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Overexpression of Mouse Nck Transforms Mouse Fibroblast NIH3T3

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We isolated a mouse *nck* cDNA from the thymus cDNA expression library. The cDNA encodes a 377 amino acid protein and displays 97% amino acid sequence identity to human oncogenic protein *nck*, which is composed almost exclusively of three src homology 3 (SH3) domains and one SH2 domain. The sequence analysis also showed that the isolated cDNA is the mouse counterpart of the human *nck* and different from the mouse *grb4*, which has been reported to be highly similar to the human *nck* and, therefore, considered as a mouse *nck*. Northern blot analysis showed that the transcript of the gene was 1.8 kb and was highly expressed in the testis, thymus, and brain but moderately in the liver and lymph node. Western blot analysis showed that the size of the protein was about 47 kDa. Overexpression of the mouse Nck transformed a mouse fibroblast cell line, NIH3T3. The results clearly indicate that normal *nck* gene has transforming ability and provide an argument against a suggested possibility that the transforming ability of the human *nck* gene is due to a mutation(s) in the gene.

Nck is an adaptor protein of intracellular signaling pathways which are initiated by activation of receptor protein tyrosine kinases. Nck isolated from a human cell line is widely expressed in all tissues and during all stages of development (Li et al., 1992). The protein lacks a catalytic domain and contains three N-terminal src homology 3 (SH3) domains followed by a SH2 domain (Lehmann et al., 1990). Other members of this class of adaptor proteins include Grb2 and Crk (van der Geer et al., 1994). The *nck* cDNA was first cloned fortuitously from a human melanoma expression library with antibodies to MUC18 (Lehmann et al., 1990) and also by virtue of its ability to bind to the phosphorylated C-terminal tail of the EGF receptor (Margolis et al., 1992). Several studies have shown that the human Nck plays important roles in signaling pathways crucial for the cell growth control. After stimulation of cells by growth factors, the human Nck becomes phosphorylated on Ser, Thr, and Tyr residues and associates with receptor protein tyrosine kinases such as EGF and PDGF receptors via its SH2 domain (Chou et al., 1992; Li et al., 1992; Meisenhelder and Hunter, 1992; Park and Rhee, 1992). In addition, overexpression of Nck isolated from human melanoma transforms mammalian fibroblasts (Chou et al., 1992; Li et al., 1992), even though the mechanism is not clear. However, it has been pointed out that the human cDNA clone of *nck* used for the transformation assay was originally isolated from a melanoma cDNA expression library (Lehmann et al., 1990) and therefore may contain a mutation(s) which activates its oncogenic potential (Li et al., 1992). This raises a question whether overexpression of normal Nck itself can transform a fibroblast cell line. Furthermore, overexpression of the human Nck in rat or mouse fibroblasts may reflect a general species difference, so that they may have an affinity or specificity different from that of the endogenous protein for its normal substrates and associated proteins (Chou et al., 1992). These suggest a possibility that the transformation induced by overexpression of the human Nck in rat or mouse fibroblasts may not reflect an actual function of a normal Nck. Therefore, it is necessary to test whether overexpression of Nck isolated from a normal tissue induces transformation of a cell line originating from the same species as the *nck* cDNA.

We have previously reported the cloning and overexpression of the mouse *nck* (Park D., 1997). The mouse Grb4 has been reported to be quite similar to the human Nck, and therefore, considered as a mouse counterpart of the protein (Margolis et al., 1992). The amino acid sequence analysis showed that the mouse Nck contained three SH3 domains followed by a SH2 domain, similar to the human counterpart, and was different from the mouse Grb4. Northern analysis also

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showed that tissue-specific expression was different from that of the previous report (Margolis et al., 1992). In addition, we also showed that overexpression of normal mouse Nck could transform the mouse fibroblast NIH3T3.

Materials and Methods

Materials

Monoclonal antibody (mAb) to PLC-γ1, B16-5, was previously described (Park and Rhee, 1992). Antiserum to mouse Nck was generated by immunizing a rabbit with Nck overexpressed in *E. coli* and was used for immunoblot analysis. The NIH3T3 cells were purchased from American Type Culture Collection, and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. C57BL/6J mice for RNA extraction from tissues were maintained in the Institute for Molecular Biology and Genetics, Seoul National University.

