, receptor-associated protein; PAI-1, plasminogen activator inhibitor type-1; uPAR, urokinase receptor; MAb, monoclonal antibody.

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gous function to urokinase receptor (uPAR). Several earlier biochemical studies proved binding of tPA to immobilized extracellular matrix proteins like laminin and fibronectin (13,14). Numerous analyses using rather traditional techniques such as immunoaffinity chromatography or ligand overlay assay have demonstrated interactions with varying affinities between tPA and certain proteins mostly from endothelial cells (15,16). Annexin II, which is basically intracellular protein, has been described as a tPA receptor (17). In spite of approval of its external surface localization by antibody-based technologies the evidence for its cell surface expression is being disputed. These studies are mostly performed along with identification of cellular binding sites for plasminogen (17,18).

Mature tPA is a complex glycoprotein with molecular mass ~67 kDa that is composed of a series of five discrete, putative, structurally autonomous domains (19). From the N-terminus of the molecule, a finger-like domain (F), an epidermal growth factor-like domain (G), two kringle domains (K1 and K2) and a protease domain (P) can be discerned.

The purpose of the present study was to determine which domain(s) of the tPA molecule are involved in the interaction with the melanoma cell surface. Firstly, using a microplate immunocapture assay we have estimated plasminogen activation by melanoma secreted tPA bound to solid phase by different monoclonal antibodies directed to individual domains of the enzyme. Then using FACS analysis and the same set of monoclonal antibodies we have determined the accessibility of respective epitopes on the cell-bound tPA molecule. Finally, we have performed binding experiments of radioiodinated melanoma-derived tPA as well as three domain deletion tPA mutants to cultured melanoma cells and estimated the IC_{50} values. The results demonstrate that the K2 domain is mainly responsible for the specific binding of the tPA to intact melanoma cells in culture.

MATERIALS AND METHODS

Cell cultures. The Bowes human melanoma cell line was a gift from Dr. D. Collen, Katholieke Universiteit, Leuven, Belgium. The stock culture was grown in MCDB#153 medium (Sigma, St. Louis, MO), supplemented with 0.5% (v/v) fetal calf serum (FCS) and antibiotics and were subcultured every two weeks by brief exposure to 0.25% (w/v) trypsin/5 mM EDTA. The cell line was periodically monitored for mycoplasma contamination and was found to be negative. The cells used in the present experiments were continuously cultured in serum-free medium. The cells under serum-free conditions retained the characteristic production of tPA (20) and binding of tPA to the cell surface (21).

tPA assay. Melanoma-derived tPA (md-tPA) present in conditioned medium from Bowes cells was assayed for the capacity to activate plasminogen by the following modification of an immunocapture method (22). Microtiter wells of polystyrene immunoplates (Costar, E.I.A./R.I.A. Plate #3590, Cambridge, MA) were coated with 50 μl of a solution of monoclonal antibodies (MAbs) against-human tPA (MAbs #3700, #3704, #3707, PAM-2, PAM-3, ESP-2; American Diagnostica, Greenwich, CT). All selected MAbs were of IgG_1 subclass. The coating solution contained 2.5 μg of IgG/ml in 0.1 M sodium carbonate, pH 9.8. After rinsing, the wells were treated with serum-free conditioned medium from melanoma cell cultures. To assay the enzyme activity bound to the wells, 40 μl of a solution of human plasminogen (23) with an assay buffer containing 50 mM Tris-HCl, pH 7.8, 60 mM NaCl, 0.01% (v/v) Triton X-100, and 20 μg/ml poly-D-lysine was added and the mixture incubated for 30 min. The plasmin produced was then assayed by its thioesterase activity after the addition of 150 μl of a solution containing 200 mM potassium phosphate (pH 7.5), 200 mM KCl, 0.1% (v/v) Triton X-100, 200 μM Z-lysine thiobenzyl ester (Peninsula Laboratories, San Carlos, CA) and 220 μM 5,5′-dithiobis (2-nitrobenzoic) acid (DTNB; Sigma). The absorbances of the wells were measured in a Labsystems Multiskan MCC/340 plate reader at 405 nm.

