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Substrate Preference of Metalloproteinases Secreted by *ts*-110 Moloney Murine Sarcoma Virus-Transformed Normal Rat Kidney Cells

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In this study, the two forms of affinity-purified transformation-associated proteins (TAPs) (68 and 64 kD) were shown to have different substrate preferences. For the 68-kD TAP, the order of substrate preference was collagen types I, III, and V; fibronectin; gelatin; and collagen IV. For the 64-kD TAP, the order of substrate preference was collagen I, III, and V and gelatin. The 64-kD TAP did not cleave collagen IV and fibronectin. We also found a 71-kD metalloproteinase in the concentrated purified TAPs that reacted only weakly with a TAP monoclonal antibody and showed this substrate preference: collagen I, III, and V; gelatin; and collagen IV. Whether this 71-kD TAP is a new member of the rat metalloproteinase family will be investigated.

We previously characterized the transformation-associated proteins (TAPs) and reported that they were overexpressed in the 6m2 line, probably because of activation by v-*mos*, but not in the normal counterparts of 6m2 (1-4). To identify the function of TAPs, we isolated and characterized two cDNA clones. One clone, 34A, has been completely sequenced and was identical to rat stromelysin-2 (transin-2) (5). The other clone, 79B3, has been partially sequenced and appears to be very similar if not identical to rat stromelysin-1 (transin-1) (unpublished data). Stromelysin-1 and -2 are matrix metalloproteinases. To further characterize the two forms of TAPs, we studied their substrate preference and antigenic reactivity with a monoclonal antibody (MAb) raised against TAPs. In this communication, we report their different substrate preferences and that a 71 kD metalloproteinase in a murine sarcoma virus (MSV)-Kirsten-transformed human osteosarcoma culture and a v-*mos*-transformed mouse culture did not react with the anti-TAP MAb.

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Abbreviations: MSV, murine sarcoma virus; TAP, transformation-associated protein; MAb, monoclonal antibody; PAb, polyclonal antibody; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; SSFM, spent serum-free medium.

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MATERIAL AND METHODS

Cell lines. The cell lines used in this study were: 1) 6m2 cells, which are normal rat kidney cells transformed by *ts*-110-Moloney murine sarcoma virus (6). These cells are temperature-sensitive for the synthesis of v-*mos* and TAPs (2, 7). Their properties have been described previously (1, 2, 4, 8). 2) KHOS-240S (referred to as KHOS) is a human osteosarcoma culture transformed by MSV-Kirsten and was isolated as a non-producer clone (9). 3) 54-5A4 is a spontaneous revertant clone of 6m2 that is not temperature sensitive for the synthesis of v-*mos* and the expression of transformation properties (10, 11). 4) FG 10-0 (referred to as FG 10) was derived from a semisolid agar colony of MSV-transformed mouse cells (12, 13). It harbors a rescuable MSV genome but does not produce infectious virus or murine leukemia virus antigens. It was supplied by Dr. Peter J. Fischinger of the National Cancer Institute. 5) RBT CL A S60 (referred to as RBT) was established from an MSV-Soehner-Dmochowski-induced bone tumor in a New Zealand black rat (14) and produced only murine leukemia viruses.

TAP-specific MAbs and Polyclonal antibodies (PAbs). The production of MAb MC by the hybridoma technique and its properties have been described previously by us (1, 2). A PAb was raised in a New Zealand rabbit by serial immunization with a preparation of TAP purified from 6m2 cells (7), with the previously described MC affinity column (2). Both MC and the PAb were capable of detecting the 64-kD and 68-kD forms of extracellular TAP by immunoprecipitation-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7).

Purification of TAPs. TAPs were purified by the method described previously (7).

Zymography. Metalloproteinase activity was assayed as described previously (7, 15, 16) with some modifications. Briefly, a slab gel containing 11% acrylamide, 0.1% SDS, and 0.11% copolymerized substrates was cast. Unheated samples were mixed with equal parts of 2X sample buffer (125 mM Tris, pH 6.8; 4% SDS; 15% glycerol; 0.01% bromphenol blue) and loaded on the gel. Electrophoresis was performed at 4°C at a constant voltage of 200 V. After electrophoresis, the gel was washed three times for 30 min each time with 2% Triton X-100 in 50 mM Tris-HCl, pH 7.4, and rinsed three times for 5 minutes in 50 mM Tris-HCl, pH 7.4. The gel was then incubated for 16 h. at 37°C in pH 7.4 buffer containing 50 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 2 μM $ZnCl_2$, 1% Triton X-100, and 0.02% sodium azide, pH 7.4. After incubation, the gel was fixed; stained with 0.2% Coomassie blue in 7% acetic acid, 50% methanol, and 43% distilled water; and destained with a 1:3:6 solution of acetic acid, methanol, and water. The following substrates were used: gelatin (bovine skin, Sigma), collagen I (rat tail, Sigma), collagen III (human placenta, Sigma), collagen IV (human placenta, Sigma), collagen V (human placenta, Sigma), and fibronectin (human, Collaborative Research Inc.). In some assays, Clostridium histolyticum collagenase D (Boehringer Mannheim Biochemicals) was included as a reference.

