The EMBO Journal vol.9 no.1 pp.191 – 196, 1990

Oncogenic activation of the human *trk* proto-oncogene by recombination with the ribosomal large subunit protein L7a

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Communicated by R.Friis

The *trk*-2h oncogene, isolated from the human breast carcinoma cell line MDA-MB 231 by genomic DNA-transfection into NIH3T3 cells, consists of the *trk* proto-oncogene receptor kinase domain fused to a N-terminal 41 amino acid activating sequence (Kozma,S.C., Redmond,S.M.S., Xiao-Chang,F., Saurer,S.M., Groner,B. and Hynes,N.E. (1988) *EMBO J.*, 7, 147–154). Antibodies raised against a bacterially produced βgal–*trk* receptor kinase fusion protein recognized a 44 kd phosphoprotein phosphorylated on serine, threonine and tyrosine in extracts of *trk*-2h transformed NIH3T3 cells. *In vitro*, in the presence of Mn^{2+}/γATP, this protein became phosphorylated extensively on tyrosine. Cells transformed by *trk*-2h did not, however, show an elevation in total phosphotyrosine. We have cloned and sequenced the cDNA encoding the amino terminal activating sequences of *trk*-2h (Kozma *et al.*, 1988). The encoded protein has a high basic amino acid content and the gene is expressed as an abundant 1.2 kb mRNA in human, rat and mouse cells. Anti-peptide antibodies raised against a C-terminal peptide recognized specifically a 30 kd protein on Western blots of human, rat and mouse cell extracts. Immuno-fluorescence revealed, in addition to granular cytoplasmic fluorescence, intense nucleolar staining. The high basic amino acid content and nucleolar staining prompted us to investigate whether the 30 kd protein could be a ribosomal protein. Western immunoblotting analysis of 2D-electrophoretically resolved ribosomal proteins indicated that the 30 kd protein is the ribosomal large subunit protein L7a. This was confirmed by comparison with a published N-terminal sequence of rat L7a (Wittmann-Liebold,B., Geissler,A.W., Lin,A. and Wool,I.G. (1979) *J. Supramol. Struct.*, 12, 425–433). Thus, the *trk*-2h oncogene consists of 41 amino acids of the human ribosomal protein L7a fused onto the *trk* proto-oncogene receptor kinase domain. Ribosomes purified from cells transformed by *trk*-2h were found to have a significant amount of the *trk*-2h oncogene product tightly associated with them, and this raises the question whether activation of this oncogene might be the result of localization of the tyrosine kinase to ribosomes.

Key words: breast carcinoma/oncogenic activation/ribosomal protein L7a/*trk* tyrosine kinase

Introduction

Cellular tyrosine kinases can be oncogenically activated by a variety of mechanisms including, truncation of the ligand-binding domain of growth factor receptors, loss or replacement of carboxyl terminal regulatory tyrosine residues and point mutations (Yarden and Ullrich, 1988). In the case of the *trk*-1h oncogene isolated from a human colon carcinoma, the oncoprotein was generated by the fusion of 231 amino acids of non-muscle tropomyosin, corresponding to the product of the first seven exons, to 398 amino acids of the amino terminally truncated c-*trk* protein (Martin-Zanca *et al.*, 1986a, 1989). The *trk*-1h encodes a 70 kd cytosolic phosphoprotein with an associated tyrosine protein kinase activity (Mitra *et al.*, 1987). No additional modifications of the truncated *trk* proto-oncogene are necessary for its activation, the carboxyl termini of the *trk*-1h and the c-*trk* protein are identical (Martin-Zanca *et al.*, 1989). The *trk* proto-oncogene encodes a novel tyrosine kinase growth factor receptor for an unknown ligand (Martin-Zanca *et al.*, 1989).

We have recently isolated a *trk*-1h related oncogene, *trk*-2h, following transfection of genomic DNA from the human MDA-MB231 breast carcinoma cell line into NIH3T3 cells. The *trk*-2h chimeric protein consists of 41 amino acids of an unknown human protein fused to an amino terminally truncated c-*trk* protein (Kozma *et al.*, 1988). *trk*-2h shares the identical truncation breakpoint within the *trk* proto-oncogene with *trk*-1h. In this communication we show that *trk*-2h encodes a 44-kd phosphoprotein exhibiting tyrosine protein kinase activity and that the N-terminal 41 amino acids are derived from the N-terminus of the human ribosomal large subunit protein L7a. Furthermore, ribosomes purified from cells transformed by the *trk*-2h oncogene have a significant amount of the oncogene product tightly associated with them.

