Fish Plasminogen Activators: Their Identification and Characterization

Kei Takahashi¹, Yuko Wakamatsu², Kenjiro Ozato², and Yuji Wakayama³

¹Department of Physiology, Shimane Medical University, Izumo 693, ²Biological Laboratory, Yoshida College, Kyoto University, Kyoto 606, and ³Division of Internal Medicine, Central Hospital, Izumo 693, Japan

ABSTRACT. Immunoblots of proteins extracted from the skin of a small viviparous fish (*Xiphophorus*) showed that a monoclonal antibody against human urokinase recognizes multiple molecular weight species of antigens. The immunoaffinity-purified antigens had serine-protease activity for the hydrolysis of a chromogenic substrate and could convert human plasminogen to plasmin in a manner similar to that for human urokinase *in vitro*. Two antigens with apparent molecular weights of 55 and 50 kilodaltons that had been purified on a fibrin-Celite column were separable on SDS-polyacrylamide gels and were characterized as major plasminogen activators on fibrin-agar indicator plates. The ¹²⁵I-tryptic peptide maps of both antigens were similar to that of human urokinase; therefore, the fish activators and human urokinase are structurally and functionally related.

Plasminogen activators are comprized of a group of serine-type proteases and convert plasminogen into active plasmin which is responsible for the dissolution of thrombi *in vivo* (13). Several activators with different molecular weight forms have been investigated (28, 29, 33) and can be classified as urokinase and tissue-type plasminogen activators by conventional discrimination with specific antibodies (29) that is based on differences in their immunological reactivities.

Recent studies have demonstrated that tumor cells, cells malignantly transformed by tumor viruses, and cells treated with tumor promotors can be induced to synthesize and secrete plasminogen activators (14, 27). These activators may have important functions in various biological processes that operate in cell migration, tissue development and metastasis (17). Extensive studies have been done on human plasminogen activators, but few have been published on those of other vertebrate species, particularly those of the lower vertebrates. If we are to understand the evolutionary biological role of the proteins, comparative studies of plasminogen activators are necessary.

We here report the identification and characterization of fish plasminogen activators in a small viviparous fish, *Xiphophorus*, determined with a monoclonal antibody against human urokinase (see refs. 15, 24) and an affinity column contain-

Abbreviations used: IgG, immunoglobulin G, F₁, interspecific hybrids between platyfish and swordtails; EDTA, ethylenediaminetetra acetic acid; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; TPCK, L-1-(tosylamide)-2-phenylethyl chloromethyl ketone; kD, kilodalton.

ing fibrin-deposited Celite matrices. The fish is a unique animal system for cancer studies because its melanomas are produced genetically by interspecific hybridization (1, 18). Two antigens with molecular weights of 55 and 50 kD were characterized as the major plasminogen activators in *Xiphophorus* skin tissue. Our results and the comparison of these two proteins with human urokinase on ^{125}I -tryptic peptide maps led us to conclude that these two fish plasminogen activators are functionally and structurally similar to human urokinase.

MATERIALS AND METHODS

Animals. The platyfish (*Xiphophorus maculatus*) have spots made up of macromelanophores throughout the body surface. These spots are determined by a macromelanophore gene, *Sp* (Fig. 1A) and are regarded as transformed but differentiated into benign cells (1, 21, 30, 31). When platyfish are hybridized with swordtails (*X. helleri*) that have no macromelanophore spots, the F_1 hybrids carrying the *Sp* gene develop extensive melanosis over their entire body surfaces (Fig. 1B), probably because of excess formation of abnormally differentiated macromelanophores (1). These melanotic lesions on the hybrid fish progress to melanoma at old age (32). Skin and dorsal fin tissues from the platyfish and from the hybrids were used for the biochemical studies described here. The sex of the fish was not considered important.