Screening cDNA library and sequencing analysis

The λgt11 cDNA expression library was constructed using mRNA isolated from the thymi of 4-6 week old 129/SVJ (Jackson Laboratory). The library was screened using mAb B16-5 which recognizes SH3 domains of PLC-γ1 and Nck. The nucleotide sequence was determined by dideoxy chain termination method (Sanger et al., 1977) using Sequenase 2.0 (United States Biochemical Co.) after subcloning the restriction fragments of the cDNA into pBluescript II (SK- and SK+) vector (Stratagene). Comparison of the nucleotide sequence with other sequences in the GeneBank was performed at the National Center for Biotechnology Information, using Blast network service.

RNA isolation and Northern blot analysis

Total RNAs from mouse tissues and cultured cells were isolated as previously described (Chomczynski and Sacchi, 1987). Twenty micrograms of RNA from each tissue was electrophoresed under denaturing condition. The RNAs were transferred onto a nylon membrane (Amersham) by overnight-capillary transfer method. Membrane was then cross-linked in an UV Stratalinker (Stratagene), prehybridized (50% formamide, 3x Denhardt's solution, 5x SSC, 0.5% SDS, and 200 μg/ml Herring sperm DNA and polyA mix), and hybridized with a riboprobe. The blot was washed, and then exposed to X-ray film (Kodak) at -80℃. The [α-P^{32}] UTP labelled riboprobe was prepared from linearized mouse Nck cDNA by *in vitro* transcription using T7 RNA polymerase (Ambion).

Transformation of NIH3T3 cells

pRc/CMV and pRc/CNCK were assayed for the transforming ability after transfection into NIH3T3 cells. For transfection, plasmid DNAs were linearized by *PvuI* digestion. In each experiment, twenty micrograms of test DNA was electroporated into 1×10^6 NIH3T3 cells using Genepulser II (BioRad). Cells were selected with 1 mg/ml G418 starting at 48 h after transfection. After G418 selection for 18 d, colonies were trypsinized, pooled, and plated in 0.3% soft agar as described below. Part of the pooled transfected cells were also expanded for immunoblotting analysis.

To assay anchorage independence, five thousand cells from pooled colonies were resuspended in 2 ml of DMEM containing 10% calf serum and 0.3% agarose, and overlaid onto 6-well petri-dish containing a bottom layer of 0.8% agarose in DMEM with 10% calf serum. The plates were incubated at 37℃ for one day and checked for even distribution of cells. After 2 wk, visible foci were counted.

Immunoblotting of Nck

Transfected cells were collected in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM NaH_2PO_4, 1.4 mM KH_2PO_4, pH 7.3), pelleted, resuspended in 500 μl of ice cold NP-40 lysis buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 μg/ml PMSF, 10 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml soybean inhibitor, 1 μg/ml pepstatin, 0.1 mM DTT, and 1% NP-40), and incubated on ice for 30 min. After centrifugation, protein concentration was determined by Bradford's assay (BioRad). Supernatant was boiled for 2 min with loading dye and analyzed by SDS-polyacrylamide gel electrophoresis. For immunoblot analysis, the separated proteins were electrotransferred to nitrocellulose paper (S&S) and incubated in a blocking solution (5% non-fat dried milk, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) with gentle agitation for 1.5 h. After incubating the blot with the Nck antiserum, bands were detected with anti-rabbit IgG conjugated with alkaline phosphatase in a blocking solution (100 mM Tris-Cl, pH 9.5, 100 mM NaCl, 10 mM $MgCl_2$) containing 165 μg/ml bromochloroindolyl phosphate (BCIP) and 330 μg/ml nitro blue tetrazolium (NBT).

