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Mechanism of Activation of the Human *trk* Oncogene

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The human *trk* oncogene was generated by a genetic rearrangement that replaced the extracellular domain of the normal *trk* tyrosine kinase receptor by sequences coding for the 221 amino-terminal residues of a nonmuscle tropomyosin. Molecular dissection of a cDNA clone of the *trk* oncogene indicated that both the tropomyosin and tyrosine kinase domains were required for proper transforming activity. Replacement of nonmuscle tropomyosin sequences with those of other tropomyosin isoforms had no deleterious effect. However, when tropomyosin sequences were replaced with those of another cytoskeletal gene, such as β-actin or β-globin, transforming activity was completely abolished. These results illustrate the important role of tropomyosin sequences in endowing the *trk* kinase with transforming properties. Functionally unrelated subdomains of the tropomyosin molecule were equally efficient in activating the *trk* gene. Moreover, the transforming activity of the *trk* oncogene was not affected when its subcellular localization was drastically altered. Therefore, tropomyosin sequences are likely to contribute to the malignant activation of the *trk* oncogene not by facilitating its interaction with defined cystoskeletal structures as initially suspected, but by allowing its kinase domain to fold into a constitutively active configuration.

Gene transfer assays have unveiled the existence of oncogenes in human malignancies. Most of these oncogenes turned out to be members of the *ras* family, so far the most frequent oncogenes present in human cancers (1). However, other classes of transforming genes have been identified by this experimental approach. One such gene is *trk*, an oncogene activated by a genetic rearrangement that occurred in a colon carcinoma patient (23, 28). This rearrangement fused a tropomyosin gene with a decapitated *trk* proto-oncogene locus. As a result of this mutation, transcription from a nonmuscle tropomyosin gene proceeded into *trk* proto-oncogene sequences, generating a hybrid transcript whose translation yielded a transforming protein in which most of the extracellular domain of the *trk* proto-oncogene product was replaced by 221 amino-terminal residues of the tropomyosin molecule (23).

Other members of the tyrosine kinase gene family have been implicated in the development of human cancer. Generation of the Philadelphia chromosome in chronic myelogenous and acute lymphocytic leukemias results from a genomic rearrangement in which a decapitated c-*abl* proto-oncogene protein is fused to the amino-terminal domain of *bcr*, the product of an unrelated locus (4, 9, 12, 17, 29). Amplification of two related genes, the epidermal growth factor (EGF) receptor and the *erb*B2 proto-oncogene, has been observed frequently in glial tumors and in breast carcinomas, respectively (15, 19, 30, 32). So far, the involvement of the *trk* locus in human malignancies has been limited to a single colon carcinoma (23). However, no systematic studies have been conducted as yet to determine the extent to which this proto-oncogene might contribute to human cancer. Recent evidence indicates that the *trk* tyrosine kinase is rather prone to malignant activation. Kozma et al. (14) have reported the isolation of two independent *trk* oncogenes that became activated during the course of gene transfer assays by genetic rearrangements similar to that which occurred in vivo. Moreover, multiple *trk* oncogenes have been generated in vitro by transfection of NIH 3T3 cells with plasmids carrying the *trk* kinase domain (27). Therefore, it is likely that the *trk* proto-oncogene may play a more significant role in the genesis of human cancer than initially suspected.

The present studies were undertaken to dissect the contribution of each of the distinct domains of the human *trk* oncogene to malignant transformation in an effort to understand the detailed mechanisms by which the *trk* proto-oncogene can be endowed with oncogenic properties.

MATERIALS AND METHODS

Expression plasmids. The expression plasmids generated in this study were constructed as follows. For the pDM series, (i) pDM18 was generated by deleting the 163-base-pair (bp) *Eco*RV-*Kpn*I DNA fragment of pDM16 (23). The 3′ overhang of the *Kpn*I site was removed prior to religation. (ii) pDM20 was generated by removing the 521-bp *Nco*I-*Sca*I DNA fragment from pDM16, followed by religation in the presence of the appropriate adaptor

5′-CATGGCTGGGATCT-3′
3′-CGACCCTAGA-5′

(iii) pDM22 was generated by deleting the 855-bp *Nco*I-*Bal*I DNA fragment of pDM16. The *Nco*I site encompassing the ATG initiator codon was regenerated by using the same adaptor used to construct pDM20. (iv) pDM35 was generated by replacing the 1.5-kbp *Sca*I-*Eco*RI DNA fragment of pDM16 by the corresponding 1.4-kbp *Hin*dIII-*Eco*RI DNA fragment of the *trk* proto-oncogene (25). This *Hin*dIII-*Eco*RI DNA fragment was obtained from a deletion mutant of pLM6 that lacked the 5′ 1,250 bp. (v) pDM37 was generated by replacing the 1,029-bp *Xmn*I-*Eco*RI DNA fragment of pDM16 by the equivalent 1,043-bp DNA fragment of pLM6 (25). For the pFC series, (i) pFC1 was generated by replacing the 1,279-bp *Nco*I DNA fragment of pDM16 with the 621-bp *Eco*RI-*Nco*I fragment of a Bal 31 deletion mutant that contained the 3′ 1,405 bp of the *trk* oncogene preceded by an

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*Eco*RI linker. This fragment was ligated with the help of an *Nco*I-*Eco*RI adaptor,

5′-AATTCTCCATGGAG-3′
3′-GAGGTACCTCTTAA-5′

that preserved the tropomyosin translational initiator. (ii) pFC2 was generated by inserting a 597-bp *Nco*I fragment of TM36 (20) into the 5′ *Nco*I site of pFC1. (iii) pFC3 was generated by inserting a 365-bp *Nco*RI fragment of pB6-6 (22) into the 5′ *Nco*I site of pFC1 with the help of the same *Nco*I-*Eco*RI adaptor used to construct pFC1. (iv) pFC4 was generated by inserting a 666-bp *Nco*I-*Taq*I DNA fragment from pB2000 (5) with the help of a synthetic adaptor,

5′-CGCCATGG-3′
3′-GGTACCGC-5′

(v) pFC7 was generated by inserting the 521-bp *Nco*I-*Sca*I DNA fragment of pDM16 into the *Nco*I site of pDM22 with the help of an appropriate adaptor,