Chemicals. Nitrocellulose sheets were purchased from Schleicher and Schüll (Dassel, G.F.R.). Peroxidase-conjugated IgG of rabbit anti-mouse IgG was obtained from Miles-Yeda (Rehovot, Israel). Monoclonal antibodies against human urokinase were produced as described elsewhere (15). A hybridoma (G62)-producing, IgG-isotype antibody was purified and was characterized (15, 24); this antibody was used throughout the study. Human thrombin was purchased from United States Biochemicals Corp. (Cleveland, OH, USA). Human fibrinogen, plasmin-free plasminogen, and a chromogenic substrate, L-pyroglyutamyl-glycyl-L-arginine-*p*-nitroanilide (S-2444) were obtained from Kabi Vitrum (Stockholm, Sweden). CNBr-activated Sepharose 4B was obtained from Pharmacia (Stockholm, Sweden) and Clite from Johns-Manville (USA). Cellulose-coated TLC plates were purchased

Fig. 1. (A), an adult platyfish that carries macromelanophore spots determined by the macromelanophore gene, *Sp* (female). Total length, 43 mm. (B), an adult F_1 offspring of the platyfish and a green swordtail (male). Total length, 65 mm.

from E. Merck (Darmstadt, F.R.G.) and sodium iodine-125 (17 Ci/mg) from New England Nuclear. Other chemicals are of reagent grade.

Preparation of antigen extract. The fish used were sterilized briefly by a rinse in 50% ethanol, then the skin and dorsal fin tissues were removed, after which the specimens were washed by centrifugation at $12,000 \times g$ for 20 min in phosphate-buffered saline (pH 7.3) containing 0.5 mM phenylmethyl sulfonyl fluoride (PMSF). The pellet formed was homogenized in 2 ml of buffer solution containing 0.25 M sucrose, 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, 3 mM CaCl_2 , 3 mM MgCl_2 and 0.5 mM PMSF, and the homogenate was centrifuged at $27,000 \times g$ for 20 min. The supernatant was collected and adjusted to 6 M urea, after which it was recentrifuged at $27,000 \times g$ for 20 min to remove undissolved material. This fish material was dialyzed against 10 mM ammonium bicarbonate (pH 7.3) then lyophilized.

SDS-polyacrylamide slab gel electrophoresis. One-dimensional 10% SDS-polyacrylamide slab gel electrophoresis followed the Laemmli's method (11).

Blot-transfer analyses of antigenic proteins. The proteins that separated on 10% SDS-polyacrylamide slab gels were blotted electrophoretically on nitrocellulose sheets (26). Antigenic proteins were determined by the method described by Glass *et al.* (4).

Affinity purification of antigens. Monoclonal antibodies were conjugated to CNBr-Sepharose 4B as described previously (15). Fibrin-deposited Celite was prepared according to the method described by Husain *et al.* (10). The material bound on the fibrin-Celite column was eluted with a buffer solution containing 10 mM sodium phosphate (pH 7.4), 1 mM EDTA, 0.3 M NaCl, 5 mM ϵ -aminohexanoic acid and 0.2 M arginine after extensive washing with the same buffer solution containing no arginine. The samples obtained were dialyzed against 10 mM ammonium bicarbonate (pH 7.3) then lyophilized.

Enzyme assay. Enzyme assays were done with 1 ml of a reaction mixture containing 50 mM Tris-HCl (pH 8.8), 38 mM NaCl and 0.3 mM S-2444 (9), after which the mixture was incubated for the desired period at 37°C. The reaction was stopped by an addition of 100 μl of 50% acetic acid. The absorbance was read spectrophotometrically at 405 nm.

Fibrin autography. Proteins that separated on SDS-polyacrylamide slab gels were examined on fibrin-agar indicator plates according to the method described by Granell-Piperno and Reich (5) in order to determine plasminogen-mediated fibrinolytic activity.

Two-dimensional tryptic peptide maps. The following procedures were done by the method described by Elder *et al.* (3). In brief, protein bands that had separated on the 10% SDS-polyacrylamide slab gels were cut out, then they were radioiodinated with ^{125}I in the gel slices by the chloramine T method (6). After extensive washing in 10% methanol, the gel slices were lyophilized. An addition of 0.5 ml of trypsin-TPCK (50 $\mu\text{g}/\text{ml}$) in 50 mM ammonium bicarbonate (pH 8.0) was made to the samples, which then were incubated at 37°C overnight, after which the supernatant were removed and lyophilized.

These samples were analyzed on cellulose-coated TLC plates (20 $\times$ 20 cm) as described previously (25). The first dimension was electrophoresis in acetic acid/formic acid/water (3:1:16, V/V, pH 3.0) at 900 V for about one hour. In the second dimension, peptides were separated by ascending chromatography in *n*-butanol/pyridine/acetic acid/water (15:12:3:10, V/V) for about four hours at room temperature. The plates were dried and the peptide spots were analyzed by autoradiography using Kodak X-Omat RP film.