Western blot analysis. Antigenic analyses of affinity-purified TAPs and metalloproteinases from the spent serum-free medium (SSFM) of cell cultures were carried out by western blotting as previously described (17).

RESULTS

Affinity-purified TAPs were analyzed for their substrate preference by zymogram analyses using several extracellular matrix proteins. As shown in Fig. 1A, the purified TAPs migrated as three sharp bands (71, 68, and 64 kD) in the rat collagen I zymogram. In the bovine-gelatin zymogram, one sharp band (71 kD, Fig. 1B) and two faint bands (68 and 64 kD, Fig 1B) were found. In the human collagen-IV zymogram, the TAPs migrated as two faint bands (71 and 68 kD, Fig. 1C). In human fibronectin zymogram, only one TAP band (68 kD) was found (Fig. 1D).

To obtain the same level of digestion as for type I rat collagen and type III and V human collagen (they had above the same level of activity when 0.4 μg of TAPs was used; data not

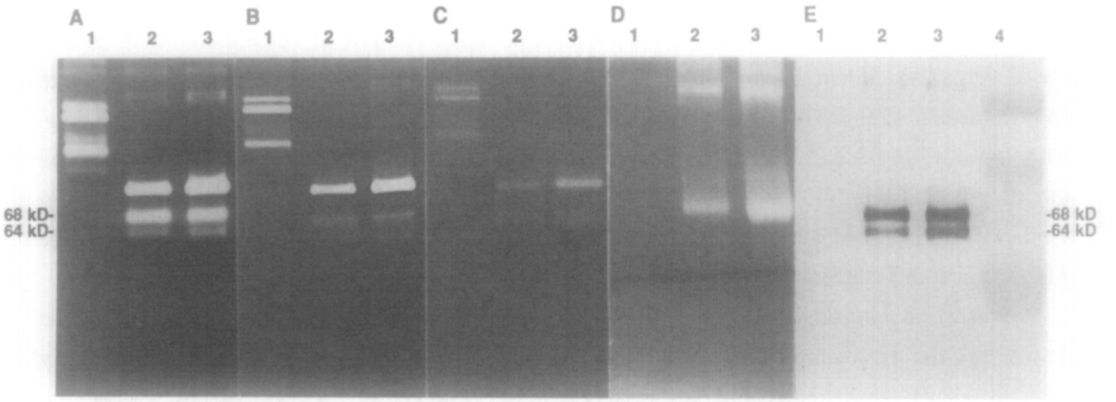

Fig. 1. Zymograms (panels A-D) and western blot (panel E) showing digestion of various substrates by the two preparations of affinity-purified TAPs. The following substrates were used: rat collagen type I (panel A), bovine gelatin (panel B), human collagen type IV (panel C), and human fibronectin (panel D). In panel E, the gel was blotted to a nitrocellulose membrane after the samples were electrophoresed, and then the membrane was incubated with MC. In all five panels, C. histolyticum collagenase D (5 ng in lane 1 of panels A, B, C, and E; 500 ng in lane 1 of panel D), affinity-purified TAPs (0.4 µg in lane 2 and 3 of panels A, B, C, and E; 1.6 µg/lane in lanes 2 and 3 of panel D) were analysed. Molecular-size markers were included in panel E as a reference.

shown), the amount of TAPs had to be increased to 1.6 µg for gelatin and to 3.2 µg for type IV human collagen.

Our initial efforts to run a human fibronectin zymogram using the same amount of TAPs as in Fig. 1A did not work. When the amount of TAPs were increased to 1.6 µg, one 68-kD band was found (Fig. 1D). C. histolyticum collagenase D was not able to digest fibronectin even at 100 times of the usual amount (5 ng/lane)(Fig. 1D).