Results

trk-2h encodes a 44 kd phosphoprotein with tyrosine kinase activity

Immunoprecipitation of RIPA-buffer extracts (see Materials and methods) of cells labelled *in vivo* with either [^{35}S]methionine or [^{32}P]orthophosphate by an antiserum raised against a bacterially produced βgal–*trk*-2h fusion protein brought down specifically a 44 kd protein from cells transformed by *trk*-2h (Figure 1; top left Yll and top right Yll). The results using extracts of *trk*-1h transformed cells are shown in Figure 1 for comparison. The *trk*-1h gene product of the expected size, 70 kd, was precipitated specifically by the antiserum (Figure 1; top left 106 and top right 106). Phosphoamino acid analysis of excised and eluted *in vivo* [^{32}P]orthophosphate labelled $p44^{trk\text{-}2h}$ and $p70^{trk\text{-}1h}$ revealed that both proteins have substantial amounts of label on tyrosine residues in addition to serine and theronine (Figure 1, bottom Yll and 106, respectively).

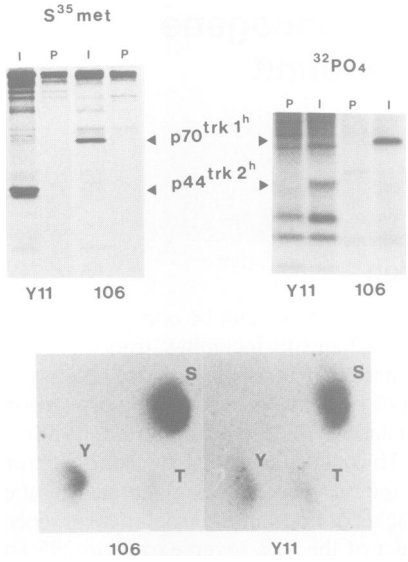

Fig. 1. Metabolic labelling of *trk*-1h and *trk*-2h oncogene products. NIH3T3 cells transformed by *trk*-1h (106) or *trk*-2h (Yll) were labelled with either [^{35}S]methionine (S^{35}met) or [^{32}P]orthophosphate ($^{32}PO_4$) and cell lysates immunoprecipitated with antiserum raised against bacterially produced βgal−*trk*-2h fusion protein. Lanes designated by a P are with pre-immune serum and by an I with immune serum. Phosphoamino acid analyses of [^{32}P]orthophosphate labelled p44$^{trk\text{-}2h}$ (Yll) and p70$^{trk\text{-}1h}$ (106) are shown at the bottom of the figure: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

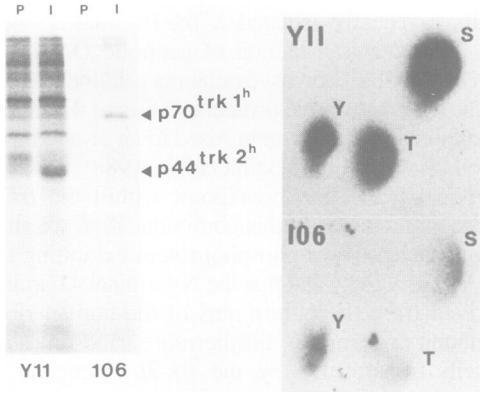

Fig. 2. *In vitro* protein kinase activity. Extracts of NIH3T3 cells transformed by *trk*-1h (106) or *trk*-2h (Yll) were precipitated with antiserum raised against the βgal−*trk*-2h fusion protein and the immunoprecipitates subject to a kinase reaction. Lanes designated by P are with pre-immune serum, by I with immune serum. Phosphoamino acid analyses of *in vitro* labelled p44$^{trk\text{-}2h}$ (Yll) and p70$^{trk\text{-}1h}$ (106) are shown on the right: S, phosphoserine; T, phosphothreonine; Y, phosphotyrosine.

The *in vitro* kinase activity associated with p44$^{trk\text{-}2h}$ is illustrated in Figure 2. In the presence of Mn^{2+} and [γ-^{32}P]ATP, immunoprecipitated p44$^{trk\text{-}2h}$ became labelled on tyrosine, serine, and threonine residues (Figure 2; left panel Yll, right panel Yll). Under identical experimental conditions p70$^{trk\text{-}1h}$ was also labelled on tyrosine, serine, and threonine. The consistent observation that *in vitro* phosphorylation on serine and threonine residues occurs concurrently with tyrosine phosphorylation is unusual for a tyrosine protein kinase and may reflect the presence of serine/threonine protein kinases non-specifically trapped in the immune complexes.