5′-ACTTTTCTTC-3′
3′-TGAAAAGAAGGTAC-5′

(vi) pFC10 was generated by inserting a 521-bp *Nco*I-*Sca*I DNA fragment of pDM16 into the 5′ *Nco*I site of pFC1 by using the adaptor described to construct pFC7. (vii) pFC13 was generated by inserting a 165-bp *Nco*I-*Bam*HI DNA fragment from pDM16 into the 5′ *Nco*I site of pFC1 by using an *Nco*I-*Bam*HI adaptor,

5′-GATCGCCATGGC-3′
3′-CGGTACCGCTAG-5′

(viii) pFC16 was generated by replacing the 1,279-bp *Nco*I DNA fragment of pDM16 with an 877-bp *Bcl*I-*Nco*I DNA fragment isolated from unmethylated pDM10-1 DNA (23) in which the *Bcl*I site has been replaced by an *Nco*I site with the help of an appropriate adaptor,

5′-GATCGCCATGGC-3′
3′-CGGTACCGCTAG-5′

(ix) pFC23 was generated by inserting a 225-bp *Ban*II-*Eco*RI DNA fragment from pLSX (31) into the 5′ *Nco*I site of pDM16 with the help of *Ban*II-*Nco*I and *Eco*RI-*Nco*I adaptors:

5′-ATGGGGG-3′
3′-TCGGTACCCCC-5′

and

5′-AATTCCCATGGG-3′
3′-GGGTACCCTTAA-5′

(x) pFC26 was generated by ligating a synthetic 93-bp *Nco*I DNA fragment corresponding to the signal peptide coding domain of pLSX into the 5′ *Nco*I site of pDM16. The second ATG codon was eliminated by site-directed mutagenesis as described before (16). The presence of the desired mutation (ATG→TTG) was determined by double-stranded DNA sequencing with modified T7 polymerase (Sequennase; United States Biochemicals). Restriction digestion, DNA fragment purification, and DNA ligation were done by standard protocols (21). Synthetic adaptors were prepared on solid support with DNA synthesizer model 380B (Applied Biosystems).

Gene transfer assays. Mouse NIH 3T3 cells were transfected with various amounts of linearized plasmid DNAs by the calcium phosphate precipitation technique (8). Transformed foci were scored 10 to 14 days after transfection.

Cell labeling, immunoprecipitation, and kinase assays. Subconfluent cultures (10-cm dishes) were preincubated for 30 min and labeled with [^{35}S]methionine (50 μCi/ml, 1,200 Ci/mmol; ICN Radiochemicals) for 3 h in methionine-free Dulbecco modified Eagle medium (DMEM) containing 10% dialyzed calf serum (Gibco). Cells were washed with phosphate-buffered saline, lysed in RIPA buffer (2), and immunoprecipitated for 1.5 h with polyclonal antibodies raised in rabbits against bacterially expressed p70^{trk} (26). The resulting immunocomplexes were precipitated with protein A-Sepharose beads (Pharmacia) and resolved by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 8% acrylamide slab gels (18). Immunocomplexes were incubated with [γ-^{32}P]ATP (25 Ci/mmol; 20 μM) in the presence of 10 mM $MnCl_2$ and 5 mM $MgCl_2$ for 10 min at 30°C, washed with RIPA buffer, and analyzed by SDS-PAGE as described before (12).

Cellular fractionation. Exponentially growing pDM16- or pFC26-transformed NIH 3T3 cells were metabolically labeled with [^{35}S]methionine for 3 h in methionine-free DMEM supplemented with 10% dialyzed calf serum (Gibco). After three washes in phosphate-buffered saline, cells were lysed in hypotonic buffer (20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, pH 7.4], 1 mM EDTA, 1 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). Cell lysates were fractionated into nuclear, soluble (S-100), and particulate (P-100) fractions by differential centrifugation as described before (3). Protein extracts were subjected to immunoprecipitation as described above.

RESULTS

Role of tropomyosin sequences in malignant activation. To understand the mechanism of activation of the *trk* oncogene, we first examined the contribution of nonmuscle tropomyosin sequences to the malignant activation of the human *trk* locus. For this purpose, we generated pDM35, a tropomyosin-*trk* gene chimera in which the first five coding exons of a human nonmuscle tropomyosin gene derived from the *trk* oncogene (23) were ligated to a truncated cDNA clone of the human *trk* proto-oncogene (25). This truncated cDNA lacked the 5′ 1,250 nucleotides of the *trk* proto-oncogene, which correspond to all but four of the nucleotides replaced by tropomyosin in the *trk* oncogene. A second chimera, pDM37, containing the seven tropomyosin exons present in the *trk* oncogene was also obtained. This construct contained 360 bp derived from the *trk* oncogene. However, these sequences are unlikely to contribute to malignant transformation since they were found to be identical to those present in the *trk* proto-oncogene.

These in vitro-generated tropomyosin-*trk* chimeras were tested in NIH 3T3 gene transfer assays along with pDM16, the expression plasmid carrying the *trk* oncogene (23), and pDM38, the corresponding plasmid carrying the nontransforming *trk* proto-oncogene (25). Both pDM35 and pDM37 induced foci of transformed cells at efficiencies (1×10^5 to 2×10^5 FFU/μg of DNA) comparable to that of pDM16 (Fig. 1). These results demonstrate that replacement of the putative extracellular domain of the human *trk* proto-oncogene by nonmuscle tropomyosin sequences is sufficient to activate its transforming potential.

