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Rat Transformation-Associated Proteins (TAP) Induced by Moloney Murine Sarcoma Virus Interact with Specific Receptors on Normal Rat Kidney Cells

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Summary: In this report, data are presented to show that transformation-associated proteins (TAP) secreted from the transformed 6M2 cells have mitogenic activities in the stimulation of DNA synthesis and proliferation of normal rat kidney (NRK-2) cells and of nonpermissively grown 6M2 cells. TAP also bound specifically to NRK-2 cells with a binding dissociation constant (K_d) of 1.4 pM. Approximately 2 x 10^5 binding sites per cell were found. Therefore, TAP may represent a set of virally-induced growth stimulatory factors.

Introduction: Growth factors, such as insulin, insulin-like growth factor (IGF), somatomedin C, epidermal growth factor (EGF), and platelet-derived growth factor (PDGF), are important in stimulating quiescent cells to enter the S phase of the cell cycle. These growth factors can act alone or synergistically with other mitogens through specific surface receptors to induce DNA synthesis and proliferation of specific target cells (1). Transformed cells, however, are not subjected to the same growth regulation as normal cells. For instance, some transformed cells require less exogenous growth factor for optimal proliferation than their normal counterparts. This phenomenon, at least in part, may be the result of production of endogenous growth factors from these transformed cells and subsequent interaction with endogenous growth factor receptors (2,3). This so-called "autocrine concept" has been extended to include the abnormal modulation of growth stimulatory and inhibitory factors, which results in altered growth control (4).

The 6M2 cell line (5) was derived from the normal rat kidney cells (NRK) infected with a temperature-sensitive mutant (ts110) of the Moloney murine sarcoma virus (Mo-MSV). The 6M2 cells express the transformed phenotypes at the permissive temperature; the phenotypes include a transformed morphology, a lower requirement for the exogenous growth factors, an increased saturation density, an increased rate of hexose uptake, anchorage-independent growth, and production of the viral transformation proteins (5-7). Of particular interest is the induction of the cellular growth factors such as

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transformation growth factor a (TGF-α) (8). However, the cells revert to untransformed phenotypes at the nonpermissive temperature with a concomitant decrease in TGF-α secretion, suggesting that a viral oncogene (v-mos) product is implicated in the control of synthesis and release of TGF-α (Todaro et al., 1980). In addition to TGF-α released from transformed 6M2 cells, our previous studies with 6M2 cells have identified a set of intracellular transformation-associated proteins (TAP) produced by the transformed 6M2 cells, with the molecular weights of 66,000, 63,000, and 60,000 (9, 10). These three intracellular TAP are converted to two polypeptides with molecular weights of 68,000 and 64,000 and subsequently secreted outside the cells. These two polypeptides (P68 and P64), which can be identified by monoclonal antibody MC, are designated extracellular TAP (10). In this paper, we report that extracellular TAP have been purified by immunoaffinity column and that purified extracellular TAP bind specifically to NRK fibroblast (NRK-2) cells and stimulate cellular proliferation.

Material and Methods

Cells and Monoclonal Antibody. NRK-2 cells have been described previously (9). The 6M2 cell line was derived from the NRK cells infected with the ts110 mutant of Mo-MSV (5). The cells were cultured in Iscove's Dulbecco modified medium (Iscove medium) with 10% inactivated fetal bovine serum (FBS). Monoclonal antibody MC has been described previously (9,10). MC was used in the form of IgG_1 purified from mouse ascites fluids.

Removal of TAP from serum-free supplement medium (SSFM) of 6M2 cells grown at 33°C. SSFM containing TAP were produced by incubating 6M2 cells at 33°C for 2 days in Iscoves medium supplemented with 5 μg of insulin and 2.5 μg of transferrin per milliliter. The medium was collected and TAP were removed from the SSFM by immunoaffinity chromatography. MC was precoupled to protein A agarose (Pharmacia Fine Chemicals, Piscataway, NJ). The MC-protein A agarose complex was incubated with SSFM at 4°C overnight and then pelletized by centrifugation. The level of TAP in the SSFM was determined by an enzyme-linked immunosorbent assay (ELISA). Alternatively, the 6M2 cells were grown at 33°C in the presence of [^{35}S]-methionine (20 μCi/ml) for 20 hr to obtain [^{35}S]-TAP. The affinity purification of [^{35}S]-TAP was as previously described (10).