The total amount of tPA bound by respective MAb was estimated in simultaneously coated plates by 15 nM ^{125}I-tPA (see below). The plates were incubated and treated as above. Bound radioiodinated tPA was recovered from the wells by solution of 5% NaOH, 1% SDS in water. The radioactivity of the samples was determined in a g-counter and tPA concentration calculated.

FACS analysis. Expression of tPA at the melanoma cell surface was analyzed by flow cytometry. Bowes cells cultured under serum-free conditions were harvested from culture flasks by phosphate-buffered saline (PBS)/5 mM EDTA containing 0.1% (w/v) NaN_3. Single cell suspension was prepared by pipetting and washed twice with PBS. Aliquots of 1.5×10^6 cells in 200 μl were incubated for 30 min on ice with the primary anti-tPA MAbs (American Diagnostica). An irrelevant mouse monoclonal IgG_1 (MOPC 21) from Sigma Immuno-

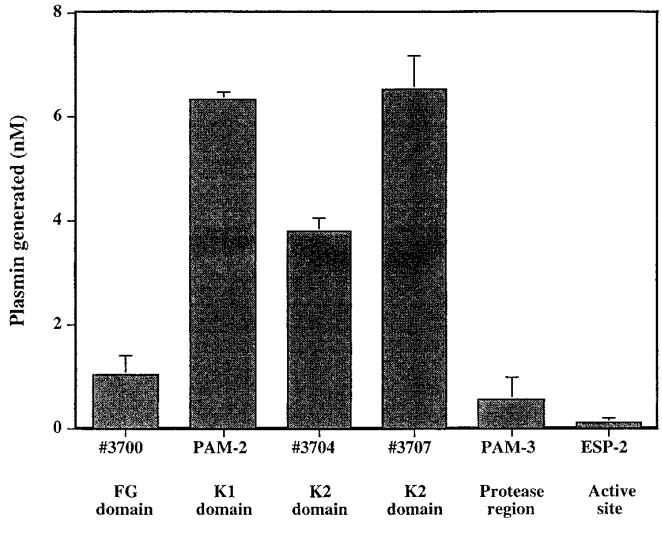

FIG. 1. Plasminogen activation by tPA bound to solid-phase by different domain-specific MAbs. Microtiter wells were first coated with a solution of anti-tPA antibodies. After blocking with albumin and washing, serum-free Bowes melanoma cell-conditioned medium (50 μl) was applied to the wells and the activator allowed to bind for 2 h. The unbound medium components were washed away and bound tPA assayed by a colorimetric assay. The total amount of tPA bound by the individual MAb to the wells was estimated by ^{125}I-tPA in simultaneously coated and treated plates. The results are expressed as the amount of generated plasmin (nM) per 10^{-4} nmol of bound tPA to the well. Each value represents the average ± S.E. of three separate experiments.