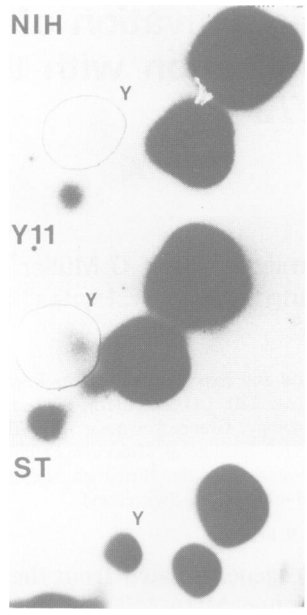

Fig. 3. Phosphotyrosine content of cells transformed by *trk*-2h. NIH3T3 cells (NIH), NIH3T3 cells transformed by *trk*-2h (Yll) and the Snyder-Theilen strain of feline sarcoma virus (ST) were metabolically labelled with [^{32}P]orthophosphate. Aliquots of RIPA-buffer lysates were hydrolysed in 6 M HCl and the phosphoamino acids resolved by 2D-electrophoresis. The area of the electrophorogram corresponding to phosphotyrosine (Y) is indicated.

Phosphoamino acid analysis of proteins extracted from cells labelled *in vivo* with [^{32}P]orthophosphate showed that transformation by *trk*-2h does not lead to a substantial elevation of phosphate on tyrosine residues (Figure 3; compare areas indicated in NIH and Yll). The dramatic increase in phosphotyrosine observed in NIH cells transformed by the ST-FeSV encoded *fes* tyrosine kinase oncogene is shown for comparison (Figure 3, ST).

The 5′ activating sequence of trk-2h is derived from the ribosomal L7a gene

We have recently cloned and sequenced the cDNA encoding the 5′ activating sequence of *trk*-2h from a human placental cDNA library. The cDNA has an open reading frame encoding a predicted protein of 266 amino acids, the most striking feature of which is a high content of the basic amino acids, lysine and arginine (23%; Kozma *et al.*, 1988). Data bank searches revealed no close similarities with other mammalian proteins.

We have prepared rabbit antipeptide antibodies against the C-terminal 14 amino acids of the unknown protein; LEKAKAKELATKLG. The antiserum recognized specifically a protein of approximately 30 000 molecular weight (30 kd) on Western blots using total protein extracts of human and mouse cells (Figure 4). Immunofluorescent staining of fixed, permeabilized mouse cells with the 30 kd protein antibody showed fairly uniform granular cytoplasmic fluorescence and intense nucleolar staining (Figure 5a). The nucleus was devoid of staining. An identical staining pattern was seen with human cells. This characteristic staining could be competed away by immunizing peptide (Figure 5b). The distinct nucleolar fluorescence and the high basic amino acid content of the 30 kd protein prompted us to analyse ribosomal proteins. Figure 6 shows the results of an experiment in which total protein extracts of human and mouse cells,

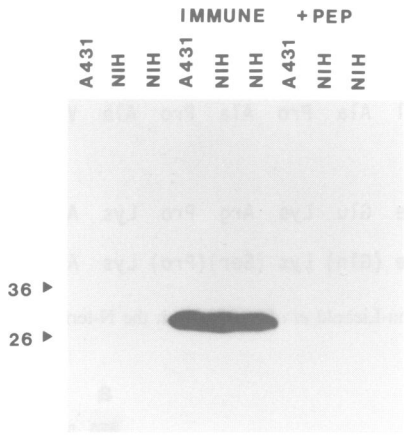

Fig. 4. Western immunoblot analysis of human (A431) and mouse (NIH) cell extracts using an antiserum raised against a C-terminal peptide of the activating protein. The first three lanes are with pre-immune serum, the middle three lanes with immune serum and the last three lanes with immune serum mixed with peptide. Molecular weight markers for 36 kd (36) and 26 kd (26) are indicated.