Protein determination. The amount of protein was determined by the Bio-Rad protein assay (2), using bovine gamma globulin as a standard.

RESULTS

Identification of antigens on immunoblots. To determine whether immunological cross-reactive proteins were present in the crude extracts prepared as described in MATERIALS AND METHODS, the proteins were separated on 10% SDS-polyacrylamide slab gels then blot-transferred to nitrocellulose sheets. Fig. 2A shows the proteins stained with Coomassie brilliant blue, and 2B the localization of the immunologically reactive proteins. Of the approximately fifty stained bands, six major ones that had molecular weights of 69, 59, 55, 50, 46 and 43 kD were immunostained. No obvious differences in protein components or in immunostained proteins were observed between the platyfish and their hybrids.

Enzyme activities of the immunoaffinity-purified antigens. We next determined whether the antigens had enzymatic activities. Since plasminogen activators are serine-proteases, we used a synthetic substrate, S-2444, and checked whether the antigens could hydrolyze it. The proteins of the platyfish and of their hybrids were purified on a monoclonal antibody-immobilized Sepharose 4B column (See ref. 15: Similar antigenic proteins were found on SDS-polyacrylamide slab gels as seen in Fig. 2B) and their protease activities were determined. Antigens prepared from both specimens showed proteolytic activity on the hydrolysis of the chromogenic substrate (Fig. 3). We calculated the specific enzyme activity for each specimen on the

Fig. 2. SDS-polyacrylamide slab gel electrophoreses of the fish proteins and their immunoblots. The fish proteins were extracted then fractionated on 10% SDS-polyacrylamide slab gels. The separated proteins were blot-transferred to nitrocellulose sheets. (A), proteins (50 μ g each) prepared from the platyfish (lane 1) and its hybrids (F_1) (lane 2). Lane M, molecular weight standards in kilodaltons. (B), immunoblots of the respective specimens in (A). In the blots, the left half was incubated with monoclonal antibodies (8 ng/ml) and the right half with mouse preimmune IgG (8 ng/ml). Numbers in the middle give the calculated molecular weights of the antigens in kilodaltons.

assumption that an increase in optical density of 0.00125/min gives one enzyme unit/ml. On this basis, the platyfish and their hybrids contained, respectively, 100 and 4,000 units/mg antigens. For comparison, the specific activities of the respective crude samples were determined; 70 and 700 units/mg proteins. Our results indicate that the hybrid fish antigens have much higher proteolytic activity than the platyfish antigens.

Molecular conversion of plasminogen by the fish antigens. The immunoaffinity-purified antigens of the hybrid fish were examined to see whether they could convert plasminogen to plasmin *in vitro*. The purified antigens were mixed with human plasminogen and incubated at 37°C for the desired period. To eliminate any non-specific cleavage of plasminogen by serine-protease activity other than that of the plasminogen activator, an excess amount of soybean trypsin inhibitor was added to the reaction mixtures. After incubation, the mixtures were analyzed on 10% SDS-polyacrylamide slab gels in the presence of the reducing reagent, 2-mercaptoethanol.

Because inactive plasminogen is a single chain form that is cross-linked internally by disulfide bonds, when a specific activation occurs during incubation with the fish antigens, the plasminogen can form an active two-chain structure (see DISCUSSION). Eventually, when reduced, it can separate plasmin into two components, A- and B-chains, on the gels. This phenomenon is demonstrated in Fig. 4 which

Fig. 3. Enzyme assays of purified fish antigens. Fish proteins were purified on an antibody-immobilized Sepharose 4B column as described elsewhere (15). The antigens purified from the hybrid fish (5 μ g, —○—○—) and from the platyfish (22 μ g, ...○...○...) were reconstituted separately in 1 ml of the reaction mixture, after which they were incubated for the desired period at 37°C. Abscissa, incubation time in minutes, ordinate, absorbance at 405 nm.