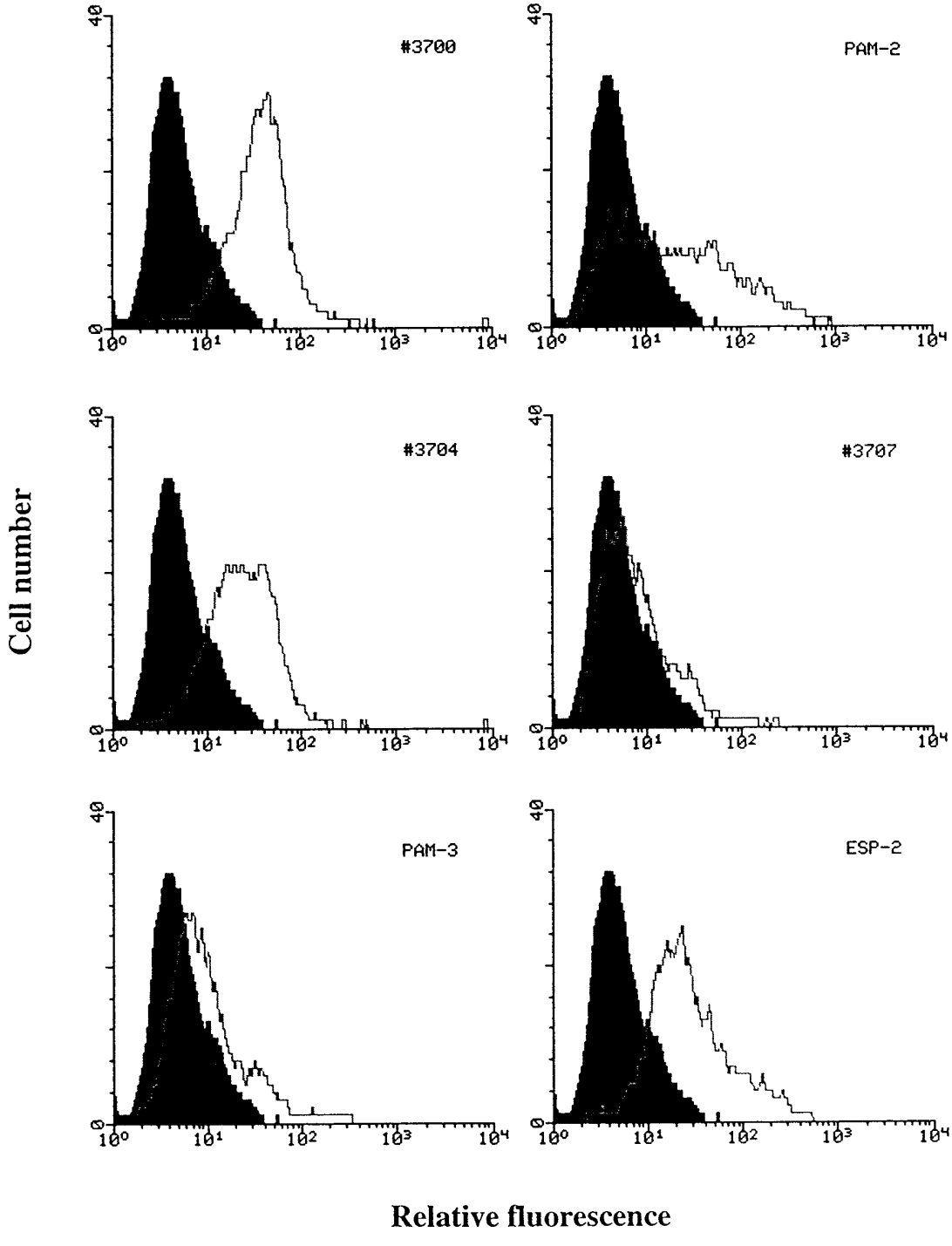

FIG. 2. Histogram of FACS analyses of Bowes melanoma cells incubated with anti-tPA antibodies. Aliquots (1.5×10^6) of Bowes cells were incubated with the individual MAbs that distinguish specific epitopes within each tPA domain. The histogram is an example of the cytofluorometric profiles of representative experiment. Relative cell numbers and immunofluorescence intensities are exhibited on vertical and horizontal axes, respectively. Black profiles represent staining by an irrelevant, isotype-matched MAb, white profiles represent staining with respective anti-tPA MAb followed by FITC-conjugated anti-mouse IgG.

chemicals (St. Louis, MO) was used as a control. The cells were washed again and 50 μl FITC-conjugated goat F(ab′)2 anti-mouse IgG(H+L) (5 μg/ml) (Protos Immunoresearch, San Francisco, CA) was added and the reaction mixtures were further incubated for 20 min on ice in the dark. After being washed, the cells were resuspended in 500 μl of PBS and analyzed by flow cytometry in a Fluorescence Activated Cell Sorter (FACScan™, Becton Dickinson, Sunny Wall, CA).