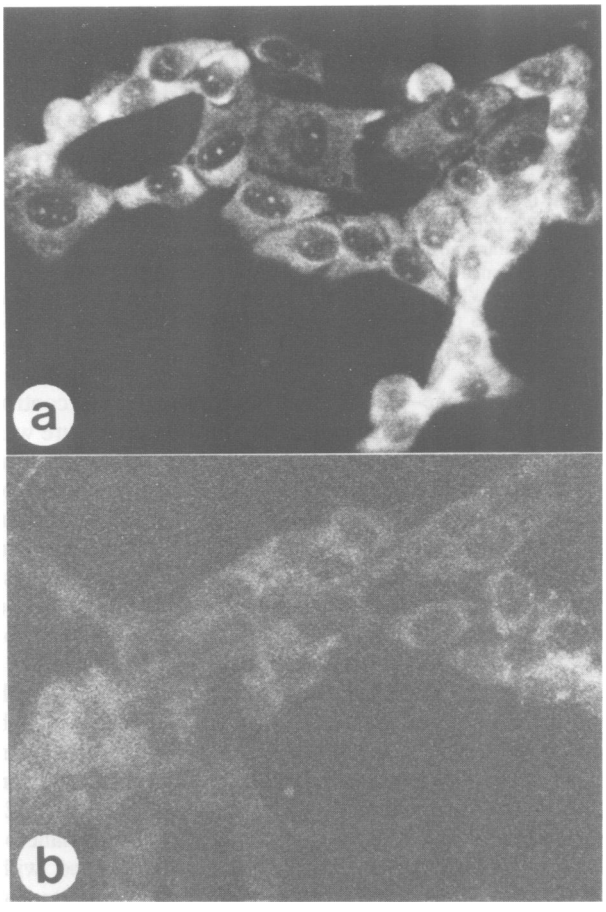

Fig. 5. Immunofluorescence observed with *trk*-2h transformed NIH3T3 cells incubated with the anti-30 kd protein serum: a, antiserum; b, antiserum mixed with peptide.

human and rat ribosomal proteins, and rat ribosomal small subunit proteins were resolved by PAGE, blotted and probed with the 30 kd antiserum. The 30 kd protein was detected specifically in all the preparations with the exception of the rat ribosomal small subunit proteins (Figure 6). This

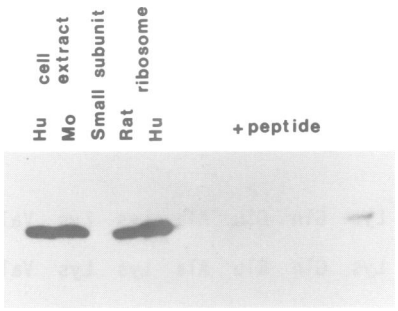

Fig. 6. Western immunoblot analysis of human (Hu) and mouse (Mo) cell extracts (~200 μg), rat and human ribosomes (~30 μg) and rat ribosomal small subunits (~20 μg) using the anti-30 kd protein serum. In the right half of the figure the antibody incubation was done in the presence of peptide.

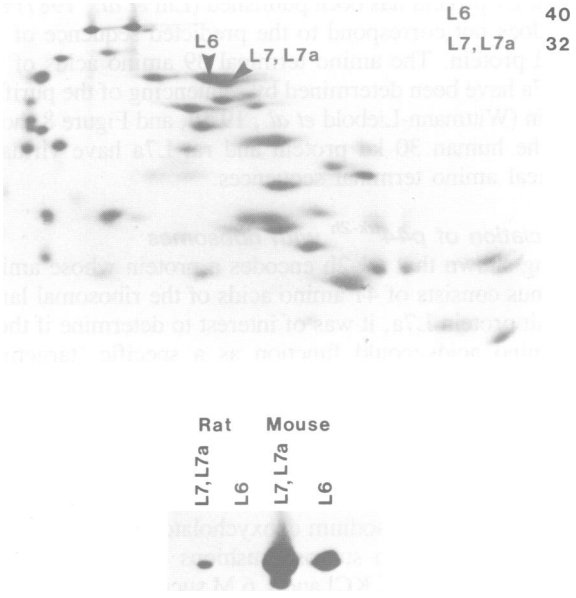

Fig. 7. Western immunoblot analysis of basic ribosomal proteins resolved by 2D-electrophoresis. The Coomassie blue spots corresponding to rat and mouse ribosomal proteins L6 and L7, L7a were excised from the 2D-gel, re-electrophoresed and immunoblotted using the anti-30 kd protein serum. The molecular weights of L6 and L7 as determined by SDS−PAGE are shown inset.

experiment suggested strongly that the 30 kd protein was associated with the ribosomal large subunits.

To identify which of the large subunit proteins reacted with the anti-peptide antiserum, we performed Western blotting experiments on ribosomal proteins separated by 2D-electrophoresis. The gel electrophoretic procedure and nomenclature proposed by McConkey *et al.* (1979) were adopted. A typical resolution pattern of the basic ribosomal proteins is shown in Figure 7. Western blot analysis of such gels using the 30 kd antiserum indicated that a protein in the vicinity of large subunit proteins L6, L7, or L7a reacted. The Coomassie stained spots corresponding to these ribosomal proteins were cut out, re-electrophoresed on an SDS gel, Western blotted and reprobed with the 30 kd antiserum. The antiserum recognized specifically the L7/L7a spots excised from 2D gels of both rat and mouse ribosomes (Figure 7, lower panel). The reaction seen in the lane corresponding to L6 is due to cross-contamination of the L6 spot with L7/L7a. This is supported by the facts that L6 has

