Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 90-96

OVEREXPRESSION OF HUMAN TRK PROTO-ONCOGENE INTO MOUSE CELLS USING AN INDUCIBLE VECTOR SYSTEM

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Received February 14, 1991

The TRK proto-oncogene encodes a tyrosine kinase receptor for an, as yet, unidentified ligand. In order to help the identification of this ligand, we have constructed an expression vector capable of overexpressing the TRK protein in an inducible fashion. We report here the characterization of the TRK proto-oncogene products obtained from this expression vector. © 1991 Academic Press, Inc.

The TRK oncogene was first identified by gene transfer assay in DNA from a colon carcinoma (1,2). It encodes a Mr 70 cytoplasmic protein with tyrosine specific kinase activity (3). The TRK oncogene was shown to be derived from a gene coding for a tyrosine kinase receptor for an, as yet, unidentified ligand (4). The recombination event responsible for the activation of the normal locus as oncogene resulted in the replacement of the extracellular, ligand binding domain by sequences from tropomyosin (4,5).

The TRK proto-oncogene encodes a polypeptide of 790 residues which is processed to give two glycoproteins of Mr 110 and 145 with intrinsic tyrosine kinase activity. The Mr 145 moiety has been shown to be a transmembrane protein (4).

Identifying the ligand of the TRK proto-oncogene product would give important insight into its physiological role. In order to identify this ligand, we thought it necessary to obtain an inducible expression system expressing very high amount of the TRK proteins. For this purpose, the cDNA encoding the TRK proto-oncogene product was inserted into the autonomously replicating bovine papilloma virus vector, pBMT3X (6) and was under the control of an inducible metallothionein promoter.

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0006-291X/91 $1.50
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We report here the construction of the expression vector and the biochemical characterization of the TRK proto-oncogene products.

MATERIALS AND METHODS

Construction of recombinant BPV vector

A 2.9 kb EcoRI fragment containing the human TRK proto-oncogene cDNA sequence (plasmid pLM6, 4) was inserted into the XhoI site of pBMT3X (6) with the help of EcoRI/XhoI adaptor. The insert was under the control of an inducible metallothionein promoter. The resulting construct (pRM2, Figure 1) was checked for proper orientation of the insert by restriction enzymes mapping. Plasmid DNA was prepared by standard procedures (7), purified on CsC12 isopycnic gradient, and used for transfection.

Cell lines and transfection

C127 cells ($2x10^5$ cells per plate) were transfected with 0.1, 1 and 5 µg of pRM2 DNA by the calcium phosphate precipitation technique (8). After 24 h, the cells were trypsinized, replated and selected by culturing in the presence of 20 µM CdC12. Cadmium resistant colonies were picked up after 2-3 weeks and grown in 10 cm plate.

Immunization of mice against TRK proto-oncogene product

Female NIH Swiss mice were injected intraperitoneally with $2x10^6$ E25-427 cells expressing the TRK proto-oncogene proteins (4). Mice were injected once a week for 3 weeks, and bled periorbitally 6 days after the last immunization. The sera thus obtained was specific to the external domain of TRK proteins