Stimulation of DNA synthesis of 6M2 cells grown at 39°C by SSFM. 6M2 cells were seeded in 24-well plates and incubated at 39°C for 16 hr, and various growth factors were added as described in the legend of Fig. 1. The cells were then labeled with [^{3}H]-thymidine for another 6 hr, as described previously (10).

Stimulating NRK-2 cell proliferation with purified TAP. NRK-2 cells (2 x 10^5 per Petri dish) were incubated with various stimulants, as described in the legend of Fig. 2. The cell cultures were refed daily and cell counts were performed on triple samples per group on day 4, 7, and 9.

Standard binding assay of TAP. NRK-2 cells (2 x 10^5 per well in 6-well plates) were incubated overnight, the growth medium was then aspirated and binding buffer (Iscoves medium with 50 mM BES, pH 7.0) with 1% bovine serum albumin (BSA) was added 2 hr prior to the assay. Then various amount of [^{35}S]-TAP, with or without 200-fold excess of purified TAP in the binding buffer with 0.2% BSA, were added into each well for 1 hr at 22°C. The cells were washed three times with the binding buffer and then lysed by 1% Sodium dodecyl sulfate (SDS) with 50 mM NaOH. The radioactivity associated with the cell lysates was determined by a scintillation counter.

Results and Discussion

The 6M2 cell line (5) provided a temperature-sensitive model for studies of cellular transformation. It was shown by Todaro, et al. (8) that the SSFM of 6M2 cells grown at 33°C contained TGF-α. Subsequently, we showed that SSFM of 6M2 cells grown at permissive temperatures also contained TAP with mitogenic activities (10). In our previous experiment, the SSFM of 6M2 grown at 33°C (permissive temperature) was more effective in the stimulation of DNA synthesis and proliferation of 6M2 cells grown at 39°C than SSFM generated at 39°C . This is probably due to the effects of TGF-α and TAP. To analyze the mitogenic activities of TAP in this study, TAP in SSFM from 6M2 cells grown at 33°C were removed by immunoprecipitation with the MC-protein A agarose complex, and the removal of TAP from the medium was confirmed by ELISA (data not shown). The SSFM containing TAP was compared with SSFM after MC removal of TAP for their ability to stimulate DNA synthesis of 6M2 cells grown at 39°C. It was found that the DNA stimulating activity was greatly reduced after the MC removal of TAP from the SSFM when compared with SSFM containing TAP (Fig. 1). This result suggests that crude SSFM may represent the mitogenic activities. It is interesting, however, that MC did not neutralize the mitogenic activities of TAP in this experiment (group 4, Fig. 1). Furthermore, immuno-affinity purified TAP were shown to stimulate the proliferation of NRK-2 cells (Fig. 2). While the addition of TAP alone induced significant cellular proliferation, the combination of insulin and TAP was shown to result

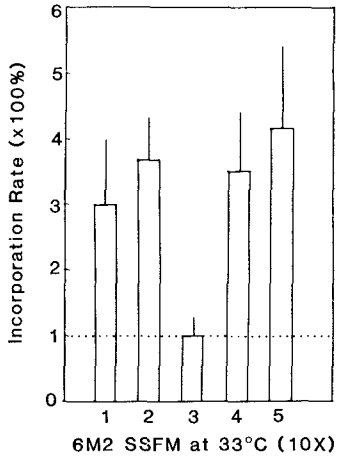
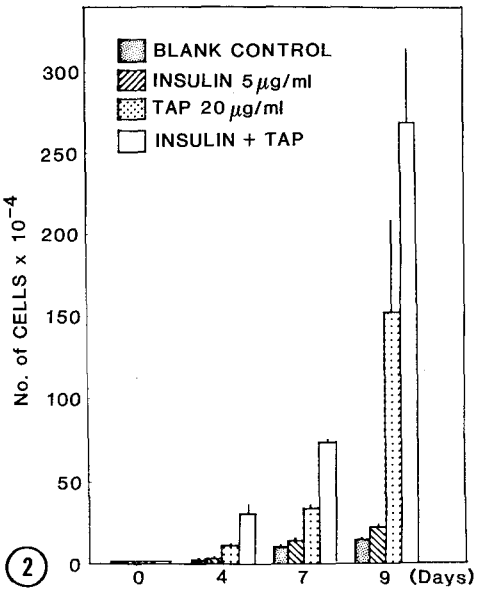