									10										20
X	Met	Pro	Lys	Gly	Lys	Lys	Ala	Lys	Gly	Lys	Lys	Val	Ala	Pro	Ala	Pro	Ala	Val	Val	Lys
L7a		Pro	Lys	Gly	Lys	Lys	Ala	Lys	Gly	Lys	Lys	Val	Ala	Pro	Ala	Pro	Ala	Val	Val	Lys
									30										40
X	Lys	Gln	Glu	Ala	Lys	Lys	Val	Val	Asn	Pro	Leu	Phe	Glu	Lys	Arg	Pro	Lys	Asn	Phe	Gly
L7a	Lys	Gln	Glu	Ala	Lys	Lys	Val	(Val)	Asn	(Pro)	Leu	Phe	(Gln)	Lys	(Ser)	(Pro)	Lys	Asn	Phe	Gly

Fig. 8. Amino acid sequence comparison of the N-terminal 39 amino acids of L7a (Wittmann-Liebold *et al.*, 1979) with the N-terminus of the 30 kd protein (Kozma *et al.*, 1988).

a molecular weight of approximately 40 000 (Figure 7; Terao and Ogata, 1975), and that the published partial sequence of L6 does not correspond to the predicted amino acid sequence of the 30 kd protein. The entire sequence of the rat L7 protein has been published (Lin *et al.*, 1987) and also does not correspond to the predicted sequence of the 30 kd protein. The amino terminal 39 amino acids of the rat L7a have been determined by sequencing of the purified protein (Wittmann-Liebold *et al.*, 1979), and Figure 8 shows that the human 30 kd protein and rat L7a have virtually identical amino terminal sequences.

Association of p44$^{trk\text{-}2h}$ with ribosomes

Having shown that *trk*-2h encodes a protein whose amino terminus consists of 41 amino acids of the ribosomal large subunit protein L7a, it was of interest to determine if these 41 amino acids could function as a specific 'targeting' sequence for ribosomes. We have purified ribosomes from [^{35}S]methionine-labelled cells transformed by either the *trk*-2h or the *trk*-1h oncogene. The purification procedure, developed for the isolation of clean ribosomes, (see Materials and methods for exact details) consisted of ultracentrifugation of a Triton X-100−sodium deoxycholate detergent extract of cells through two sucrose cushions containing 0.7 M sucrose with 100 mK KCl and 1.6 M sucrose with 500 mM KCl, respectively, in extraction buffer lacking detergents. After centrifugation, the detergent extract, the 0.7 M sucrose and 1.7 M sucrose cushions were carefully collected and the pellet resuspended in cold RIPA buffer. Aliquots, corresponding to one fifth of each of the above four fractions, were diluted where necessary (1.7 M sucrose) and brought to 1% with Triton X-100 and deoxycholate where necessary (0.7 M sucrose and 1.6 M sucrose) and immunoprecipitated with an excess of an antiserum recognizing the *trk* kinase domain.

The results of this experiment are shown in Figure 9. The majority of p44$^{trk\text{-}2h}$ was found distributed among the detergent extract supernatant (sup) and the 0.7 M sucrose cushion (0.7 M) fraction. A small amount was found in the 1.6 M sucrose (1.6 M) fraction but a significantly larger portion was detected in the ribosomal pellet (pel). We interpret this as indicating a significant tight association of p44$^{trk\text{-}2h}$ with ribosomes. The results of three control experiments favour this conclusion: (i) an identical experiment with [^{35}S]methionine labelled extracts of cells transformed by p70$^{trk\text{-}1h}$ revealed most of the oncoprotein in the sup and 0.7 M fraction and none in the pellet. (ii) Western immunoblot analysis of equal aliquots of the above four fractions using the ribosomal large subunit protein L7a specific antibody showed that L7a was mostly in the pellet

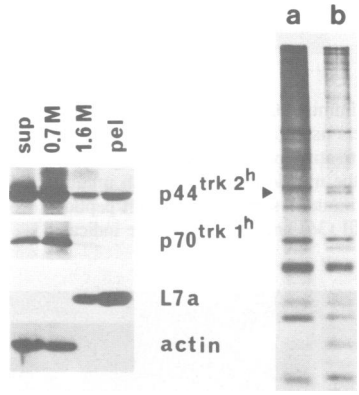

Fig. 9. Association of p44$^{trk\text{-}2h}$ with ribosomes. Four fractions were obtained after ultracentrifugation: (sup) detergent extract supernatant, (0.7 M) 0.7 M sucrose and 100 mM KCl, (1.6 M) 1.6 M sucrose and 500 mM KCl, (pel) ribosomal pellet. p44$^{trk\text{-}2h}$ and p70$^{trk\text{-}1h}$ were detected by immunoprecipitation of [^{35}S]methionine labelled proteins and the ribosomal protein L7a and actin were detected by Western immunoblotting. Lanes a and b are aliquots of [^{35}S]methionine labelled ribosomal pellets prepared from *trk*-1h and *trk*-2h transformed cells respectively.