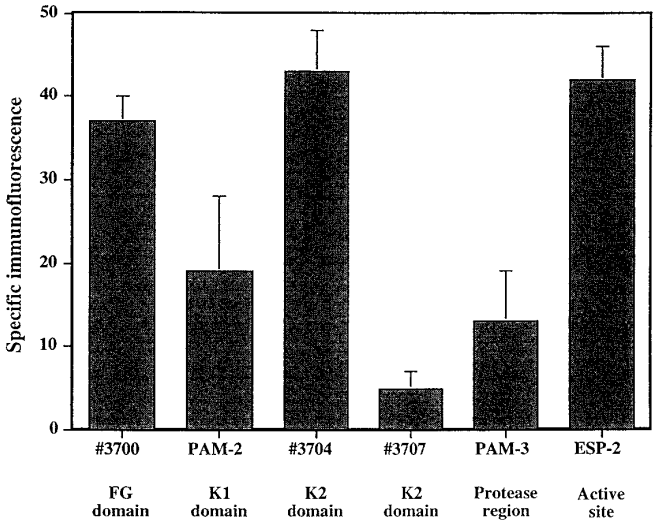

FIG. 3. Comparison of specific immunofluorescence determined for the epitopes within individual domains of surface-bound tPA. Values of specific fluorescence were calculated as the difference between mean fluorescence value measured for the respective domain-specific MAb and mean fluorescence value estimated for the irrelevant isotype-matched MAb from the analysis data obtained as shown in Fig. 3. Each value represents the average ± S.E. of three separate experiments.

tPA and tPA domain deletion mutants. Single-chain tPA (isolated from Bowes melanoma cells) was purchased from American Diagnostica. Recombinant tPA (Actilyse) was obtained from Genentech, San Francisco, CA. The tPA domain deletion mutants GK1K2P, FGK1P and K2P were produced in CHO cells after transfection with expression plasmids as described (24). The recombinant tPA mutants were purified by immunoaffinity chromatography using a monoclonal antibody against the protease domain of tPA coupled to agarose. The enzymatic activity of the tPA mutants was analyzed in detail (25).

Radiolabeling of tPA. Md-tPA and tPA variants were iodinated by the Iodogen method according to the manufacturer's instructions (Pierce Chemical Co., Rockford, IL). Radiolabeled proteins were bound on a 1 ml column of Zn-chelate-Sepharose and washed extensively with 0.02 M Tris-HCl, pH 7.4, 1 M NaCl and 0.01% (v/v) Tween 80, and then eluted with the same buffer containing 100 mM imidazole (Merck, Darmstadt, Germany), resulting in specific radioactivity of between 5000 and 9500 cpm/ng of protein.

Binding studies. For binding studies of radiolabeled tPA to Bowes cells, 1 ml aliquots (10^6 cells) of single cell suspension were prepared in MCDB#153 medium containing 2% (w/v) BSA. Cells were then incubated with 10^6 cpm of respective radiolabeled ligand in the presence or absence of the competitor, human tPA (Actilyse) used in the range of concentration from 37.5 nM to 22.5 μM. Cell incubations were performed for 2 h at +4°C and then the cells were washed twice with 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM $CaCl_2$ plus 0.2 % (w/v) BSA and once with the same buffer without BSA. Finally cells were resuspended in the same buffer without BSA and cell-bound radioactivity was measured in a g-counter. The concentration of competitor needed to block ligand binding by 50% (IC_{50}) was calculated from the inhibition curves using a computerized non-linear fitting program (Prism, Graphpad Software Inc., San Diego, CA) as described (26).

RESULTS

Previously we observed that tPA secreted by various human melanoma cells is bound to the cell surface where it effectively mediates generation of cell-bound plasmin (3). Based on these studies we used the Bowes cell line for further investigations (21) as these cells did not produce α_2M or PAI-1, inhibitors which could interfere with the tPA activity on the cell surface. This was in accordance with our study where we did not observe internalization of α_2M in human melanoma cell cultures (27). No binding of the receptor associated protein (RAP), a ligand for LRP/α_2M receptor, was found (data no shown). Use of serum-free cultures of Bowes cells enabled us also to avoid interference of plasminogen and the extracellular matrix proteins present in serum which were known to bind tPA (13, 14).