Results

Cloning of nck and sequencing analysis

We isolated a cDNA fragment encoding the mouse *nck* by screening a thymus expression library with a mAb, B16-5, which recognizes SH3 domains of PLC-γ1. We determined the DNA sequence of 1.8 kb cDNA fragment cloned in pBluescript II SK- (Fig. 1). The cDNA consisted of 167 bp 5′-untranslated region, 1131 bp coding region, and 496 bp 3′-untranslated region including a part of poly (A) sequence. The coding region encoded a 377 amino acid protein which is composed almost exclusively of three SH3 domains and one SH2 domain. The amino acid sequence was compared and shown to be very similar to that of the human Nck

(Lehmann et al., 1990) (Fig. 2). Over the entire coding region, the predicted amino acid sequence displayed 97.1% identity to the human Nck allowing a few conservative substitutions. It is noteworthy that almost all differences in amino acid sequence lay between the SH2/SH3 domains, and that the sequence of the SH2 domain matched perfectly to that of the human Nck. On the other hand, Grb4, which has been shown to be quite similar to the human Nck (Margolis et al., 1992), exhibit 74% identity (Fig. 2) even though only the sequence for C-terminal part of the Grb4 is available. The differences between the human Nck and the mouse Grb4 sequences are dispersed in all regions of the proteins. Therefore, a protein encoded by the cDNA clone we isolated, but not Grb4, represents a genuine mouse counterpart of the human Nck.

Tissue distribution of Nck

The expression of the mouse *nck* was tested by Northern blot analysis using a riboprobe prepared from the mouse *nck* cDNA (Fig. 3A). The size of *nck* transcript was 1.8 kb long and matched well to the size of the isolated full length cDNA clone. The transcript was detected in all mouse organs tested. However, the expression level was highest in testis followed by the thymus, brain, liver and lymph node. Western blot analysis showed a 47 kDa Nck protein (Fig. 3B). Distinctive tissue distribution of Nck suggests that Nck may have unique functions in different tissues even though it may also function as a common signal transducing molecule.

The expression pattern was different from that previously reported by Li et al. (1992). The size of mRNA for mouse *nck* was reported to be 2.4 kb, which is much larger than our estimation, and the expression levels of the gene among tissues were completely different from our results. It is likely that the probe employed by Li et al. might not be the mouse *nck* cDNA. Unfortunately, they did not provide any evidence suggesting that the clone they isolated was actually the mouse *nck* gene (including sequencing data). It seems that the cDNA probe used by Li et al. was mouse Grb4 mRNA rather than mouse Nck mRNA. The sequence analysis indisputably showed that our cDNA clone, a fragment of which was used as a probe for the Northern analysis, is the mouse *nck*. In addition, the cDNA isolated by Li et al. should contain approximately 600 bp 5′-untranslated region, which is not unlikely but unusually long, to produce a 2.4 kb transcript, assuming poly (A) tail to be about 200 bp long.

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  1  MAEGVVVVAK FDYVAQQEQE LDIKKNERLW LLDDSKSWWR
 41  VRNSMNKTGF VPSNYVERKN SARKASIVKN LKDNLGIGKV
 81  KRKTSVPDTE SPADDSFVDP GERLYDLNMP AFVKFNYMAE
121  REDELSLIKG TKVIVMEKCS DGWWRGSYNG QIGWLPCNYV
161  TEEGDSPLGD HVGSLSEKLA AVVNNLNTGQ VLHVVQALYP
201  FSSSNNEELN FEKGDVMDVI EKPENDPEWW KCRKINGMVG
241  LVPKNYVTIM QNNPLTSGLE PSPPQCDYIR PSLTGKFAGN
281  PWYYGKVTRH QAEMALNERG HEGDFLIRDS ESSPNDFSVS
321  LKAQGKNKHF KVQLKETVYC IGQRKFSTME ELVEHYKKAP
361  IFTSEQGEKL YLVKHLS
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Fig. 1. Amino acid sequence of the mouse Nck. Three SH3 domains and one SH2 domain (bold letters) are indicated.