with some in the 1.6 M sucrose fraction. This control demonstrates that the ribosomal purification procedure was successful and that the conditions are harsh, causing partial dissociation of the ribosomes. (iii) Western immunoblot analysis of aliquots identical to those above using an actin specific antibody revealed that the potentially 'sticky' protein actin was confined to the sup and 0.7 M fractions. Thus, under experimental conditions rigorous enough to cause partial dissociation of ribosomes, a significant amount of p44$^{trk\text{-}2}$ was found tightly associated with ribosomes.

Evidence for stochiometric association of p44$^{trk\text{-}2h}$ with ribosomes is also presented in Figure 9. Lanes a and b correspond to aliquots of ribosomes purified as above from *trk*-1h and *trk*-2h transformed cells respectively, lysed in electrophoretic sample buffer and resolved by SDS−PAGE. The *trk*-2h protein is seen as an additional band in lane b. Comparison of the intensity of the p44$^{trk\text{-}2h}$ band with ribosomal proteins of similar molecular weight indicate that approximately equal amounts of these proteins are present in the ribosomes.

Discussion

We have demonstrated that the *trk*-2h oncogene encodes a chimeric protein consisting of the first 41 amino acids of the human ribosomal large subunit protein L7a fused to the truncated human *trk* proto-oncogene tyrosine kinase domain.

These 41 amino acids correspond to the first two exons of the L7a gene (Huxley *et al.*, 1988; N.E.Hynes, unpublished). This rearrangement and concomitant oncogenic activation occurred during transfection. Recently, Giallongo *et al.* (1989) have shown that the mouse *surf*-3 gene encodes the mouse ribosomal large subunit protein L7a. Comparison of the amino acid sequence of mouse and human L7a revealed only one amino acid difference (Huxley *et al.*, 1988; Kozma *et al.*, 1988).

The N-terminal 41 amino acids derived from the ribosomal protein L7a endow $p44^{trk\text{-}2h}$ with the ability to associate strongly with ribosomes. Under conditions stringent enough to cause some dissociation, a significant portion of $p44^{trk\text{-}2h}$ remains associated with the ribosomes (Figure 9). It is unclear how this association is established and if there is any displacement of L7a. The stoichiometric data presented in Figure 9 would argue against displacement of L7a since there appear to be no differences in the intensities of the ribosomal bands in the region of L7a. Taken together, the data in Figure 9 also indicate that in *trk*-2h transformed cells a small amount of the over-expressed $p44^{trk\text{-}2h}$ is sufficient to saturate the available association site(s) on the ribosomes. Under identical experimental conditions neither the *trk*-1h encoded oncoprotein (Figure 9) nor the *gag-fgr* oncoprotein encoded by the TPI isolate of feline sarcoma virus (Ziemiecki *et al.*, 1984; Kappes *et al.*, 1989) was found associated with ribosomes (data not shown). We are presently investigating the kinetics of association in order to establish if $p44^{trk\text{-}2h}$ becomes associated with the ribosomes in the cytoplasm during or after synthesis, or if association occurs in the nucleolus. The latter would imply that the 41 amino acids of L7a contain a functional nucleolar localizing signal. Preliminary immunofluorescence experiments using antisera specific for the *trk* tyrosine kinase domain of $p44^{trk\text{-}2h}$ revealed cytoplasmic and perinuclear staining of *trk*-2h transformed cells with no apparent accumulation in the nucleolus.

The ribosomal localization raises the possibility that this 'targeting' is crucial for transformation. A ribosomal localization may bring the tyrosine kinase in a favourable environment with respect to potential substrates. Preliminary experiments looking at the *in vivo* and *in vitro* phosphorylation of ribosomal proteins have not revealed striking differences between *trk*-2h transformed cells and control NIH3T3 cells. Western blotting experiments using anti-phosphotyrosine antibodies have also failed to show any differences. Potentially important targets may be associated with ribosomes only fleetingly. One interesting possibility envisages that the ribosome-associated protein kinase could phosphorylate target proteins during their synthesis. This possibility could be tested in an *in vitro* protein synthesizing system utilizing ribosomes isolated from *trk*-2h transformed cells.