Fig. 4. Molecular cleavage of human plasminogen by the fish antigens. Sample of human plasminogen was incubated at 37°C for four hours with purified human urokinase (44,000 units/mg proteins) or with the antigens purified from the hybrid fish (see Fig. 3). All the reaction mixtures contained 20 μ g of soybean trypsin inhibitor to ensure that there was no further breakdown of the cleavage products. The cleavage products were analyzed by 10% SDS-polyacrylamide slab gel electrophoreses. Lane M, molecular weight standards in kilodaltons, lane 1, human plasminogen alone (13 μ g), lane 2, plasminogen with urokinase (3 units), lanes 3 and 4, plasminogen with the purified antigens (4,000 units/mg antigens) of 6 and 17 units. Arrows indicate the location of native plasminogen (100 kD) and soybean trypsin inhibitor (20 kD). Arrowheads show the cleavage products of plasminogen. Note that the plasminogen preparation contained doublet bands (see Discussion).

shows that antigens had preferential activity for the cleavage of plasminogen (Fig. 4, lanes 3 and 4) that is similar to that of urokinase (Fig. 4, lane 2). Our results are strong evidence that the fish antigen preparation contained proteins with plasminogen-activating activity.

Affinity purification of plasminogen activators on a fibrin-Celite column. To determine which proteins having plasminogen activating activity in the antigen preparations (Fig. 2B), the proteins were purified on a fibrin-Celite affinity column then analyzed by 10% SDS-polyacrylamide slab gel electrophoreses and examined on fibrin-agar indicator plates (see MATERIALS AND METHODS). The 55 and 50 kD proteins predominantly enriched the bound fraction on the affinity column (Fig. 5). Similar electrophoresed gels were subjected to examine on fibrin-agar indicator gels to determine whether these two proteins have plasminogen-mediated fibrinolytic activity. Apparently, they did (Fig. 5, inset B), and they were definitively positive on immunoblots (data not shown) evidence that both activators are antigens of interest (see Fig. 2B).

Fig. 5. Purification and characterization of the plasminogen activators. Proteins prepared from the hybrid fish were chromatographed on a fibrin/Celite column. The flow-through and bound fractions (marked by bars) were collected separately, dialyzed against 10 mM ammonium bicarbonate (pH 7.0) then lyophilized. Abscissa: fraction number, ordinate: absorbance at 280 nm. Inset, (A), 10% SDS-polyacrylamide slab gel electrophoresis of the fractionated proteins. Lane M; molecular weight standards in kilodaltons, lane 1; total proteins (10 μ g), lane 2; proteins in the flow-through fractions (10 μ g), and lane 3; proteins in the bound fractions (10 μ g). (B), the determination of plasminogen-mediated fibrinolytic activity of the purified proteins (lane 3 of (A)) on fibrin-agar indicator plates. Proteins with approximate molecular weights of 55 and 50 kD are marked with asterisks. The other protein bands were not positive in this assay.

Comparison of 125 I-tryptic peptide maps of the fish plasminogen activators and human urokinase. Are these fish plasminogen activators structurally similar to human urokinase? The proteins that separated (Fig. 5, inset A, lane 3) as well as the urokinase (55 kD, data not shown) present on the SDS-polyacrylamide slab gels were cut out after being stained with Coomassie brilliant blue, then the gel slices were radioiodinated separately. Tryptic peptide maps were made as described in MATERIALS AND METHODS, and are shown in Fig. 6. To facilitate the comparison of the maps, the peptide spots were numbered. The total numbers of peptide spots identified for each of the three proteins, the 55 kD (band A) and 50 kD (band B) fish activators and the 55 kD urokinase, were 17, 19 and 14. Of those analyzed, peptide No. 2 and 3, 6 to 11 and 15 were common to all three proteins. Peptide No. 4 and 16 were not present in the urokinase, and No. 18 through 20 were absent from both bands A and B. Our results show that the three proteins have considerable similarity.

Fig. 6. ^{125}I -tryptic peptide mapping of fish plasminogen activators and human urokinase. ^{125}I -labeled tyrosine-containing tryptic peptides from the fish activators and urokinase were analyzed as described in MATERIALS AND METHODS. To compare the three proteins, we numbered the peptide spots (see Text). (A): the 55 kD plasminogen activator (fish); (B): the 50 kD plasminogen activator (fish), and (C): the 55 kD human urokinase. Arrowheads indicate additional peptides that were not considered. Faint but visible spots are encircled by dots. Abscissa: electrophoretic dimension, ordinate: chromatographic dimension.