Metalloproteinases produced by two rat cultures (54-5A4 and RBT), a mouse culture (FG10), and a human culture (KHOS) were included in the zymogram studies for comparison with the metalloproteinases TAPs produced by 6m2 cells. As shown in Fig. 2A, the SSFM of all cell

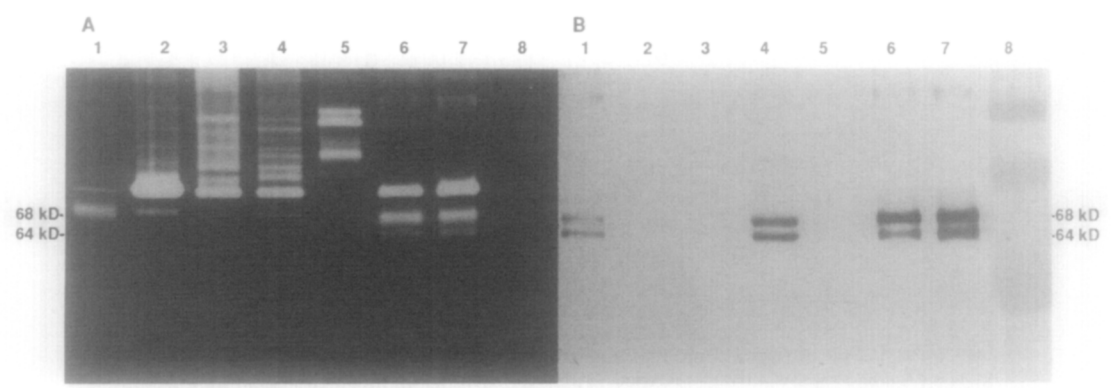

Fig. 2. Zymogram with rat collagen I as a substrate. (A) Zymogram of metalloproteinases released by various cell lines and affinity-purified TAPs. Samples of 2-day SSFM (0.1 µg/lane) and affinity-purified TAPs (0.4 µg/lane) were analyzed as described in Fig. 1A. The lanes contain: 1, 54-5A4; 2, KHOS; 3, FG10; 4, RBT; 5, C. histolyticum collagenase (5 ng); 6 and 7, affinity-purified TAPs; 8, molecular-size markers. (B) The corresponding western blot was reacted with MC.

lines degraded collagen I, and three metalloproteinases of 71, 68 and 64 kD were found in 6m2 cells. The SSFM of KHOS, FG10, and RBT had one major band of 71 kD and one faint band of 68 kD (Fig. 2A). When the amount of TAPs produced by 6m2 and 54-5A4 cells was increased to four times that of Fig. 1A, one band (68 kD) appeared in the human-fibronectin zymogram (Fig. 3A). A similar 68 kD band was found in the medium of 54-5A4 cells (Fig. 3A). The metalloproteinase in KHOS cells (52 kD) degraded fibronectin (Fig. 3A), unlike the TAPs from 6m2 cells or SSFM of other cell cultures. The enzymes in FG10 and RBT cells did not degrade fibronectin at this concentration.

Western blot analysis was carried out to confirm the identity of the metalloproteinase bands, and the results are shown in Figs. 1E, 2B, and 3C. In every case, MC reacted strongly with the 64- and 68-kD bands but only weakly with the 71-kD band. In lane 2 of Fig. 3C, MC did not react with the 52-kD metalloproteinase in KHOS cells, which cleaved fibronectin (lane 3 of Fig. 3A).

Although the western blot analyses confirmed that MC reacted with two equally intense bands (68 and 64 kD) of purified TAPs, zymograms showed different bands of activity. For example, in Fig. 1, the enzymes in all cell lines degraded type I collagen to produce a strong band of 71 kD, but this band did not react with MC. Furthermore, even though the 68 and 64 kD bands had the same intensity on a western blot (Fig. 2B), they did not produce equally intense bands (68 and 64 Kd) in the corresponding zymogram (Fig. 2A).

To determine whether the 71-kD purified TAP could be recognized by MC at higher concentrations, we assayed various amounts (0.5-2 μg) of TAPs with MC. The results (Fig. 4B) showed that when the amount of affinity-purified TAPs was increased to 2 μg, MC reacted weakly with the 71-kD band.