Overexpression of Nck and test of transforming ability

It has been postulated that the the human *nck* cDNA isolated from melanoma cells may contain mutations which activate its oncogenic potential (Li et al., 1992). In addition, a possibility cannot be excluded that transformation of rat or mouse fibroblasts induced by overexpression of the human Nck (Chou et al., 1992; Li et al., 1992) may be due to an altered affinity or specificity of the Nck for its regulators and effectors different from those of endogenous protein. Therefore, it is necessary to test whether the overexpression of normal mouse Nck can transform mouse fibroblasts. NIH3T3, a mouse fibroblast line, was transfected with the *nck* cDNA isolated from mouse thymus. The expression of the Nck was driven by CMV promoter (pRc/CMV vector). After the transfection and G418 selection, resistant colonies were pooled and overexpression of Nck was confirmed by immunoblotting whole cell lysates with antiserum raised against a GST-Nck fusion protein. The transfected cells with pRc/CNCK expressed approximately 2.5 times-higher

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MAEEVVVVAK FDYVAQQEQE LDIKKNERLW LLDDSKSWWR VRNSMNKTGF
***G****** ********** ********** ********** **********

VPSNYVERKN SARKASIVKN LKDNLGIGKV KRKTSVPDTE SPADDSFVDP
********** ********** ***N****** ***T****TE **********

GERLYDLNMP AYVKFNYMAE REDELSLIKG TKVIVMEKCS DGWWRGSYNG
********** *F******** ********** ********** **********

QVGWFPSNYV TEEGDSPLGD HVGSLSEKLA AVVNNLNTGQ VLHVVQALYP
*I**L*C*** ********** ********** ********** **********

FSSSNNEELN FEKGDVMDVI EKPENDPEWW KCRKINGMVG LVPKNYVTVM
*****N**** ********** ********** ********** ********I*
                   ** ********** ***NARQ*** *******V*L

QNNPLTSGLE PSPPQCDYIR PSLTGKFAGN PWYYGKVTRH QAEMALNERG
********** ********** ********** ********** **********
SDG*...A*H *AT**IS*TG **AS*R***R E****N**** ***C******

HEGDFLIRDS ESSPNDFSVS LKAQGKNKHF KVQLKETVYC IGQRKFSTME
********** ********** ********** ********** **********
V********* ****S***** ***S*R**** ****VDS*** ****R*HS*D

ELVEHYKKAP IFTSEQGEKL YLVKHLS  377aa  Human NCK
********** ********** *******  377aa  Mouse NCK
********** *****H**** ***RA*Q  157aa  Grb4
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Fig. 2. Comparison of the amino acid sequences of mouse and human Nck. An available sequence of the mouse Grb4 is also compared to these sequences. The sequence homology of the mouse Nck and Grb4 to the human Nck is 98.2% and 74%, respectively. Asterisks indicate identical amino acids between mouse and human sequences. SH domains are in bold letters.

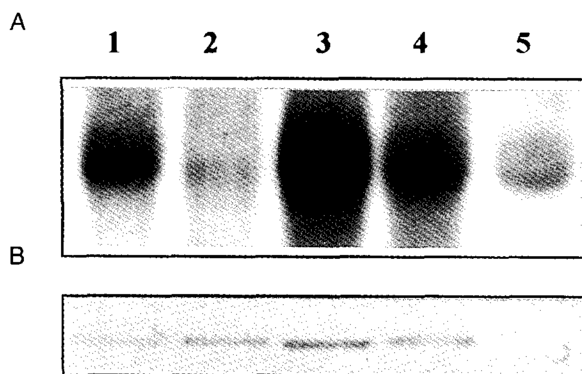

Fig. 3. Nck expression in different tissues. A. Northern blot analysis: Twenty micrograms of total RNA from various mouse tissues was hybridized with riboprobe prepared from the *nck* cDNA. The size of *nck* transcript is about 1.8 kb. B. Western blot analysis: Total cell lysate from tissues was immunoblotted with antiserum raised against GST-NCK fusion protein. The size of Nck protein is about 47 kDa. Lanes: 1, brain; 2, lymph node; 3, testis; 4, thymus; 5, liver.