DISCUSSION

We identified and characterized the plasminogen activators present in the platyfish and in its swordtail-hybrid offspring. These proteins are the first characterized plasminogen activators found in a lower vertebrate. Our monoclonal antibody raised against human urokinase (15, 24) was able to pick up 6 major antigens on the immunoblots (Fig. 2B). We also demonstrated that the two fish antigens with approximate molecular weights of 55 and 50 kD have plasminogen-mediated fibrinolytic activity on fibrin-agar indicator plates (Fig. 5, inset B), and that the two activators can be purified on a fibrin-Celite affinity column. The assay system, in which the active plasmin generated by the plasminogen activators is capable of lysing fibrin, was quite specific, since the depletion of plasminogen did show no fibrinolytic bands (data not presented).

These fish antigens purified on an affinity column containing monoclonal antibody-conjugated Sepharose 4B showed serine-protease activities in the hydrolysis of a synthetic chromogenic substrate (Fig. 3, discussed later), and were capable of converting human plasminogen into two polypeptide components, A- and B-chains (Fig. 4, discussed later). Therefore, both these 55 and 50 kD activators appear to be functionally similar to human urokinase.

We then determined whether plasminogen was specifically converted to plasmin on incubation with the fish plasminogen activators. During *in vitro* activation of inactive plasminogen with an apparent molecular weight of about 100 kD, site-specific proteolytic cleavage at Arg 560-Val 561 position would form an active two-chain structure (12, 34–36).

The amino acid sequence of the antibody-combining site was determined to be CQGDSGG (24), and the epitope structure is present in other serine proteases, such as trypsin (24), and chymotrypsin (manuscript in preparation); therefore, our monoclonal antibody would be immunoreactive with those particular proteins. To eliminate this possibility, an excess amount of soybean trypsin inhibitor was added to the reaction mixture. This inhibitor was effective during long incubation. In fact, plasminogen was split selectively into discrete molecular components. In the presence of a reducing reagent, A (about 80 kD) and B chains (about 29 kD) can be separated on SDS-polyacrylamide slab gels (Fig. 4, lanes, 3 and 4). The manner in which the A and B chains of plasmin were generated, was quite similar to that during incubation with human urokinase (Fig. 4, lane 2, see ref. 36).

Our plasminogen preparation contained two components of a high and low molecular weight (Fig. 4, lane 1). These polypeptide species probably represent Glu- and Lys-Plasminogens. A short segment of polypeptide with an approximate molecular weight of about 6 kD can be deleted from the Glu-plasminogen by proteolytic cleavage toward the NH₂-terminal (12). On activation, similar cleavage sites in both plasminogens would participate as described above; therefore, if plasminogen activators were responsible for the site-specific cleavages, the two types should be simultaneously convertible to two molecular species of A-chain and one of B-chain plasmin. Our assays of the gels strongly supports this. Accordingly, the fish plasminogen activators acted equally on the two species of plasminogen.

Because our fish plasminogen activators had molecular weights similar to human urokinase and a similar function as described above, we concluded that they are urokinase-type plasminogen activators.

To determine whether the fish plasminogen activators (the 55 and 50 kD components) are structurally similar to human urokinase, we purified the proteins to electrophoretic homogeneity on SDS-polyacrylamide slab gels. The proteins in the corresponding bands were radioiodinated separately as described in MATERIALS AND METHODS. The ¹²⁵I-labeled proteins in the gel slices were trypsinized, after which the tryptic peptides released were analyzed on TLC plates (Fig. 6).

On the basis of the number of lysine and arginine residues present (7, 23), the theoretical yield for the radioiodinated urokinase is thirteen tryptic peptides (Fig. 1 in ref. 24), assuming that all the tyrosine residues were radioiodinated by the chloramine T method. The total number of peptides identified for the urokinase is 14, which agrees well with the theoretical number. Nine peptides (No. 2, 3, 6–11 and 15) were common to all three proteins, and the similarity of the maps suggests that they are structurally related. The similarity between bands A and B is closer than in urokinase because fifteen peptides (No. 1–4, and 6–16) were common to these bands. Further comparative studies of the structure of fish and human plasminogen activators should prove useful for understanding the evolutionary origin of the plasminogen activator system.

Several studies have suggested that malignantly transformed cells have high protease activities and have plasminogen activators (8, 16, 19, 20, 22, 27, 33). We,

therefore, presume that the high serine-protease contents of the hybrid fish antigens (Fig. 3) probably are associated with the development of extensive malanosis (see MATERIALS AND METHODS). In contrast, antigens purified from the platyfish contained relatively latent activity. The role of fish plasminogen activators in the etiology of genetically determined melanoma calls for further investigation.