$p44^{trk\text{-}2h}$ has an associated tyrosine kinase activity, however, transformed cells do not exhibit substantially elevated levels of phosphotyrosine, a characteristic common to cells transformed *in vitro* by oncogenes of the *src* tyrosine protein kinase family. Cells transformed by *trk*-1h, the other characterized member of the *trk* family, also do not exhibit elevated levels of phosphotyrosine (Mitra *et al.*, 1987). The v-*fms*, v-*erbB* and v-*ros* oncogenes, derived from growth factor receptor genes (Downward *et al.*, 1984; Sheer *et al.*, 1985; Neckameyer *et al.*, 1986), also fail to illicit elevated levels of total cellular phosphotyrosine in transformed cells (Barbacid and Lauver, 1981; Reynolds *et al.*, 1981; Kamps and Sefton, 1988). This may reflect a general difference in activity of tyrosine kinases derived from growth factor receptors as compared to the *src* family tyrosine kinases and may implicate a role for a serine/threonine protein kinase activity in transformation.

The *trk* proto-oncogene appears particularly susceptible to activation during transfection and Oskam *et al.* (1988) have characterized 13 different *trk* oncogenes. Each has retained its tyrosine protein kinase activity and an intact C-terminus and has acquired a new amino terminus. These chimeric molecules exhibit different biochemical properties, probably conferred by the N-terminal activating sequences. What properties do these activating sequences need to have in order to bestow oncogenicity? Of the two characterized *trk* proto-oncogene activating genes, one encodes tropomyosin (*trk*-1h) and the other the ribosomal protein L7a (*trk*-2h). Both chimeric oncogenes are driven from strong promoters allowing a high level of oncogene product. Retrovirally-transduced oncogenes are also driven from strong promoters, and this may be the primary characteristic of an activating sequence. An additional property of such promoters may be the possibility of unscheduled expression of the *trk* proto-oncogene kinase in an inappropriate cell type or developmental stage. Indeed, expression of the *trk* proto-oncogene appears to be tightly controlled; it is expressed at low levels in some human cells of haematopoietic and mesenchymal origin, whereas human fibroblasts are negative (Martin-Zanca *et al.*, 1986b).

In addition to affecting the expression of the *trk* proto-oncogene kinase, the 5′ activating species could affect other properties including intracellular localization, stability, conformation, affinity for substrates and regulatory molecules and the enzymic activity itself. In a recent study aimed at determining the contribution of tropomyosin sequences to *trk* kinase oncogenic activation, Coulier *et al.* (1989) concluded that these sequences bestow a constitutively active conformation on the kinase domain. A proper appreciation of the activation process must await further characterization of the transformation mechanisms exemplified by the *trk* family of oncogenes.

Materials and methods

Cells: culture, metabolic labelling and immunoprecipitation

NIH3T3 cells expressing either the *trk*-1h (106) or *trk*-2h (Yll) oncogene were grown in DMEM supplemented with 10% fetal calf serum. Cells were metabolically labelled with [^{35}S]methionine (50 μCi/ml; 2 h) in methionine-free medium or [^{32}P]orthophosphate (1 mCi/ml; 5 h) in phosphate-free medium. Cells were washed on ice in PBS, lysed in RIPA buffer (20 mM Tris−HCl, pH 7.3, 1% Triton X-100, 1% Na deoxycholate, 0.1% SDS) containing 100 μM orthovanadate. Lysates were clarified by centrifugation in an Eppendorf bench centrifuge and incubated on ice for 60 min with antiserum. Antigen−antibody complexes were isolated using protein A-bearing *Staphylococcus aureus* (Cowan I strain) bacteria. After extensive RIPA buffer washing, precipitated proteins were solubilized by boiling for 2 min in electrophoresis sample buffer (80 mM Tris, pH 6.8, 10% glycerol, 2% SDS, 2% mercaptoethanol), and the labelled proteins resolved by SDS−PAGE (Laemmli, 1970). [^{35}S]methionine labelled proteins resolved by SDS−PAGE were visualized by fluorography (Bonner and Laskey, 1974). [^{32}P]orthophosphate labelled proteins were detected directly by exposure of dried gels to Kodak XAR-5 film. For *in vitro* kinase assays, cells were lysed in 10 mM PO_4 buffer, pH 7.2, containing 150 mM NaCl, 1% Triton X-100, 100 μM orthovanadate and 1 mM PMSF, and immunoprecipitates washed with the same buffer. The kinase reaction was carried out at 30°C for 10 min in 20 mM Tris−HCl, pH 7.3, containing

20 mM $MnCl_2$ and 5 μCi [γ-^{32}P]ATP (6000 Ci/mmol, Amersham). Phosphoamino acid analyses were carried out according to Hunter and Sefton (1980).