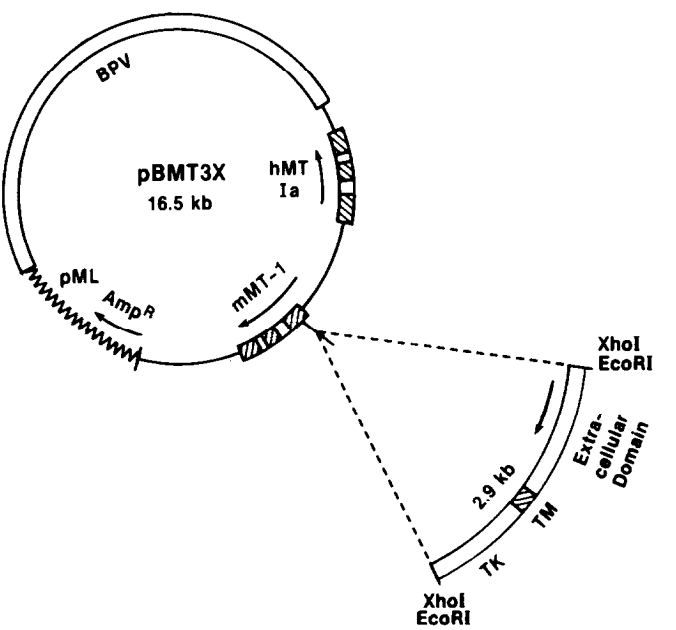


Figure 1. Schematic representation of pRM2 expression vector. Expression plasmid pRM2 was constructed as described in Materials and Methods. Sequences derived from Bovine Papilloma Virus are indicated (BPV). Wavy line represents pBR322 derived sequences. The human (hMT) and mouse (mMT-1) metallothionein genes are indicated. Sequences coding for the extracellular, transmembrane (TM) and tyrosine kinase (TK) domains of TRK were placed under the control of the mouse metallothionein gene promoter.

as it was able to immunoprecipitate them and not the TRK oncogene protein $p70^{TRK}$ (data not shown).

Other antibodies used in this study are anti-$p70^{TRK}$ antibodies raised against the bacterially expressed TRK oncogene product (3) and anti-C-terminal antibodies raised against a synthetic peptide corresponding to the 14 carboxy terminal residues of the human TRK proteins (4).

Cell labelling, immunoprecipitation and kinase assays

Subconfluent cultures (10 cm dishes) were preincubated for 30 min and labelled with [^{35}S]methionine (50 µCi/ml, 1200 Ci/mmol, ICN Radiochemicals) for 3 h in methionine-free Dulbecco's modified Eagle's medium (DMEM) containing 10% dialyzed calf serum (Gibco). Cells were washed with phosphate buffered saline (PBS), lysed in RIPA-buffer (9) and immunoprecipitated for 1.5 h using rabbit anti-$p70^{TRK}$ or anti-C-terminal sera, or polyclonal antibodies raised in mice against the external domain of the TRK proto-oncogene product. The resulting immunecomplexes were precipitated with protein A-Sepharose beads (Pharmacia) and resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 8% slab gels (10). To study the kinase activity, immunecomplexes were incubated with [γ^{32}P]-ATP (25 Ci/mmol; 20 µM) in the presence of 10 mM MnC12 and 5 mM MgC12 for 10 min at 30°C, washed with RIPA-buffer and analyzed by SDS-PAGE as described (11).