Acknowledgements. We are grateful to Dr. Masaaki Tanifuji (Daigo Nutritive Chemicals Ltd. Osaka) who kindly supplied us the urokinase. We also thank Mrs. Toshiko Hara for preparing the typescript. This research was supported in part by a Grant-in-Aid from The Mochida Memorial Foundation for Medical and Pharmaceutical Research.

References

1. ANDERS, A. and F. ANDERS. Etiology of cancer as studied in the platyfish-swordtail system. *Biochim. Biophys. Acta* **514**, 61–95, 1978
2. BRADFORD, M.M. A rapid and sensitive method for the quantitation of microgram of protein utilizing the principle of protein-dye binding. *Anal. Chem.* **72**, 248–254, 1976
3. ELDER, J.H., R.A. PICKET II, J. HAMPTON and R.A. LERNER. Radioiodination of proteins in single polyacrylamide gel slices. Tryptic peptide analysis of all the major members of complex multicomponent systems using microgram quantities of total protein. *J. Biol. Chem.* **252**, 6510–6515, 1977
4. GLASS II, W.F., R.C. BRIGGS and L.S. HNILICA. Identification of tissue-specific nuclear antigens transferred to nitrocellulose from polyacrylamide gels. *Science* **211**, 70–72, 1981
5. GRANELL-PIPERNO, A. and E. REICH. A study of proteases and protease-inhibitor complexes in biological fluids. *J. Exp. Med.* **148**, 223–234, 1978
6. GREENWOOD, F.C., W.M. HUNTER and J.S. GLOVER. The preparation of ¹²⁵I-labeled human growth hormone of high specific radioactivity. *Biochem. J.* **89**, 114–123, 1963
7. GÜNZLER, W.A., G.J. STEFFENS, F. OTTING, S-M.A. KIM, E. FRANKUS and L. FLÖHE. The primary structure of high molecular mass urokinase from human urine. The complete amino acid sequence of the A-chain. *Hoppe-Seyler's Z. Physiol. Chem.* **361**, 1155–1165, 1982
8. HARVEY, S., J. MINOWADA, H. TAKITA, L. KOVER and G. MARKUS. Urokinase-like plasminogen activators of unusually high molecular weight secreted by a cell line derived from a human lung cancer case. *J. Biol. Chem.* **257**, 5645–5651, 1982
9. HÉRION, P. and A. BOLLEN. Purification of urokinase by monoclonal antibody affinity chromatography. *Biosci. Rep.* **1**, 373–379, 1983
10. HUSAIN, S.S., B. LIPINSKI and V. GUREWICK. Rapid purification of a high-affinity plasminogen activator from human blood plasma by specific adsorption on fibrin/Celite. *Proc. Natl. Acad. Sci. USA* **78**, 4265–4269, 1981
11. LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**, 680–685, 1970
12. MARKUS, G., J.L. EVERS and G.H. HOBITA. Comparison of some properties of native (Glu) and modified (Lys) human plasminogen. *J. Biol. Chem.* **253**, 733–739, 1978
13. MATSUO, C., D.C. RIJKEN and D. COLLEN. On the thrombolytic properties of human tissue plasminogen activator and urokinase. in *Urokinase: Basic and Clinical Aspects*, Vol. **48**, eds. P.M. Mannuci and A. D'Angelo, Academic Press, New York, pp 55–61, 1982
14. NAGAMINE, Y., M. SUDOL and E. REICH. Hormonal regulation of plasminogen activator mRNA production in porcine kidney cells. *Cell* **32**, 1181–1190, 1984
15. NAKAMURA, M., K. TAKAHASHI, H. NAORA and T. TSUNEMATSU. A monoclonal antibody against human urokinase: Characterization of the epitope and its localization in human kidney. *Cell Struct. Funct.* **9**, 167–179, 1984
16. OSSOWSKI, L., J.P. QUIGLEY and E. REICH. Fibrinolysis associated with oncogenic transformation. Morphological correlates. *J. Biol. Chem.* **249**, 4312–4320, 1974
17. OSSOWSKI, L. and E. REICH. Antibodies to plasminogen activator inhibit human tumor