In continuing series of experiments we have recently demonstrated that a MAb directed to the K2 domain of tPA as well as an anti-catalytic MAb prevented very efficiently activation of plasminogen on the surface of Bowes cells (21). These data raised the possibility that the K2 domain could play an important role in the process of tPA-mediated plasminogen activation at cell surface.

Now using a set of six selected MAbs, first, employing an immunocapture assay we have investigated plasminogen activation by Bowes cell-secreted tPA bound to a solid phase via each individual MAb. We proposed that the immobilized MAb to a certain extent mimics the natural receptor for tPA and in this way allows us to asses the enzymatic activity of tPA in different stereospecific orientations. The MAbs were adsorbed to the surface of multiwell plates and nonspecific binding was prevented by BSA as described in "Materials and Methods". Serum-free conditioned medium from the melanoma cells, containing tPA, was applied to the wells and after extensive washing human plasminogen was added. The generated plasmin was quantitated using a specific substrate. The total amount of tPA bound by the respective MAb was estimated with ^{125}I-tPA applied in the same volume as above to the simultaneously prepared plates. Fig. 1 demonstrates that the efficiency of surface-bound tPA to generate plasmin is dependent on the antibody used. The tPA was enzymatically the most effective when it was anchored via the K1 or the K2 domain. In the case of the latter domain two different monoclonal antibodies were employed. The MAb #3704 was less effective by >35 % than the MAb #3707. Significantly less enzymatic activity of tPA was obtained if it was anchored via the FG domain, the protease region or the active site.

Then using FACS analysis we probed the accessibility of the epitopes within tPA produced by Bowes cells and spontaneously bound to the surface of these intact cells. The histogram of a representative FACS analysis is shown in Fig. 2 (see Legend to the figure). Fig. 3 demonstrates the values of specific fluorescence calculated as the difference between mean fluorescence value estimated for domain-specific MAb and mean