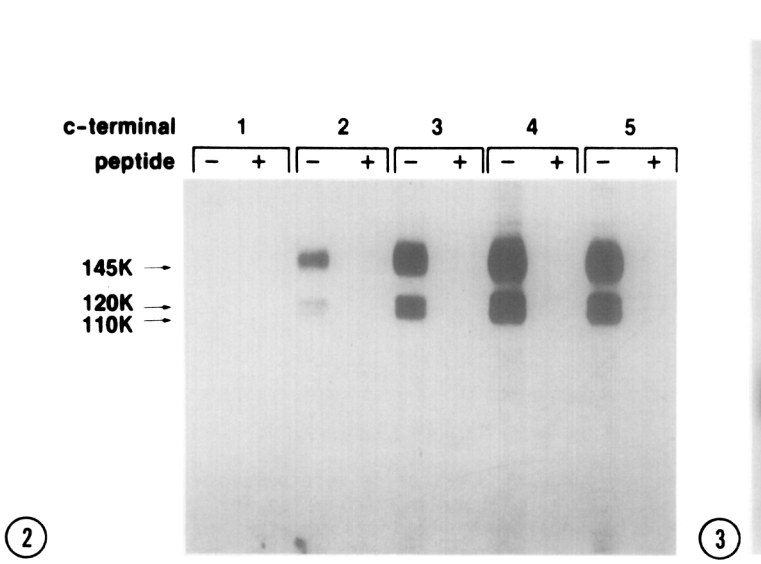
RESULTS

Isolation of TRK-proto-oncogene expressing clones

C127 cells were transfected with plasmid pRM2 (see Materials and Methods), and selected for heavy metal resistance in the presence of 20 µM CdC12. 15 cadmium resistance clones were isolated and screened for expression of TRK-proto-oncogene product. Immunoprecipitation of metabolically labelled cell extracts with TRK-specific antibodies showed that all 15 clones epxressed the TRK-specific proteins (data not shown). One of these clones, designated M3-21, was selected for all subsequent studies. In order to induce high expression of TRK proteins, M3-21 cells were grown in the presence of increasing amounts of CdC12 (0, 20, 50 and 70 µM) for 3-4 weeks. Cells were metabolically labelled and TRK proteins were immunoprecipitated with anti-C-terminal antibodies and resolved on polyacrylamide gels. As shown in Figure 2, there was at least a 5 fold increase in the amount of TRK-specific proteins synthesised in the presence of 50 µM CdC12. Higher concentration of cadmium, i.e. greater than 70 µM CdC12, seemed to be toxic to the cells.

Characterization of TRK-proto-oncogene products expressed in C127 transfected cells

Metabolically labelled cell extract from pRM2 transfected C127 cells were submitted to immunoprecipitation and analysed on SDS-polyacrylamide gel electrophoresis. All the sera tested (i.e. anti $p70^{TRK}$, anti-C-terminal, or anti external domain, (see Materials and Methods) were able to immunoprecipitate TRK-specific proteins. However, and in contrast to what have been previously reported in NIH 3T3 cells (4), pRM2 transfected C127 cells express 3 protein species with apparent relative molecular mass of 110, 120 and 145 (Figure 2).



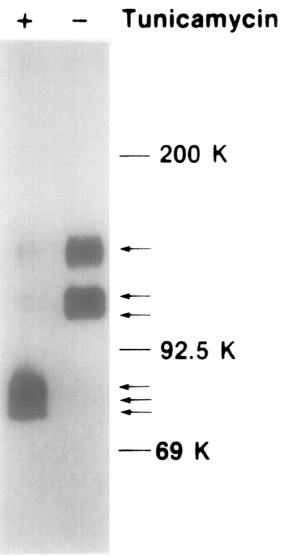


Figure 2. Induction of TRK proteins expression. Immunoprecipitation of [^{35}S]methionine-labelled cell extracts from (1) C127 cells or C127 cells transfected with pRM2 (M3-21) and grown (2) in the absence or in the presence of (3) 20 μM, (4) 50 μM and (5) 70 μM CdC12 with anti-C-terminal antibodies in either the absence (-) or the presence (+) of 10 μg of competing peptide. Relative molecular weights are shown on the left.

Figure 3.Tunicamycin treatment of TRK expressing cells. [^{35}S]methioneine-labelled M3-21 cell extracts grown either in the absence (-) or presence (+) of 10 μg/ml tuniamycin were incubated with anti-C-terminal antibodies. The resulting immunoprecipitatates were analysed as described. Arrows to the right indicate the positions of the various forms of the TRK proteins. Molecular weight markers include myosin (200,000), phosphorylase b (92,500) and bovine serum albumine (69,000).

This does not seem to be a characteristic of C127 cells since the same results were obtained when Balb/c 3T3 cells were transfected with pRM2. Tunicamycin treated M3-21 cells synthesized three unglycosylated proteins ranging in size from Mr 80 to 85 (Figure 3) as against one band in previously published work (4). However, in both cases, the extra bands could represent alternative initiation site usage, or incompletely processed proteins, due to overexpression of these peptides in our system.

In order to study the relationship between the 3 glycosylated protein bands (Mr 110, 120 and 145) , a pulse chase experiment was conducted. M3-21 cells were pulse-labelled with [^{35}S]-Methionine for 30 minutes and chased with non-radioactive amino-acids for different time intervals. As shown in Figure 4, the Mr 110 and 120 bands were visible immediately after the pulse period. These two bands gradually decreased in intensity with time of chase and by 150 minutes disappeared completely. The disappearance of the two lower bands coincided with the gradual appearance of the Mr 145 band. These results indicate that gp110 and gp120 represent the immature forms of the protein, which are further glycosylated to give the mature product, gp145.