Contribution of specific domains to malignant transformation. We next investigated the contribution of each of the three distinctive domains of the *trk* oncogene—tropomyosin sequences, transmembrane region, and tyrosine kinase catalytic domain—to its transforming properties. For this pur-

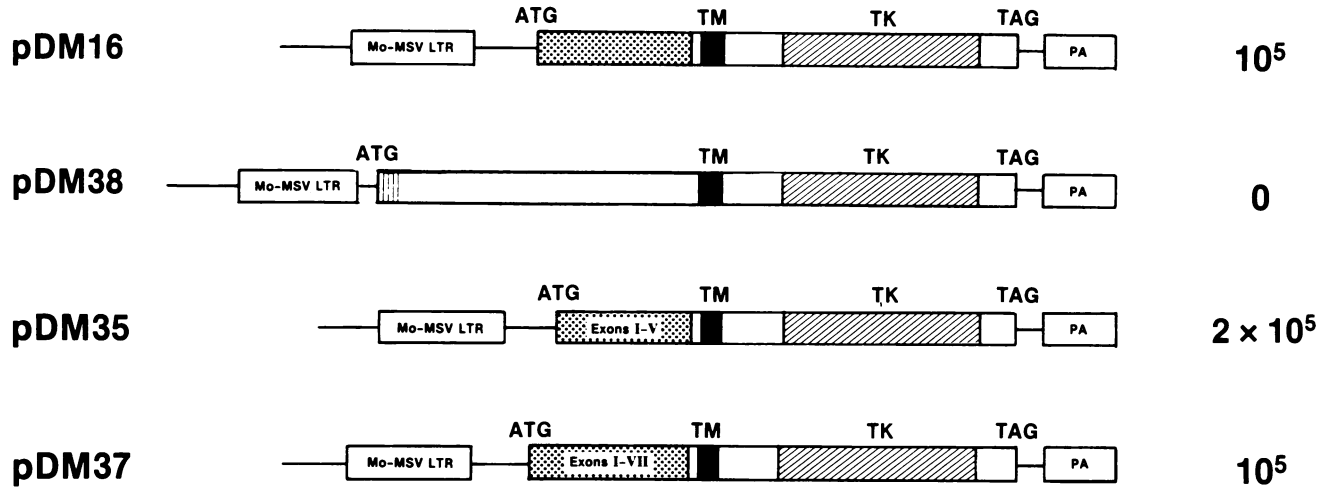

FIG. 1. In vitro activation of the transforming properties of the *trk* proto-oncogene. Expression plasmids containing the *trk* oncogene (pDM16) and *trk* proto-oncogene (pDM38) cDNA clones have been described (23, 25). pDM35 and pDM37 plasmids were constructed as described in Materials and Methods. Thick bars represent coding sequences. Sequences derived from human nonmuscle tropomyosin (stippled bars) and the *trk* proto-oncogene (open bars) are shown. The putative signal peptide (vertically shaded bars) transmembrane (TM, solid bars), and kinase catalytic (TK, diagonally shaded bars) domains are superimposed. Coding sequences were placed between a 5′ Mo-MSV LTR and a 3′ SV40 polyadenylation signal (PA) and tested in gene transfer assays with NIH 3T3 cells as recipients. Their respective transforming activities are shown on the right as number of foci induced per microgram of transfected DNA.

pose, we generated four plasmids that either lacked the tropomyosin or transmembrane regions or had deletions within the consensus tyrosine kinase sequences. Each of these plasmids contained identical punctuation signals. They are driven by a Moloney murine sarcoma virus (Mo-MSV) long terminal repeat (LTR) (6) and have an efficient ATG translational initiator (encompassed within an *Nco*I recognition site), and their coding sequences are followed by the simian virus 40 (SV40) polyadenylation signal (7). The schematic representation of these plasmids is depicted in Fig. 2. Precise information about their generation is given in Materials and Methods.

(i) pFC1 is a tropomyosin deletion mutant that codes for a 45-kilodalton (kDa) truncated form of the *trk* oncogene

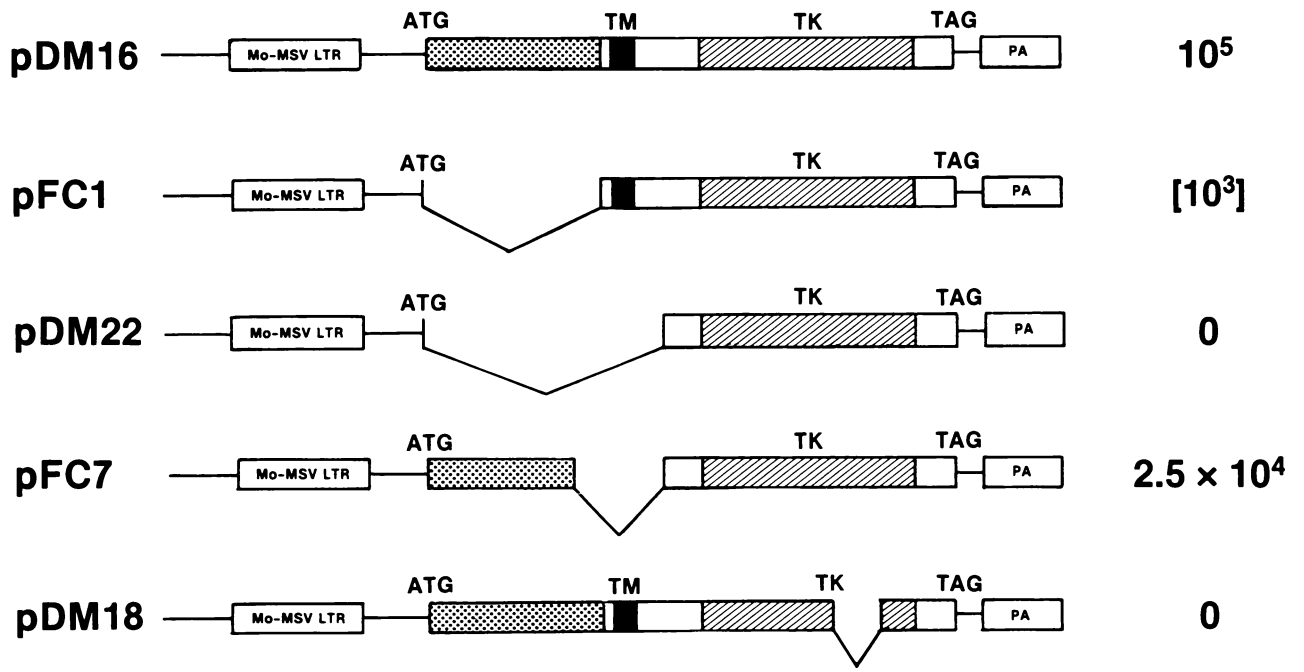

FIG. 2. Contribution of *trk* oncogene domains to malignant transformation. Construction of deletion mutants pFC1, pDM22, pFC7, and pDM18 is described in Materials and Methods. Each clone contains the initiator codon (ATG) of the *trk* oncogene. Symbols are those described in the legend to Fig. 1. Deleted sequences are indicated by thin lines pointing down. Coding sequences are contiguous in all the clones. Transforming activities of these plasmids in NIH 3T3 gene transfer assays are given on the right as number of foci induced per microgram of transfected DNA. Brackets indicate partially transformed morphology (see text).