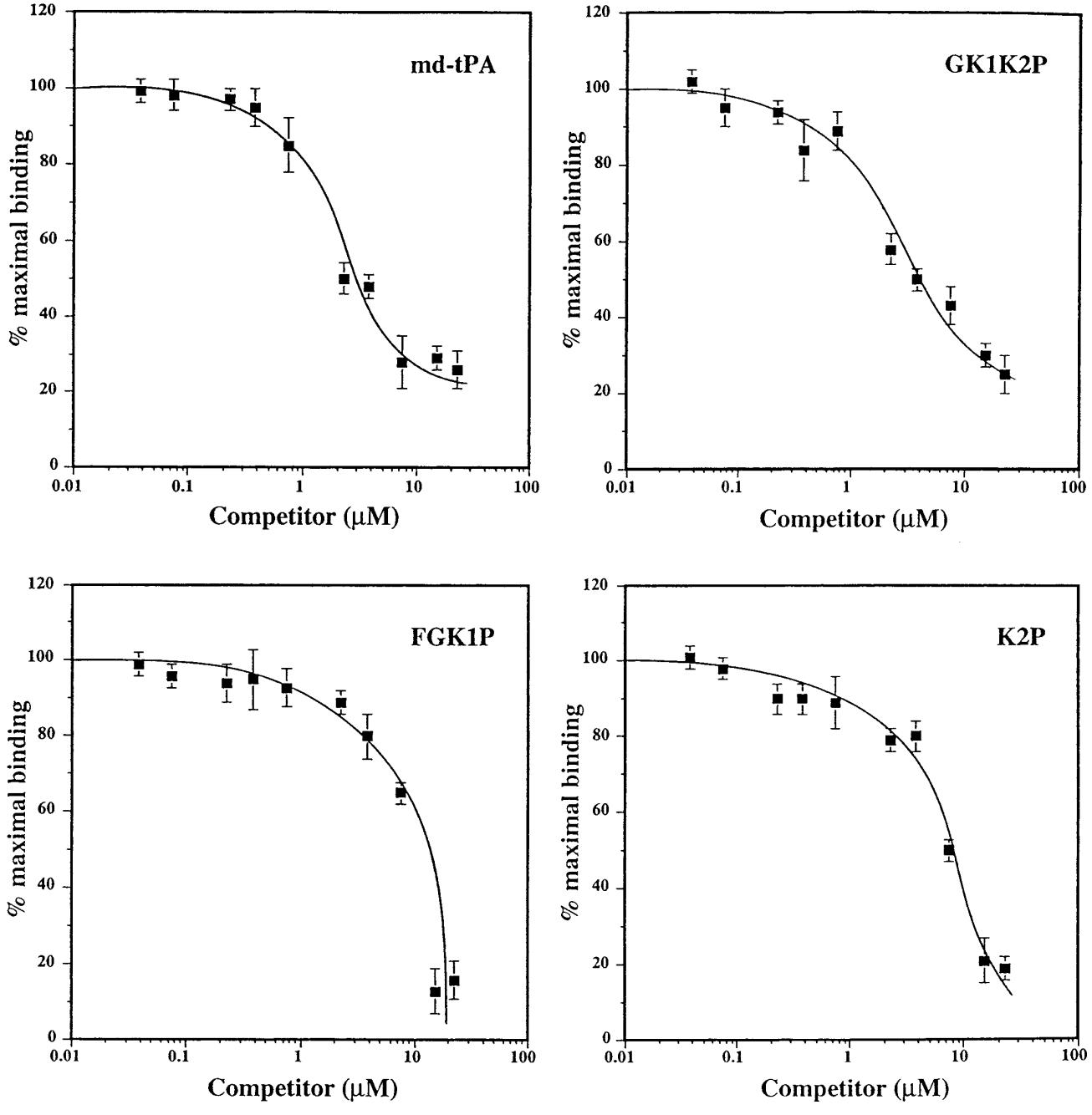

FIG. 4. Effect of unlabeled recombinant tPA on the binding of melanoma-derived ^{125}I-labeled tPA and ^{125}I-labeled domain deletion tPA variants to Bowes cells. 1×10^6 cells in 1 ml aliquots were incubated for 2 h at 4°C with 100 pM ^{125}I-labeled respective ligand: md-tPA, GK1K2P, FGK1P or K2P in the presence of unlabeled control tPA (Actilyse). At the end of the incubation the cells were washed and cell-bound radioactivity was determined. Data are expressed as percentage of the binding in control sample without competitor. Each point represents the average ± S.E. of four replicate determinations in a single study.

fluorescence value for an irrelevant isotype-matched MAb. The data show that epitopes within FG domain, part of the K2 domain, recognized by MAb #3704, and the active site epitope were the most exposed on cell-surface bound tPA. The K1 domain epitope and the protease region epitope appeared only partially exposed. The second epitope in the K2 domain, recognized by MAb #3707, was the least exposed of all analyzed epitopes.

The affinities of the binding of md-tPA and three tPA domain deletion variants by the capacity of control recombinant tPA to displace the binding were deter-

mined. Fig. 4 demonstrates the results. Both md-tPA and two variants studied could be displaced with similar concentration of the competitor as comparable IC_{50} values, varying from 2.3 ± 0.25 μM for md-tPA to 3.6 ± 0.6 μM for GK1K2P and 7.5 ± 0.45 μM for K2P, respectively were estimated. The interaction of FGK1P variant with cell surface exhibited completely different characteristics as IC_{50} value was not gained within the used competitor concentrations (from 37.5 nM to 22.5 μM). The theoretically calculated value by curve fitting suggested an IC_{50} >100 μM. These data indicated that the variant lacking the K2 domain interacts with low specificity and reinforced the importance of the K2 domain in the binding of tPA to melanoma cell surface.