Western immunoblotting

The procedure used was a modification of the original described by Towbin *et al.* (1979). Proteins were electrophoretically transferred onto nitrocellulose membranes (BA85, Schleicher and Schüll) and blocked for 1 h at 37°C in freshly autoclaved PBS containing 0.5% calf skin gelatin. The filters were rinsed twice in PBS containing 0.25% Triton X-100, 0.02% gelatin (PTG) and reacted with antiserum for 1 h at 37°C. Filters were rinsed four times with PTG and incubated with [^{125}I]protein A (Amersham) at 0.1 μCi/ml for 30 min at 37°C. Filters were washed in several changes of PTG at 37°C, sealed in plastic bags and exposed to Kodak XAR-5 film.

Antibody production

Antipeptide antibodies were prepared in rabbits using peptide coupled to keyhole limpet haemocyanin by gluteraldehyde. Preparation of the *trk*-kinase domain specific antiserum was as follows: the pBD2 bacterial expression vector (Bröker, 1986) was used to construct a plasmid encoding a fusion protein consisting of the amino terminal 375 amino acids of βgal fused to amino acids 36–440 of *trk*-2h (βgal–*trk*-2h protein). The approximately 87 kd βgal–*trk*-2h fusion protein was isolated from total protein extracts of IPTG induced bacteria by preparative SDS gel electrophoresis followed by electro-elution. Rabbits were immunized by multiple intradermal injections of 100–200 μg of the βgal–*trk*-2h fusion protein. Chicken smooth muscle actin antiserum was a gift of Dr T.Kreis, EMBL, Heidelberg.

Immunofluorescence

Semi-confluent cells were washed twice in PBS containing Ca^{2+} and Mg^{2+}, fixed and permeabilized in acetone:methanol (1:1, kept at −20°C) for 2 min and air-dried. Two-fold dilutions of antipeptide antiserum in complete culture medium were spotted on and incubated for 60 min at 37°C in a CO_2-incubator. After several washes with water, the second antiserum, FITC conjugated goat anti-rabbit diluted in culture medium was applied for 60 min at 37°C. Cells were washed with water and examined under UV light after mounting in 10% Mowiol (Hoechst) containing 5% DABCO (diazobicyclo-octane) as an anti-bleaching agent.

Ribosome preparation

Purified ribosomes were prepared using the method described by Siegmann and Thomas (1987). Confluent plates of cells (typically 3 × 14 cm diameter) were washed on ice with PBS, followed by hypotonic buffer (5 mM Tris–HCl, pH 7.5, 1.5 mM KCl, 2.5 mM $MgCl_2$). Cells were lysed in hypotonic buffer containing 1% Triton X-100, 1% Na deoxycholate. Lysates were clarified by centrifugation at 2000 *g* for 5 min and layered onto a two-step gradient in a Beckman SW40 centrifugation tube (4 ml of 20 mM Tris–HCl, pH 7.5, containing 1.6 M sucrose, 500 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol overlayered with 4 ml of the above buffer containing 0.7 M sucrose and 100 mM KCl). Centrifugation was at 35 000 r.p.m. in the SW40 rotor for 18 h at 4°C. The detergent extract supernatant, the 0.7 M and the 1.6 M sucrose fractions were removed carefully and the pellet resuspended in RIPA buffer. Equal aliquots of each fraction were immunoprecipitated with antiserum specific for the *trk* tyrosine kinase domain and analysed by SDS–PAGE followed by DMSO-PPO fluorography.

Ribosomal proteins were resolved by two dimensional polyacrylamide gel electrophoresis according to McConkey *et al.* (1979).

Acknowledgements

The authors are grateful to the Ludwig Institute for Cancer Research for supporting this work. We thank Drs Dionisio Martin-Zanca and Mariano Barbacid (Frederick Cancer Research Facility) for anti p70^{trk} serum and the 106-63 cell line expressing p70^{trk}; Dr George Thomas (FMI, Basel) for information, advice and laboratory facilities; Dr Joachim Krieg (The Salk Institute, San Diego) for excellent 2D-gels of ribosomal proteins; Michel Siegmann (FMI, Basel) for patient help in preparing ribosomes and Drs Robert Friis and Bernd Groner for encouragement. We are grateful to Carol Widmer, Marianne Bischoff and Theres Tüscher for preparing the manuscript and Peter Wegmüller for photography.

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Received on March 13, 1989; revised on November 3, 1989