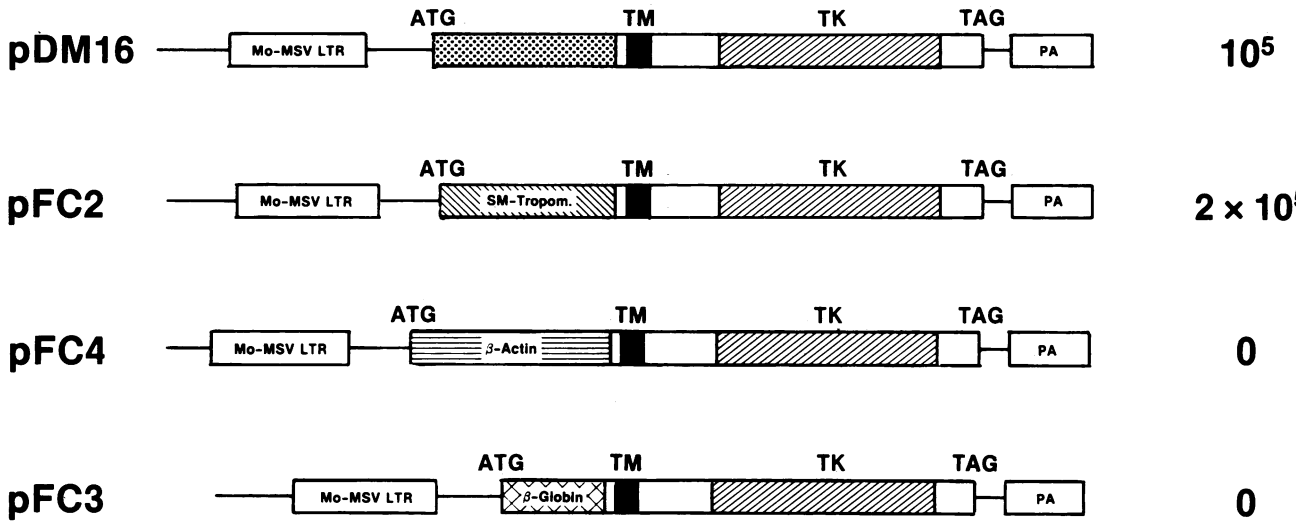

FIG. 3. Malignant activation of *trk* oncogene sequences by different genes. Chimeric clones were constructed by insertion of smooth muscle tropomyosin (SM-Tropom), β-actin, or β-globin sequences into the *Nco*I site of pFC1 as described in Materials and Methods. Each clone contains the initiator codon (ATG) of the *trk* oncogene. Other symbols are described in the legend to Fig. 1. Transforming activities of these plasmids in NIH 3T3 gene transfer assays are given on the right as number of foci induced per microgram of transfected DNA.

product $p70^{trk}$ in which the 221 amino-acid-long tropomyosin domain has been replaced by a linker-derived tetrapeptide, Met-Gln-Asn-Ser. (ii) pDM22 is similar to pFC1 except that it also lacks the transmembrane domain. This plasmid codes for a 36-kDa protein that corresponds to the carboxy-terminal moiety of $p70^{trk}$. p36 also contains the four amino-terminal residues of $p70^{trk}$, Met-Ala-Gly-Ile, which were regenerated by the corresponding linker. (iii) pFC7 lacks the transmembrane domain. This plasmid is a pDM22 derivative generated by the addition of cDNA sequences coding for the first five nonmuscle tropomyosin exons. (iv) pDM18 codes for a protein lacking 55 amino acid residues within the tyrosine kinase catalytic domain of $p70^{trk}$. This deletion encompasses Tyr-503, the residue corresponding to the autophosphorylation site of $pp60^{v\text{-}src}$, Tyr-416 (10).

The transforming activity of each of these deletion mutants was tested in NIH 3T3 gene transfer assays. As shown in Fig. 2, removal of the tropomyosin sequences from the *trk* oncogene drastically reduced (about 100-fold) its ability to transform NIH 3T3 cells. More importantly, pFC1-transfected NIH 3T3 cells did not have the refractile morphology characteristic of those transformed by the *trk* oncogene. Instead, these cells exhibited a rather normal phenotype, although they had a tendency to pile up in dense cultures. NIH 3T3 cells expressing the pFC1-coded 45-kDa protein grew poorly in agar (<5% efficiency) and were at least 10-fold less tumorigenic in nude mice than their *trk*-transformed counterparts (data not shown). Thus, tropomyosin sequences are required to activate the full transforming potential of the *trk* oncogene.

Removal of the transmembrane domain in conjunction with the tropomyosin sequences completely abolished transforming activity. NIH 3T3 cells transfected with microgram amounts of pDM22 did not exhibit detectable phenotypic changes, in spite of expressing high levels of the truncated 36-kDa *trk* molecule. This pDM22-coded p36 protein possessed an efficient in vitro tyrosine kinase activity, indicating that kinase activity alone is not sufficient to confer transforming properties to the human *trk* oncogene.

pFC7, the mutant lacking the transmembrane domain, retained full transforming activity, indicating that membrane association of the *trk* oncogene product is not required for transformation. These observations were not unexpected, since we have previously shown that $p70^{trk}$ is mostly located in the cytoplasm (26). Finally, the internal deletion in the *trk* kinase domain of pDM18 completely eliminated the transforming activity of this plasmid. G418-resistant NIH 3T3 colonies expressing levels of pDM18-coded p62 protein similar to those of $p70^{trk}$ in transformed NIH 3T3 cells were isolated. Immunoprecipitates obtained by incubating cell extracts derived from these morphologically normal pDM18-transfected cells with anti-$p70^{trk}$ antibodies failed to show detectable tyrosine kinase activity in the deleted p62 molecule (data not shown). These results indicate that kinase activity, although not sufficient, is absolutely required to endow the *trk* gene with transforming properties.