DISCUSSION

Several lines of evidence suggest that cell surface activation of plasminogen in human tumor cells as well as in certain normal counterparts depends on appropriate assembly of the components taking part in the process at the cell surface (28,29). Most types of human adherent cells use urokinase to generate plasmin. Melanoma cells, as well as neuroblastoma cells, are known to produce mainly tPA (29) and bind it to their surfaces in a process where a lysine-binding mechanism appears to be involved (21) but a clear characterization of a specific receptor protein, as in the case of uPA (30) has not been accomplished.

The present study was undertaken to identify the domains on the tPA molecule that interact with its binding site on human melanoma cells. We employed three different approaches. In the first one MAbs were used to mimic the tPA-binding protein and it was evident that anchoring the activator via both kringles accelerates plasminogen activation much more efficiently than via binding to the FG domain or protease domain.

In accordance with this result, the FACS analysis revealed that the epitopes recognized within the FG domain or active site did not favor tPA binding as they were most exposed on cell surface bound tPA. Both techniques provide evidence that part of K2 recognized by MAb #3707 could contain a binding motif responsible for the interaction of tPA with the cell surface. The third approach demonstrated that interaction of tPA with its surface binding protein has an affinity at least 3 orders of magnitude lower than that of the interaction of uPA with its receptor, uPAR (~3 μM for tPA versus 2 nM for uPA). Also the tPA variant lacking the K2 domain exhibited significantly lower affinity than the K2P variant. Taken together, these results as well as our previous observation that the binding of tPA was prevented by e-aminocaproic acid, provide further evidence that tPA interacts with the cell surface via the K2 domain involving the lysine-binding site. A number of previous studies from different laboratories have elucidated the structure-function relationships of individual domains of human tPA and on the basis of molecular modeling; the lysine binding site of the tPA molecule has been localized to the K2 domain (31,32). Our results at the cellular level using melanoma cells are in accordance with these studies.

In conclusion, our present results provide evidence in favor of a role of the K2 domain in the interaction of tPA with its binding protein at the surface of human melanoma cells. The results also suggest that the K1 and protease region can support the interaction. The present results may be helpful not only for a better understanding of the precise function of tPA in the process of cell surface plasminogen activation but may assist in the design of further studies to isolate and identify the putative tPA cell surface receptor. These type of data should yield important insight into the progression of human melanomas and malignant tumors in general.

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REFERENCES

1. Bu, G., Williams, S., Strickland, D. K., and Schwartz, A. L. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89,** 7427–7431.
2. Lijnen, H. R., and Collen, D. (1991) *Thromb. Haemostasis* **66,** 88–100.
3. Bizik, J., Lizonova, A., Stephens, R. W., Grofova, M., and Vaheri, A. (1990) *Cell Reg.* **1,** 895–905.
4. Meissauer, A., Kramer, M. D., Schirrmacher, V., and Brunner, G. (1992) *Exp. Cell Res.* **199,** 179–190.
5. Alizadeh, H., Ma, D., Berman, M., Bellingham, D., Comerford, S. A., Gething, M. J. H., Sambrook, J. F., and Niederkorn, J. Y. (1995) *Curr. Eye Res.* **14,** 449–458.
6. De Vries, T. J., Quax, P. H. A., Denijn, M., Verrijp, K. N., Verheijen, J. H., Verspaget, H. W., Weidle, U. H., Ruiter, D. J., and Van Muijen, G. N. P. (1994) *Am. J. Pathol.* **144,** 70–81.
7. De Vries, T. J., Van Muijen, G. N. P., and Ruiter, D. J. (1996) *Melanoma Res.* **6,** 79–88.
8. Camani, C., and Kruithof, E. K. O. (1995) *J. Biol. Chem.* **270,** 26053–26056.
9. Kuiper, J., Van't Hof, A., Otter, M., Biessen, E. A. L., Rijken, D. C., and van Berkel, T. J. C. (1996) *Biochem. J.* **313,** 775–780.
10. Morton, P. A., Owensby, D. A., Wun, T. C., Billadello, J. J., and Schwartz, A. L. (1990) *J. Biol. Chem.* **265,** 14093–14099.
11. Bu, G., Maksymovitch, E. A., and Schwartz, A. L. (1993) *J. Biol. Chem.* **268,** 13002–13008.
12. Willnow, T. E., Orth, K., and Herz, J. (1994) *J. Biol. Chem.* **269,** 15827–15832.
13. Salonen, E. M., Zitting, A., and Vaheri, A. (1984) *FEBS Lett.* **172,** 29–32.
14. Salonen, E. M., Saksela, O., Vartio, T., Vaheri, A., Nielsen, L. S., and Zeuthen, J. (1985) *J. Biol. Chem.* **260,** 12302–12307.
15. Barnathan, E. S., Kuo, A., van der Keyl, H., McCrae, K. R.,