From these experiments we conclude that stromelysin-1 (68-kD TAP) degraded collagen types I, III and V and fibronectin better than gelatin and type-IV collagen. By comparison, stromelysin-2 (64-kD TAP) also degraded collagen types I, III, and V, but was weak in degrading gelatin and did not degrade human fibronectin or type IV collagen. 71-kD TAP strongly degraded

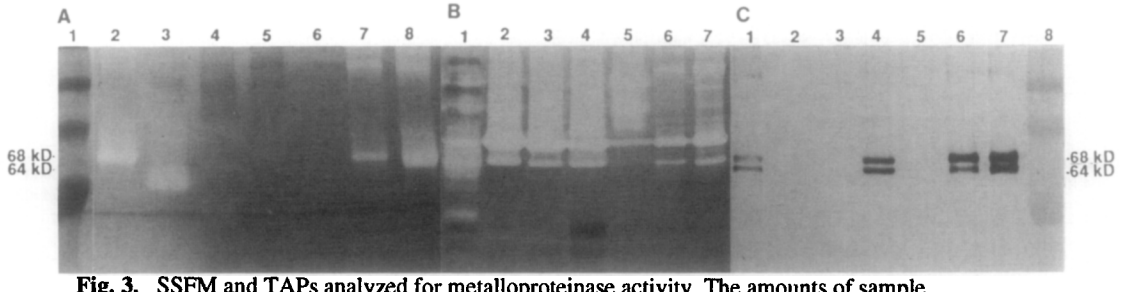

Fig. 3. SSFM and TAPs analyzed for metalloproteinase activity. The amounts of sample analyzed were: 0.4 μg of protein from the SSFM of each cell culture, 0.5 μg of C. histolyticum collagenase D, and 1.6 μg of affinity-purified TAPs. (A) Zymogram in which human fibronectin was used as a substrate, 1, Molecular-size markers; 2, 54-5A4 SSFM; 3, KHOS SSFM; 4, FG10 SSFM; 5, RBT SSFM; 6, C. histolyticum collagenase D; 7 and 8, affinity-purified TAPs. (B) Zymogram in which bovine gelatin was used as a substrate, 1, 54-5A4 SSFM; 2, KHOS SSFM; 3, FG10 SSFM; 4, RBT SSFM; 5, C. histolyticum collagenase D; 6 and 7, affinity-purified TAPs. (C) Corresponding western blot, Lane arrangement is identical to Fig. 3B for lanes 1-7. Lane 8 contains molecular-size markers as a reference.

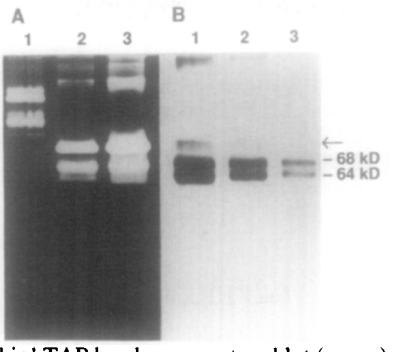

Fig. 4. Appearance of a third TAP band on a western blot (arrow). Using 2 μg of purified protein, a 71-kD band with the strongest enzymatic activity in rat collagen I zymograms was detected with MC. (A) Rat collagen-I zymogram, same as Fig. 1A. (B). Different amounts of affinity-purified TAPs on a western blot. 1, 2 μg; 2, 1 μg; 3, 0.5 μg.

collagen I, III, and V and gelatin, but was weak in degrading collagen IV and did not degrade human fibronectin.

DISCUSSION

Our results show that although rat stromelysins-1 and -2 have 78.1% amino-acid homology (5), and react with MC (2), they differed in their ability to degrade substrates. This observation is consistent with the previous reports that their respective genes (stromelysin-2 and stromelysin-1) responded differently to the same inducing regents, such as transforming growth factor-β and phorbol-ester tumor promoters (18). Another interesting observation emerged as a result of the simultaneous zymogram/western blot assays of metalloproteinases produced by cells of different animal species. Only the rat metalloproteinases produced by v-*mos*-transformed rat cells (6m2, RBT, and 54-5A4) reacted with MC on the western blots (Fig. 2). The mouse FG10 metalloproteinase putatively activated by v-*mos* (4) and the human metalloproteinase activated in human KHOS cells by K-*ras* did not react with MC (Figs. 2 and 3).

These results confirmed that MC recognizes a highly specific epitope found only on rat metalloproteinases activated in rat cells by v-*mos* (2, 5). Such epitopes apparently were either absent in mouse and human metalloproteinases or not accessible. It is possible that a different metalloproteinase was activated in mouse FG10 cells by v-*mos*, which also did not react with MC on western blots (Figs. 2B and 3C) or in previous enzyme-linked immunosorbent assays (ELISAs) (1). In the case of human metalloproteinase, the results of simultaneous zymograms and western blots revealed that a major metalloproteinase of 71 kD was produced (Fig. 2A). The 71-kD human metalloproteinase failed to react with MC in the simulatenous western blot (Figs. 2B and 3C) and also did not react with MC in a separate ELISA (results not shown). Whether the same v-*mos* oncogene activated different mouse proteinases in FG10-mouse cells and what mechanism was involved remains to be studied further.

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