Malignant activation by other genes. In view of the important role that the nonmuscle tropomyosin domain plays in the activation of the *trk* oncogene, we examined whether other cytoskeletal or noncytoskeletal genes may have similar effects. For this purpose, we generated three plasmids by inserting smooth muscle tropomyosin (pFC2), β-actin (pFC4), and β-globin (pFC3) cDNA sequences into the pFC1 deletion mutant, which lacks nonmuscle tropomyosin sequences. In each case, the inserted sequences were introduced at the *Nco*I site containing the initiator ATG, allowing us to maintain a common and efficient translational initiator for each of these constructs. pFC2 was generated by inserting a 597-bp *Nco*I DNA fragment of TM36, a cDNA clone that codes for the 199 amino-terminal residues of a human smooth muscle tropomyosin (20). pFC4 was obtained by inserting a 666-bp *Nco*I-*Taq*I DNA fragment of a chicken β-actin cDNA clone (5) into the *Nco*I site of pFC1 with the help of a synthetic *Taq*I-*Nco*I adaptor. The deduced amino acid sequence of this chicken cDNA clone is identical to that of the corresponding region of the human β-actin gene (13). Finally, pFC3 resulted from inserting a 365-bp *Nco*I-*Eco*RI fragment coding for the 122 amino-terminal residues of the human β-globin gene product (22).

As shown in Fig. 3, the chimeric smooth muscle tropomyosin-*trk* clone was as efficient in transforming NIH 3T3 cells as the wild-type human colon carcinoma isolate containing

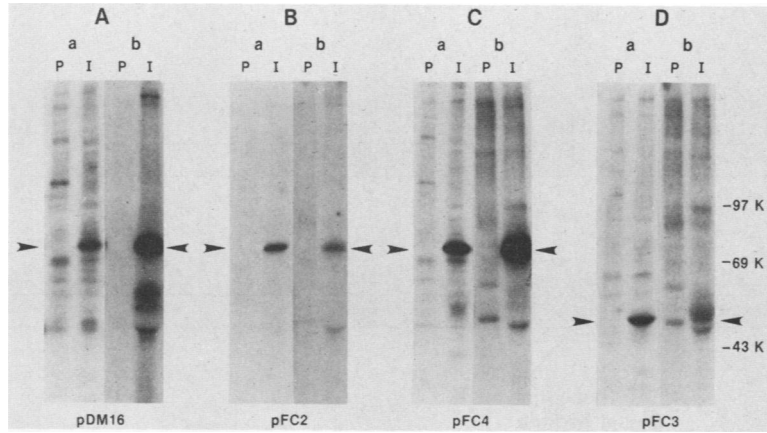

FIG. 4. Expression and protein kinase activity of chimeric *trk* proteins. (a) Immunoprecipitation of [^{35}S]methionine-labeled cell extracts from NIH 3T3 cells transfected with (A) pDM16, (B) pFC2, (C) pFC4, and (D) pFC3 plasmid DNAs with either preimmune (P) or immune (I) anti-p70^{trk} antiserum. (b) Protein kinase activity in each of the above immunoprecipitates. [^{35}S]methionine- (a) or ^{32}P-labeled (b) immunocomplexes were analyzed by 8% polyacrylamide-SDS PAGE as described in the text. Arrows indicate the migration of (A) wild-type p70^{trk}, (B) p70^{SMtrk}, (C) p68$^{actin\text{-}trk}$, and (D) p50$^{globin\text{-}trk}$. Molecular mass markers (in kilodaltons) include phosphorylase b (97 kDa [K]) bovine serum albumin (69 kDa), and ovalbumin (43 kDa).

the nonmuscle tropomyosin isoform. However, neither the β-actin nor the β-globin chimera exhibited detectable transforming activity. Cotransfection of these plasmids with pSV2*neo* allowed us to isolate morphologically normal NIH 3T3 cells expressing high levels of their corresponding products, p68$^{actin\text{-}trk}$ and p50$^{globin\text{-}trk}$ (Fig. 4). These cells did not grow in agar and were not tumorigenic in vivo. Yet both the pFC4-coded p68$^{actin\text{-}trk}$ and pFC3-coded p50$^{globin\text{-}trk}$ molecules exhibited an in vitro tyrosine protein kinase activity comparable to that of transforming p70^{trk} (Fig. 4). These results suggest that the failure of β-actin and β-globin sequences to activate the transforming properties of the