Larsen, G. R., and Cines, D. B. (1988) *J. Biol. Chem.* **263,** 7792–7799.

16. Russell, M. E., Quertermouse, T., Declerck, P. J., Collen, D., Haber, E., and Homcy, C. J. (1990). *J. Biol. Chem.* **265,** 2569–2575.
17. Hajjar, K. A., Jacovina, A. T., and Chacko, J. (1994) *J. Biol. Chem.* **269,** 21191–21197.
18. Hembrough, T. A., Li, L., and Gonias, S. (1996) *J. Biol. Chem.* **271,** 25684–25691.
19. Pennica, D., Holmes, W. E., Kohr, W. J., Hrkins, R. N., Vehar, G. A., Ward, C. A., Bennet, W. F., Yelverton, E., Seeburg, P. E., Heyneker, H. L., Goeddel, D. V., and Collen, D. (1983) *Nature (London)* **301,** 214–221.
20. Kruithof, E. K. O., Schleuning, W. D., and Bachmann, F. (1985) *Biochem. J.* **226,** 631–636.
21. Bizik, J., Stephens, R. W., Grofova, M., and Vaheri, A. (1993) *J. Cell. Biochem.* **51,** 326–335.
22. Stephens, R., Leung, K. C., Pöllänen, Salonen, E. M., and Vaheri, A. (1987) *J. Immunol. Meth.* **105,** 245–251.
23. Deutsch, D. G., and Mertz, E. T. (1970) *Science* **170,** 1095–1097.
24. Bakker, A. H. F., Weening-Verhoeff, E. J. D., and Verheijen, J. H. (1995) *J. Biol. Chem.* **270,** 12355–12360.
25. Bakker, A. H. F., van de Greef, W., Rehberg, E. F., Marotti, K. R., and Verheijen, J. H. (1993) *J. Biol. Chem.* **268,** 18496–18501.
26. Kuiper, J., Rijken, D. C., De Munk, G. A. W., and van Berkel, T. J. C. (1992) *J. Biol. Chem.* **267,** 1589–1595.
27. Bizik, J., Vaheri, A., Saksela, O., Kalkkinen, N., Meri, S., and Grofova, M. (1986) *Int. J. Cancer* **37,** 81–88.
28. Pöllänen, J., Stephens, R. W., and Vaheri, A. (1991) *Adv. Cancer Res.* **57,** 273–328.
29. Vaheri, A., Tapiovaara, H., Myöhänen, H., and Bizik, J. (1994) *N. Y. Acad. Sci.* **737,** 409–418.
30. Roldan, A. L., Cubellis, M. V., Masucci, M. T., Behrendt, N., Lund, L. R., Dano, K., Appella, E., and Blasi, F. (1990) *EMBO J.* **9,** 467–474.
31. De Vos, A. H., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L., and Kossiakoff, A. A. (1992) *Biochemistry* **31,** 270–279.
32. De Serrano, V. S., and Castellino, F. J. (1993) *Biochemistry* **32,** 3540–3546.