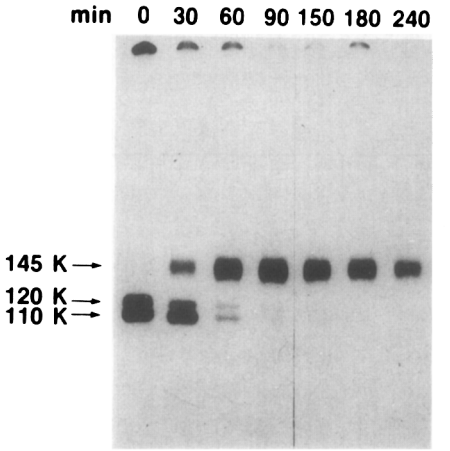


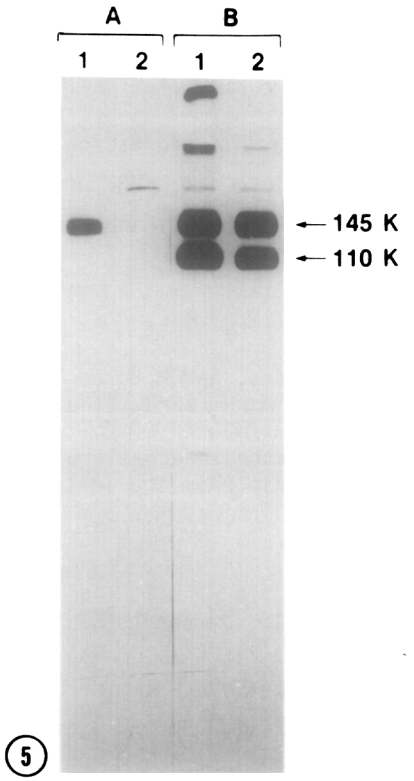
Figure 4. gp145^{TRK} is the mature product. M3-21 cells were pulsed with [^{35}S] methionine for 30 minutes, and chased with cold amino acids for the indicated period of time. Cells extracts were immunoprecipitated with anti-C-terminal antibodies and analysed by SDS-PAGE. The migration of gp110^{TRK}, gp120^{TRK} and gp145^{TRK} are indicated by arrows.

In order to confirm that the mature product, gp145^{TRK} was inserted in the proper orientation, cells were labelled with [^{35}S]-Methionine and incubated with either anti-p70^{TRK} or anti-external domain antibodies, either as a monolayer or as cell lysate. As shown in Figure 5, anti-external domain antibodies were able to immunoprecipitate gp145^{TRK} after incubation with the monolayer, whereas anti-p70^{TRK} antibodies did not bring down the protein. In the cell lysate, both antibodies recognized all 3 proteins efficiently. These results indicate that gp145^{TRK} is a transmembrane protein and that the putative extracellular domain is indeed expressed on the outside.

We next investigated the tyrosine kinase activity of the TRK proteins expressed in C127 cells. Lysates from M3-21 cells grown in the absence or in the presence of varying amount of cadmium were incubated with anti-p70^{TRK} antibodies and the resulting immunecomplexes were incubated with [γ^{32}P]-ATP in the presence of divalent cations. As shown in Figure 6, all three forms of the protein (gp110^{TRK}, gp120^{TRK} and gp145^{TRK}) were phosphorylated.

DISCUSSION

The TRK proto-oncogene codes for a putative growth factor receptor of an, as yet, unidentified ligand, with a tyrosine specific protein kinase activity (4). One approach towards the understanding of the biological function of the protein is by identifying its ligand. In this study, we attempted to develop cell lines overexpressing a functional TRK proto-oncogene product under the control of an inducible promoter, in order to facilitate the search of the ligand. Our results show that we can transfect C127 cells with pRM2, a bovine papillomavirus based vector containing the complete coding sequence