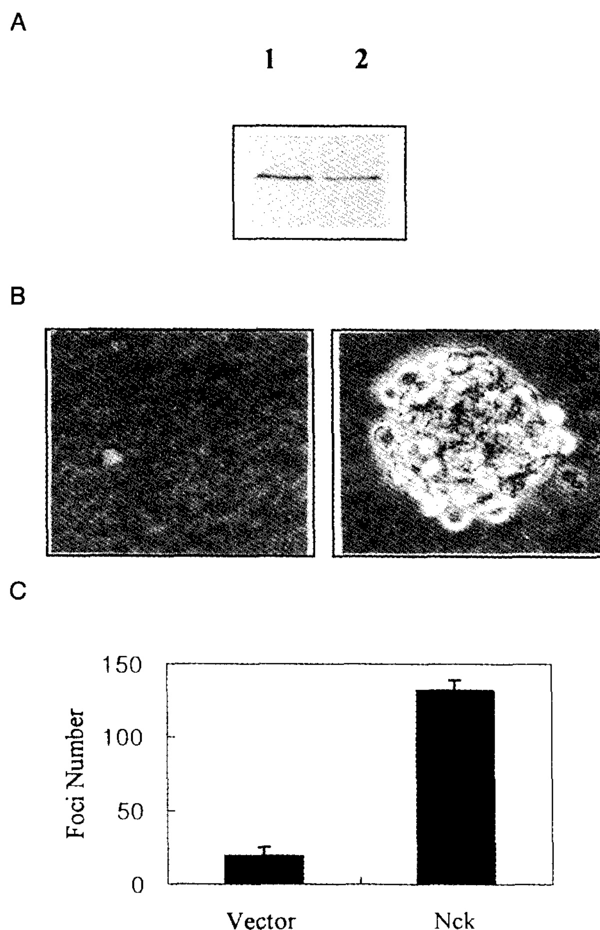

Fig. 4. Overexpression of Nck transforms a mouse fibroblast NIH3T3. Anchorage-independent growth of the transfected NIH3T3 cells was tested by soft-agar assay. Colonies formed after the transfection were pooled and used for the assay. The transfected cells were trypsinized, resuspended in DMEM containing 10% calf serum and mixed with agarose to make 0.4% agarose suspension which were overlaid on 0.8% agarose/media. Colonies were photographed and counted 14 d after plating. A. Comparison of the Nck expression levels between pooled vector and pRc/CNCK transfectant. Lanes: 1, vector; 2, pRc/CNCK transfectant. B. Photographs of non-transformed cell (left) and transformed cell (right). C. Numbers of the transformed colony formed after assay.

level of Nck proteins, compared to control cells transfected with the vector pRc/CMV (Fig. 4A).

The effect of overexpression of Nck on cell transformation was analyzed by testing anchorage independence for growth, which is a typical parameter of cell transformation. To assay anchorage independence, five thousand cells from pooled transfected cells were plated onto soft agar medium. After two weeks of incubation, visible foci were counted. In three independent experiments, vector transfected cells formed about 18 foci and the Nck transfectants formed about 132 foci (Fig. 4B, C), showing that normal Nck can transform the mouse fibroblasts.

Discussion

It has been shown that an overexpression of the human Nck led to a transformation of mammalian fibroblasts in the absence of elevated phosphotyrosine level (Chou et al., 1992; Li et al., 1992). The mechanism of the human Nck-induced transformation is not clear, partly because the available cDNA clone of the human Nck was originally isolated from a melanoma expression cDNA library (Lehmann et al., 1990). It has been reported that the mouse c-Crk itself, also an adaptor type SH2- and SH3-containing protein, is not oncogenic even when expressed at high levels (Ogawa et al., 1994). However, deletion of the second SH3 domain of c-Crk can activate its transforming potential. In human, two distinct c-Crks, one with two SH3 domains (Crk-II) and the other with one SH3 domain (Crk-I), were isolated from embryonic lung cells (Matsuda et al., 1992). Similar to the mouse c-Crk, overexpression of c-Crk-I, but not c-Crk-II, induced transformation of rat fibroblasts (Matsuda et al., 1992). It is, therefore, thought that the second SH3 domain of c-Crk negatively regulates the transforming ability of the protein i.e., lack of it induces a transformation through increasing tyrosine phosphorylation of certain proteins (Ogawa et al., 1994). Based on these circumstances, it can be postulated that the human *nck* cDNA isolated from melanoma cells may contain mutations which activate its oncogenic potential (Li et al., 1992). In addition, a possibility cannot be excluded that transformation of rat or mouse fibroblasts induced by overexpression of the human Nck (Chou et al., 1992; Li et al., 1992) may be due to an altered affinity or specificity of the Nck for its regulators and effectors different from those of endogenous protein. Therefore, it is required to test whether the overexpression of a normal mouse Nck can transform mouse fibroblasts. In this study, we clearly showed that overexpression of the native Nck itself is enough to transform the fibroblasts. This strongly argues against a possibility