Figure 1. Stimulation of DNA synthesis of 6M2 cells grown at 39°C by concentrated Serum Supplement free Medium (SSFM) (10 x) of 6M2 grown at 33°C. The 6M2 cells grown at 39°C (2×10^4 per well in 24-well plate) were stimulated for 16 hr as follows: 1. SSFM without treatment; 2. SSFM treated with protein A agarose, 3. SSFM treated with MC-protein A agarose, 4. SSFM treated with MC; and 5. SSFM treated with an anti-HBs monoclonal antibody (11G12), which does not react with transformation-associated protein (TAP). Then the cells were labeled with [^{3}H]-thymidine (2 μCi/well) for 6 hr. and then washed three times with cold 10% trichloric acid and counted in a scintillation counter. The stimulation of thymidine incorporation for each group (eight samples each group) is expressed as the percentage ± standard deviation.



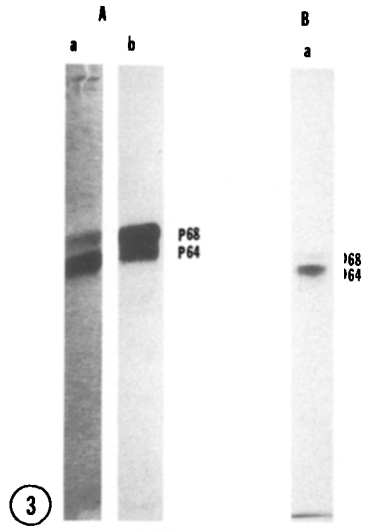


Figure 2. The stimulation of the proliferation of NRK-2 cells by purified transformation-associated protein (TAP). NRK-2 cells were treated as follows: 1. Blank control: Iscove medium with fetal bovine serum, 2. Insulin 5 μg/ml in Iscove medium, 3. TAP 20 μg/ml in Iscove medium; and 4. Insulin (5 μg/ml) plus TAP (20 μg/ml) in Iscove medium. The cells were counted on day 4, 7, and 9. The stimulation of proliferation of NRK-2 cells was expressed as the cell number ± standard deviation.

Figure 3. The purity of transformation-associated protein (TAP). Panel A: TAP (lane a) and [^{35}S] TAP (lane b) were purified by affinity chromatography and used in the receptor binding assay. Panel B: After the receptor binding assay, NRK-2 cells were washed three times with binding buffer (0.2% bovine serum albumin in Iscove medium with 50 mM BES, pH. 7.0) and lysed in Sodium dodecyl sulfate (SDS)-sample buffer, followed by centrifugation. The supernatant was run on SDS 9% polyacrylamide gel electrophoresis (lane a).

in the more potent growth stimulation. However, in separate experiments (not shown), TAP alone were incapable of inducing transformed phenotypes in NRK-2 cells or stimulating the colony formation of NRK-2 cells in soft agar. This feature distinguishes TAP from the TGF α and β (8,11).

To investigate further the possible mechanisms by which TAP stimulate the DNA synthesis and proliferation of NRK-2 cells, the binding of TAP to the cells was studied. [^{35}S]-labeled TAP was prepared from 6M2 cells at 33°C and purified by immunoaffinity column chromatography. Electrophoretic analysis of purified [^{35}S]-TAP showed only the two polypeptides of 68,000 and 64,000 daltons, either by autoradiography (Fig. 3A, lane b) or staining with Commassie blue (Fig. 3A, lane a), suggesting a homogenous preparation of TAP. The binding of various concentrations of [^{35}S]-TAP to NRK-2 cells was then determined. The binding of [^{35}S] TAP to NRK-2 cells was dependent on the concentration of the ligand added (Fig. 4A). Specificity of binding was determined by the addition of a 200-fold excess of unlabeled purified TAP. Saturation at 22°C occurred at