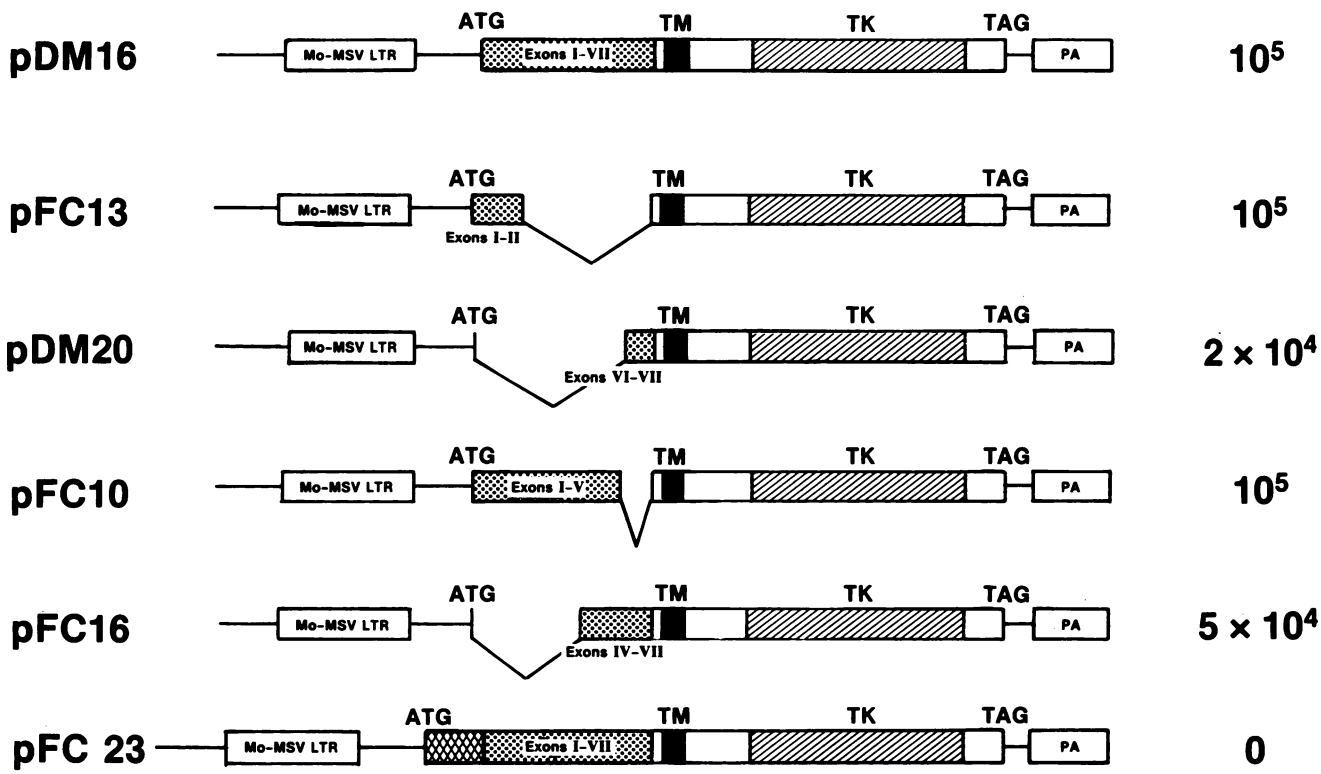

FIG. 5. Contribution of tropomyosin domains to malignant transformation. Plasmids were generated as described in Materials and Methods. Tropomyosin sequences present in pFC13 encompass amino acid residues 1 to 56; those present in pDM20 include residues 178 to 221, and those present in pFC16 are residues 136 to 221. The cross-hatched areas denote EGF receptor cDNA-derived sequences. Deleted sequences are indicated. Other symbols are described in the legend to Fig. 1. Transforming activities of these plasmids in NIH 3T3 gene transfer assays are given on the right as number of foci induced per microgram of transfected DNA.