- metastasis. *Cell* **15**, 611–619, 1983
18. OZATO, K. and Y. WAKAMATSU. Multi-step regulation of oncogene expression in fish hereditary melanoma. *Differentiation* **24**, 181–190, 1983
 19. RIJKEN, D. and D. COLLEN. Purification and characterization of the plasminogen activator secreted by human melanoma cells. *J. Biol. Chem.* **254**, 7035–7041, 1981
 20. ROBLIN, R.O., P.L. YOUNG and T.E. BELL. Concomitant secretion by transformed SVW 138-VA-13-2RA cells of plasminogen activator(s) and substance(s) which prevent their detection. *Biochem. Biophys. Res. Commun.* **82**, 165–172, 1978
 21. SCHWAB, M. and E. SCHOLL. Neoplastic pigment cells induced by N-methyl-N-nitrosourea (MNU) in *Xiphophorus* and genetic control of their terminal differentiation. *Differentiation* **19**, 77–83, 1981
 22. SISKIN, E.E., I.B. WEINSTEIN, C.H. EVANS and J.A. DiPAOLO. Plasminogen activator synthesis accompanying chemical carcinogen-induced *in vitro* transformation of Syrian hamster and quinea-pig fetal cells. *Int. J. Cancer* **24**, 331–335, 1980
 23. STEFFENS, G.J., W.A. GÜNZLER, F. OTTING, E. FRANKUS and L. FLÖHE. The complete amino acid sequence of low molecular mass urokinase from human urine. *Hoppe-Seyler's Z. Physiol. Chem.* **363**, 1043–1058, 1982
 24. TAKAHASHI, K. and H. NAORA. A monoclonal antibody against human urokinase: The epitope structure and sequence homology with a human tissue-type plasminogen activator. *Cell Struct. Funct.* **18**, 195–208, 1985
 25. TAKAHASHI, K., P.-K. CHAN, R.K. BUSCH and H. BUSCH. Identification and purification of Namalwa nuclear RNP antigen 52/5.3. *J. Cancer Res. Clin. Oncol.* **185**, 67–75, 1983
 26. TOWBIN, H., T. STAEBELIN and J. GORDON. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **74**, 4350–4354, 1979
 27. UNKELESS, J., K. DANØ, G.M. KELLERMAN and E. REICH. Fibrinolysis associated with oncogenic transformation. Partial purification and characterization of the cell factor, a plasminogen activator. *J. Biol. Chem.* **249**, 4295–4305, 1974
 28. VETTERLEIN, D., P.L. YOUNG, T.E. BELL and R. ROBLIN. Immunological characterization of multiple molecular weight forms of human cell plasminogen activators. *J. Biol. Chem.* **254**, 575–578, 1979
 29. VETTERLEIN, D., T.E. BELL, P.L. YOUNG and R. ROBLIN. Immunological quantitation and immunoadsorption of urokinase-like plasminogen activators secreted by human cells. *J. Biol. Chem.* **255**, 3665–3672, 1980
 30. VIELKIND, V. Genetic control of cell differentiation in platyfish-swordtail melanomas. *J. Exp. Zool.* **194**, 197–204, 1976
 31. VIELKIND, J., U. VIELKIND and F. ANDERS. Melanotic and amelanotic melanomas in *Xiphophorus* fish. *Cancer Res.* **11**, 868–875, 1971
 32. WAKAMATSU, Y. Two types of melanomas in a new experimental system of platyfish-swordtail hybrids. *Develop. Growth Differ.* **22**, 731–740, 1980
 33. WILSON, E.L., M.B. BECKER, E.G. HOAL and E.B. DOWDLE. Molecular species of plasminogen activators secreted by normal and neoplastic human cells. *Cancer Res.* **48**, 933–938, 1980
 34. WIMAN, B. Primary structure of the B-chain of human plasmin. *Eur. J. Biochem.* **74**, 129–137, 1977
 35. WIMAN, B. and P. WALLEN. On the primary structure of human plasminogen and plasmin. Purification and characterization of cyanogen-bromide fragments. *Eur. J. Biochem.* **57**, 387–394, 1975
 36. WIMAN, E. and P. WALLEN. Activation of human plasminogen by an insoluble derivative of urokinase. Structural changes of plasminogen in the course of activation and demonstration of a possible intermediate compound. *Eur. J. Biochem.* **14**, 25–31, 1973

(Received for publication, August 18, 1986)