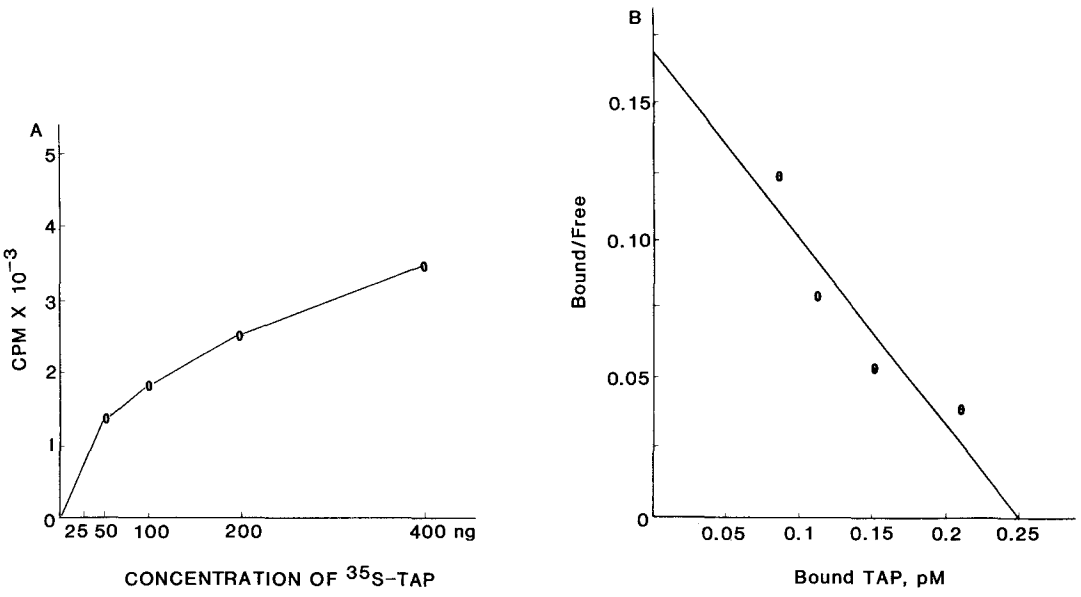


Figure 4. Transformation-associated protein (TAP)-specific receptors on normal rat kidney (NRK)-2 cells. Panel A: The specific binding curve of [^{35}S]-TAP to NRK-2 cells. NRK-2 cells (5 X 10^5) were seeded onto the 35 mm wells of 6-well plate in Iscoves medium with 10% fetal bovine serum (FBS) 24 hr and in Iscove's medium without FBS overnight prior to the assay. The cells were counted as 7.5 x 10^5 per well prior to the assay. The cells were then blocked by binding buffer with 1% bovine serum albumin for 1 hr. [^{35}S]-TAP was added to the cells, with or without 200-fold excess purified TAP as a competitor for another hr at 22°C. Finally, the cells were washed three times with binding buffer, lysed by 50 mM NaOH with 1% Sodium dodecyl sulfate, and counted in a scintillation counter. The specific binding curve of [^{35}S]-TAP to NRK-2 cells was derived from the subtraction of the nonspecific binding curve (with TAP competitors) from the total binding curve (without TAP competitors). Panel B: Scatchard plot of [^{35}S]-TAP binding to 7.5 x 10^5 NRK-2 cells at 22°C.

a concentration of about 200 ng/ml. The binding data were plotted according to the method of Scatchard (12) (Fig. 4B), and least squares analysis yielded the following binding parameters for the interaction between TAP and NRK-2 cells: K_d = 1.4 pM and 2 x 10^5 binding sites per cell.

Because the TAP fraction used in the binding experiments consisted of two proteins, P68 and P64, it was of interest to determine if a preferential binding of one or the other of the proteins would occur. Therefore, cells with bound [^{35}S] TAP were washed, lyzed, and subjected to electrophoretic analysis and fluorography (13). The resulting autoradiogram showed that the P64 was the predominant component associated with the NRK-2 cells, although P68 was also detectable (Fig. 3B). The data suggest two possibilities: first, the 64,000 dalton polypeptide is preferentially bound by the cells, and second, both polypeptides are bound, but P68 is processed to the P64 component. Experiments are currently underway to determine which of the two possibilities are correct.

Taken together, our data indicate that specific binding sites on the NRK-2 cells exist for TAP. Along with their secretion and mitogenic activities, TAP closely resemble, polypeptide factors with growth-promoting activities and act on the cells in an autocrine fashion. However, since the transformed state of 6M2 cells correlates closely with the expression of the mos gene and the production of TAP in this cell system, the mitogenic activities of TAP probably play an important role in the establishment or maintenance of the transformation state of 6M2 cells at the permissive temperature.

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