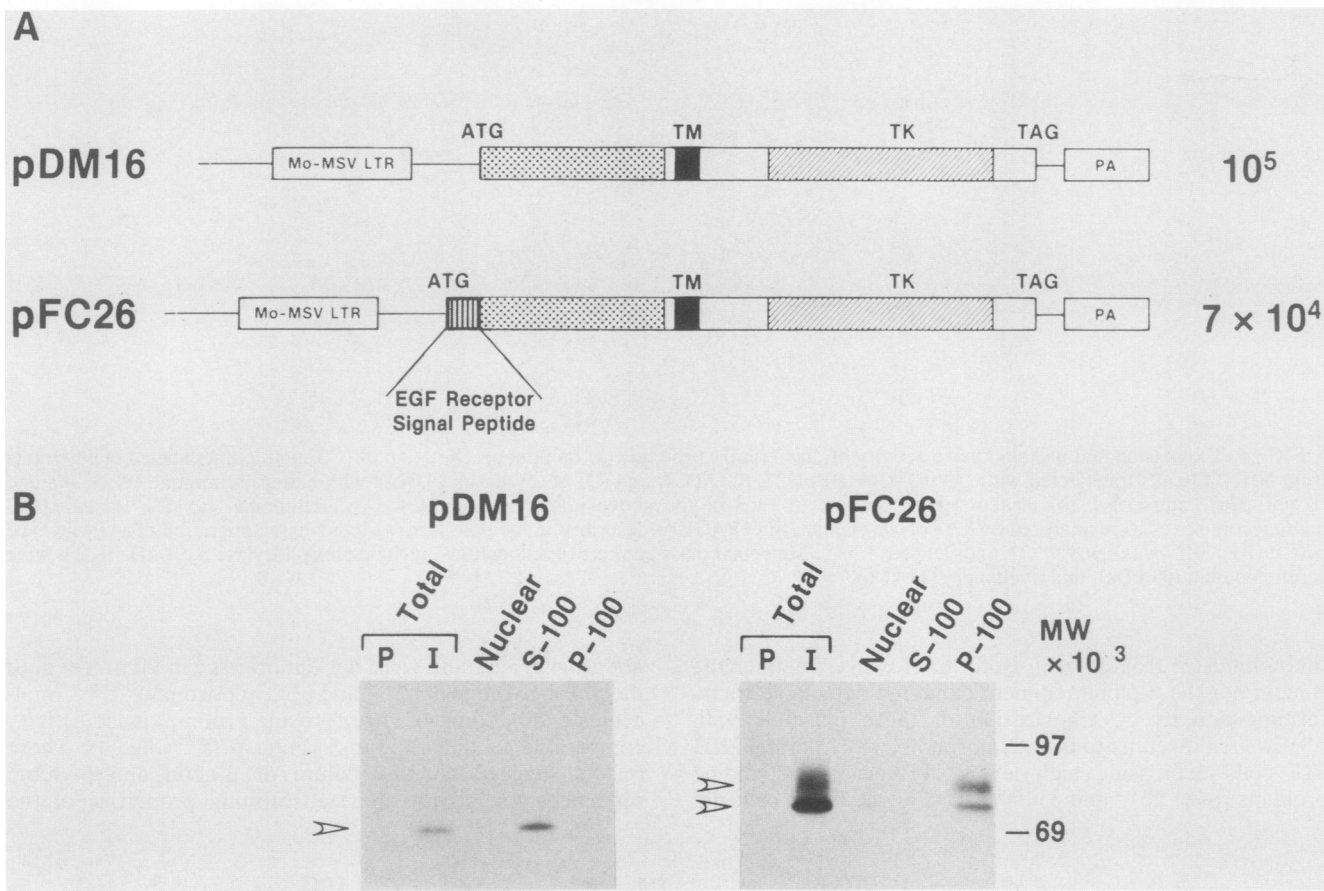

FIG. 6. Effect of subcellular compartmentalization on the transforming activity of p70^{trk}. (A) Schematic representation of plasmids pDM16 and pFC26. Symbols are described in the legend to Fig. 1. Transforming activities of these plasmids in NIH 3T3 gene transfer assays are given on the right as number of foci induced per microgram of DNA. (B) Subcellular fractionation of NIH 3T3 cells transformed by pDM16 and pFC26 DNAs. Cells were labeled with [^{35}S]methionine and subjected to subcellular fractionation as described in Materials and Methods. Extracts from unfractionated (Total) cells were immunoprecipitated with either preimmune (P) or immune (I) anti-p70^{trk} antiserum. Extracts from nuclear, soluble (S-100), or particulate (P-100) fractions were immunoprecipitated with anti-p70^{trk} antiserum. Immunocomplexes were analyzed by 8% polyacrylamide SDS-PAGE. Arrows denote the migration of pDM16-coded p70^{trk} and pFC26-encoded gp75^{trk} and gp80^{trk} proteins. Molecular mass markers are shown for phosphorylase b (97 kDa) and bovine serum albumin (69 kDa). Transforming activities of these plasmids in NIH 3T3 gene transfer assays are given on the right as the number of foci induced per microgram of transfected DNA.

human *trk* locus is not due to inactivation of its tyrosine kinase activity, at least as determined by in vitro assays. More importantly, they document that contribution of tropomyosin sequences to the activation of the *trk* oncogene is a rather selective event that cannot be accomplished by just any subset of foreign sequences.

Function of tropomyosin sequences. Tropomyosin sequences, regardless of their developmental origin, may activate the *trk* oncogene by allowing its kinase domain to interact with cytoskeleton-associated substrates whose unscheduled phosphorylation may trigger morphologic transformation. If so, only a specific subset of tropomyosin sequences might be required to activate the *trk* oncogene. A series of deletion mutants were generated either by removing internal sequences from a functional *trk* oncogene clone or by inserting the corresponding sequences in the tropomyosin deletion mutant pFC1. Their basic structures are outlined in Fig. 5. Each of these deletion mutants efficiently transformed NIH 3T3 cells in spite of the fact that they did not carry common subsets of tropomyosin sequences. pFC13, which contained exons I and II, was as efficient in transforming NIH 3T3 cells as pDM20, which contained exons VI and VII. Similar transforming efficiencies were obtained with pFC10 (exons I to V) and pFC16 (exons (IV to VII). These findings raised the possibility that tropomyosin sequences activate the transforming potential of the *trk* tyrosine kinase by providing the appropriate conformation rather than by allowing its interaction with tropomyosin-related substrates. This hypothesis was strengthened by the fortuitous observation that addition of an unrelated 75-amino-acid-long polypeptide (derived from the amino-terminal domain of the mature EGF receptor) (31) completely abolished the transforming activity of the wild-type *trk* oncogene (Fig. 5). Since these amino acid residues did not affect the stability, subcellular distribution, or in vitro tyrosine kinase activity of p70^{trk} (data not shown), it is likely that they blocked transformation by disrupting its tertiary structure.

Subcellular location of p70^{trk}. Next, we evaluated the importance of the subcellular localization of p70^{trk} and therefore its accessibility to specific substrates to exert its transforming activity. For this purpose, sequences coding for the signal peptide of the EGF receptor (31) (encompassed in a synthetic 93-bp *Nco*I DNA fragment) were introduced at the 5′ *Nco*I site of pDM16, which contains the *trk* oncogene initiator codon. Insertion of this fragment created two potentially functional ATGs. To avoid expression of wild-type

$p70^{trk}$, the physiological translational initiator of the *trk* oncogene was eliminated by site-directed mutagenesis. The resulting plasmid, pFC26, behaved like an efficient oncogene when transfected into NIH 3T3 cells (Fig. 6). Immunoprecipitation of pFC26-transformed cells with anti-$p70^{trk}$ antibodies revealed the synthesis of two forms of $p70^{trk}$ which migrated with apparent molecular weights of 75,000 and 80,000 (Fig. 6). Both of these molecules migrated as a single 70-kDa band in the presence of tunicamycin (data not shown), suggesting that they represent glycosylated forms of $p70^{trk}$. Such glycosylation could have taken place at the single Asn-Ser-Thr *N*-glycosylation site present in the *trk* oncogene. In addition, both $gp75^{trk}$ and $gp80^{trk}$, but not $p70^{trk}$, cofractionated with the particulate fraction (Fig. 6). These results demonstrate that the addition of a signal peptide resulted in the efficient translocation of $p70^{trk}$ into the endoplasmic reticulum. More importantly, they indicate that $p70^{trk}$ has transforming activity independent of its subcellular location. Whether membrane-bound $p70^{trk}$ has access to the same substrate(s) as the cytoplasmic form remains to be determined. However, these findings make it unlikely that tropomyosin sequences contribute to transformation by facilitating the interaction of the *trk* kinase with a specific subset of cytoskeletal proteins.

DISCUSSION

Malignant activation of the *trk* oncogene in a human colon carcinoma resulted from a genetic event(s) in which most of the sequences coding for the extracellular domain of the *trk* proto-oncogene product were replaced by seven of the eight exons of a nonmuscle tropomyosin gene (23). It is likely that the generation of the *trk* oncogene also required the contribution of at least some of the tropomyosin regulatory sequences, since the *trk* proto-oncogene is not normally expressed in NIH 3T3 cells or in any of the colon carcinoma cell lines we have examined (24). Comparison of the nucleotide sequence of the *trk* oncogene with that of its normal allele (25) suggested that no further modifications were required to activate this human locus. This hypothesis was conclusively demonstrated here when fully transforming *trk* oncogenes were generated in vitro by ligating tropomyosin sequences to a nontransforming, decapitated *trk* proto-oncogene cDNA clone. These results indicate that *trk*, unlike most receptor-derived oncogenes, does not require additional changes in the carboxy-terminal region for malignant activation.