that the human *nck* cDNA isolated from melanoma cells contains mutations which activate its oncogenic potential, and that transformation is due to an altered affinity or specificity of the Nck for its regulators and effectors caused by an exogenic transformation.

The mechanism of the transformation by Nck may be different from that of other adaptor molecules such as Crk or Grb2. Overexpression of v-Crk in rodent fibroblasts resulted in increased tyrosine phosphorylation of several proteins (Chou et al., 1992). In contrast, although overexpression of the human Nck in rat or mouse fibroblast induced transformation of cells, there was no difference in overall levels of phosphotyrosine in the transformants and parental cell lines (Chou et al., 1992). Even though a possibility of small, but beyond the level of detection, change cannot be excluded, signaling mediated by Nck may be different from the case of Crk and Grb2.

It has been reported that the human Nck is phosphorylated by stimulation of cells with PDGF or EGF and to form a complex with these receptors via its SH2 domain (Chou et al, 1992; Li et al., 1992; Meisenhelder et al., 1992; Park et al., 1992). In case of Crk, deletion of the SH2 domain completely abolishes its ability to elevate phosphotyrosine levels and to transform rodent fibroblast (Mayer and Hanafusa, 1990). The Nck protein also binds to Sos and interacts with Ras pathway *in vitro* (Hu et al., 1995). These results coincide with the case of Crk and Grb2. Thus, it seems that Nck plays a role in mitogenic signal transduction as an adaptor molecule bringing effectors that bind to its SH3 domains to the activated receptor protein tyrosine kinases. However, recent studies show that Grb2, Src family tyrosine kinases, and p85 subunit of phosphatidyl inositol 3'-kinase, but not Nck, physically associate with the upstream signaling molecule Flk2/Flt3 (fetal liver kinase 2), which was recently identified as a receptor tyrosine kinase expressed in the brain, placenta, testis, and primitive hematopoietic cells (Dosil et al., 1993). C3G, another guanine nucleotide-releasing protein (GNRP), has been identified as a binding partner of Crk and Grb2, but not of Nck (Tanaka et al., 1994). In addition, Grb2 with mutations in either its SH2 or SH3 domain or Crk-I with an SH3 domain mutation effectively inhibited the activation of Erk-1 by oncogenic Abl, while Nck SH2 and SH3 mutants had little or no effect (Tanaka et al., 1995). These observations also suggest that Nck might function differently from Crk or Grb2 in mitogenic signal transduction.

Recently, several proteins have been revealed to associate with Nck via its SH3 domain. These include a serine/threonine kinase (Chou and Hanafusa, 1995), the guanine nucleotide exchange factor Sos (Hu et al., 1995), p21 activated kinase (Bagrodia et al., 1995), Wiskott-Aldrich syndrome protein (Rivero-Lezcano et al., 1995), p125Nap1 (Kitamura et al., 1996), PRK2 (Quilliam et al., 1996), and NIK (Su et al., 1997) with diverse functional characteristics. Our results that overexpression of Nck itself can induce transformation of NIH3T3 fibroblasts provide the bases for further studies on elucidation of the role of Nck and investigation of its binding partners via its SH2 and SH3 domains concerning the regulation of post-receptor signals controlling mitogenesis and transformation.

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