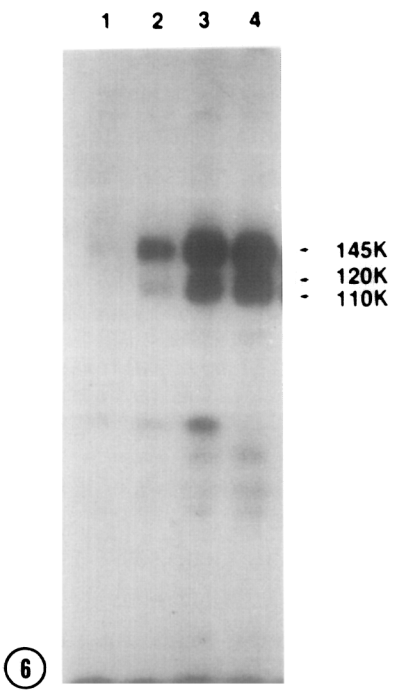


Figure 5. gp145^{IRK} is a transmembrane protein. [^{35}S]methionine-labelled cells were either (A) incubated as a monolayer with (1) anti-external domain or (2) anti p70^{TRK} antibodies, or (B) lysed first in RIPA buffer and then incubated with (1) anti-external domain or (2) anti p70^{TRK} antibodies. Immune complexes were analysed on SDS-PAGE. The migration of gp10^{TRK} and gp145^{TRK} are indicated by arrows.

Figure 6. The TRK proto-oncogene products have in vitro kinase activity. M3-21 cells grown (1) in the absence or in the presence of (2) 20 μM, (3) 50 μM, or (4) 70 μM CdC12 were immunoprecipitated with anti p70^{TRK} antibodies. Immune complexes were incubated with [γ-^{32}P]ATP and analysed by SDS-PAGE. The migration of gp110^{TRK}, gp120^{TRK} and gp145^{TRK} are indicated by arrows.

for the TRK proto-oncogene proteins under the control of the mouse metallothionein promoter, and the human methallothionein gene under the control of its own promoter. We were able to obtain stable transformants expressing the TRK proteins, and cultivation of these cell lines in the presence of increasing concentration of cadmium ions allowed us to induce a very high expression of TRK specific peptides.

Three TRK-specific proteins of Mr 110, 120 and 145, respectively, were detected in pRM2-transfected C127 cell lines, in contrast to what have been previously reported (4). The presence of an extra protein of Mr 120 is attributable to incomplete cleavage of the signal peptide, probably due to the overexpression of the proteins in this system. Pulse-chase experiment as well as tunicamycin treatment of the cells demonstrated that the Mr 110 and 120 proteins are glycosylated precursors of a more glycosylated form of Mr 145. The latter

species is a transmembrane protein having its N-terminus at the outside of the cell, and is thought to be the mature form of the TRK proto-oncogene products.

We have thus developed an inducible eukaryotic expression system able to produce appreciable amount of a mature, functional, TRK proto-oncogene. This system should facilitate the identification of the ligand of the TRK protein and give access to its normal function.

ACKNOWLEDGMENTS

We thank D.Martin-Zanca for helpful discussions, and M.Barbacid for enthusiastic support. We also thank G.Pavlakis for kindly providing us with the pBMT3X vector.

RM was supported by a fellowship from the Centre International de Recherche sur le Cancer. FC was the recipient of a fellowship from the Institut National de la Santé et de la Recherche Médicale.

REFERENCES

1) Pulciani S., Santos E., Lauver A.V., Long L.K., Aaronson S.A. and Barbacid M. (1982) Nature 300, 539-542.

2) Martin-Zanca D., Hughes S.H. and Barbacid M. (1986) Nature 319, 743-748.

3) Mitra G., Martin-Zanca D. and Barbacid M. (1987) Proc. Natl. Acad. Sci. USA, 84, 6707-6711.

4) Martin-Zanca D., Oskam R., Mitra G., Copeland T. and Barbacid M. (1989) Mol. Cell. Biol. 9, 24-33.

5) Coulier F., Martin-Zanca D., Ernst M. and Barbacid M. (1989) Mol. Cell. Biol. 9, 15-23.

6) Pavlakis G.N. and Hamer D.N. (1983) Recent Prog. Horm. Res. 39, 353-385.

7) Maniatis T., Fritsch E.F. and Sambrook J. (1980) in : Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

8) Graham F.L. and van der Eb A.J. (1973) Virology 52, 456-467.

9) Barbacid M., Beemon K, and Devare S.G. (1980) Proc. Natl. Acad. Sci. USA, 77, 5158-5162.

10) Laemmli U.K. (1970) Nature 277, 680-685.

11) Konopka J.B. and Witte O.N. (1985) Mol. Cell. Biol. 5, 3113-3123.