Molecular dissection of the three distinctive domains of the *trk* oncogene—tropomyosin sequences, transmembrane domain, and kinase catalytic region—helped to define their specific contributions to its transforming properties. Elimination of all tropomyosin sequences had a drastic effect on the ability of the *trk* oncogene to transform NIH 3T3 cells. Cells expressing a tropomyosin-deleted 45-kDa *trk* protein did not exhibit significant morphologic alterations. However, they overgrew after they reached confluence and had some tumorigenic properties when injected into nude mice. This limited transforming activity was completely abolished by removal of the transmembrane domain. Expression of the pDM22-coded p36 molecule did not have any detectable phenotypic consequences in spite of exhibiting in vitro tyrosine kinase activity. In contrast, the transforming properties of the *trk* oncogene remained unaltered when the transmembrane domain alone was eliminated from the wild-type $p70^{trk}$ molecule. These results indicate that this transforming protein does not require any domains that favor its interaction with cellular membranes, an observation in agreement with its cytoplasmic subcellular location (26). As expected, deletions within the tyrosine kinase catalytic domain of $p70^{trk}$ had a drastic effect. Removal of 55 amino acid residues including Tyr-503, the residue corresponding to $pp60^{v\text{-}src}$ Tyr-416, completely abolished its biological activity, demonstrating that a functional tyrosine kinase is absolutely required for the transforming activity of $p70^{trk}$.

The requirement of tropomyosin sequences for the malignant activation of the *trk* kinase could be explained by three different hypotheses. $p70^{trk}$ might exert its transforming properties by phosphorylating certain cytoskeletal proteins that may normally interact with nonmuscle tropomyosin. Alternatively, it could be hypothesized that the tropomyosin domain activates the *trk* kinase by drastically altering its subcellular localization. Whereas the product of the *trk* proto-oncogene, $gp140^{proto\text{-}trk}$, is tightly associated with the plasma membrane, both $p70^{trk}$ and pFC7-coded $p58^{trk}$ are mostly cytoplasmic, since tropomyosin lacks the appropriate sequences to allow translocation into the endoplasmic reticulum. This change in subcellular localization may allow the *trk* kinase to interact with nonphysiological cytoplasmic substrates whose unscheduled phosphorylation may trigger malignant transformation. Finally, tropomyosin might contribute to transformation by deregulating the *trk* catalytic domain, providing it with a constitutively active conformation.

The first hypothesis, although conceptually attractive, may not be correct. The *trk* kinase can be efficiently activated by different tropomyosin isoforms or even by independent tropomyosin domains. Tropomyosin molecules have very distinctive domains with highly specialized functions, such as actin binding, troponin binding, and head-to-tail polymerization (34). These functions are conferred to each of the isoforms by a complex array of exons which are selectively incorporated into the different tropomyosin transcripts by predefined patterns of alternative splicing (33). Thus, it is unlikely that if tropomyosin activates the *trk* oncogene by allowing the specific interaction of its kinase with defined cytoskeletal substrates, such activation could be accomplished by functionally unrelated domains of the tropomyosin molecule.

The second hypothesis proposes that tropomyosin plays a rather nonspecific role by simply disrupting the transmembrane location of the *trk* proto-oncogene product. This hypothesis is also likely to be incorrect, since the *trk* kinase could not be endowed with transforming properties by either β-actin, another cytoskeletal gene, or β-globin. The products of the actin-*trk* and globin-*trk* chimeras retained a functional in vitro tyrosine kinase activity and were no longer anchored within cell membranous structures (unpublished observations). Therefore, displacement of the *trk* proto-oncogene kinase from its physiological environment is not sufficient to activate its transforming properties.

Available evidence points towards a mechanism by which tropomyosin sequences bestow the kinase catalytic domain with a conformation that favors its constitutive activation. First of all, the *trk* oncogene does not appear to require a defined subcellular location to exert its transforming activity. Addition of a functional signal peptide to its amino-terminal end redirects its subcellular location from the cytoplasm to cellular membranes by allowing its translocation into the endoplasmic reticulum. Whether the cytoplasmic and membrane-associated forms of $p70^{trk}$ recognize different substrates remains to be determined. Preliminary results with antiphosphotyrosine antibodies (in collaboration

with P. Comoglio) indicate that only the products of the transforming *trk* chimeras are efficiently phosphorylated on tyrosine residues. This raises the possibility that tropomyosin may alter the conformation of the kinase domain, allowing phosphorylation of critical tyrosine residues required for proper in vivo enzymatic activity.

The conformational model is further strengthened by recent results demonstrating that malignant activation of the *trk* oncogene can be accomplished by sequences other than tropomyosin. Transformation of NIH 3T3 cells with either the intact *trk* proto-oncogene cDNA clone or pDM22, the nontransforming deletion mutant of the *trk* oncogene that codes for a 36-kDa tyrosine kinase, has led to the generation of more than a dozen new *trk* oncogenes (27). One of the *trk* oncogenes derived from the *trk* proto-oncogene cDNA clone exhibits a deletion in the external domain, suggesting that activation of the *trk* kinase can be accomplished by structural changes without intervention of foreign sequences (unpublished observations). Moreover, the *trk* oncogenes derived from pDM22 code for *trk* molecules of at least 65 kDa, indicating that they have taken up significant amounts of new coding sequences. Molecular analysis of their respective transcripts indicates that none of them are derived from tropomyosin loci (27). Finally, neither of the two new *trk* oncogenes independently isolated by Kozma et al. (14) carries tropomyosin sequences. These observations indicate that malignant activation of the *trk* proto-oncogene can be accomplished by sequences other than those for tropomyosin. Molecular analysis of these activating sequences should make it possible to define the common structural features required to activate the transforming potential of the *trk* proto-oncogene.

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