Identification of Grb4/Nckβ, a Src Homology 2 and 3 Domain-containing Adapter Protein Having Similar Binding and Biological Properties to Nck*

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Adapter proteins made up of Src homology (SH) domains mediate multiple cellular signaling events initiated by receptor protein tyrosine kinases. Here we report that Grb4 is an adapter protein closely related to but distinct from Nck that is made up of three SH3 domains and one SH2 domain. Northern analysis indicated that both genes are expressed in multiple tissues. Both Nck and Grb4 proteins could associate with receptor tyrosine kinases and the SH3-binding proteins Pak, Sos1, and Prk2, and they synergized with v-Abl and Sos to induce gene expression via the transcription factor Elk-1. Although neither protein was transforming on its own, both Nck and Grb4 cooperated with v-Abl to transform NIH 3T3 cells and influenced the morphology and anchorage-dependent growth of wild type Ras-transformed cells. Nck and Grb4 therefore appear to be functionally redundant.

Growth factor binding to receptor protein tyrosine kinases (R-PTKs) induces their dimerization and trans-phosphorylation, creating docking sites for proteins containing SH2 and PTB protein interaction domains (1). Many of these phosphotyrosine-binding proteins are effector enzymes, e.g. phospholipase C, the protein phosphatases SHP-1 and SHP-2, and p120 RasGAP (1). However, R-PTKs also bind to a number of adapter proteins that lack enzymatic activity and contain SH3 in addition to SH2 domains. Through SH3 domains, adapter proteins can bind to proline-rich motifs in downstream effectors, often recruiting them to multiprotein complexes at the plasma membrane. The adapter protein Grb2, for example, has the domain structure SH3-SH2-SH3 and can bind to proline-rich sequences in the Ras guanine nucleotide exchange factor, Sos, recruiting it to the plasma membrane, where it triggers the Ras-ERK pathway (2, 3).

Another abundantly expressed adapter protein, Nck, consists of three juxtaposed SH3 domains and a C-terminal SH2 domain (4). The SH2 domain of Nck has been reported to bind a variety of growth factor receptors, including those for EGF (5), PDGF (6), vascular endothelial growth factor (7), and hepatocyte growth factor (8), as well as the insulin receptor substrate, IRS-1 (9), Eph (10, 11), p62 (12), and focal adhesion kinase (13). The SH3 domains of Nck can interact with proline-rich motifs in multiple binding partners, including the Ser/Thr protein kinases Pak14-17, Prk218, casein kinase I (19), and Nck interacting kinase (20). Nck has also been found to interact with the protein tyrosine kinase Ab1 (21), Sos (22), the Wiskott-Aldrich syndrome protein (18, 23), a Drosophila protein tyrosine phosphatase dPTP61F (24), c-Cbl (25), and Sam68 (26). Although the physiological significance of most of these interactions is undetermined, several of the above SH3 binding partners (Pak, Prk2, and the Wiskott-Aldrich syndrome protein) appear to be downstream effectors of Rho family GTPases (18, 27). Because Rho proteins have been implicated in cytoskeletal reorganization (29), Nck may be responsible for coupling R-PTK activation to cytoskeletal regulation. In support of this notion, the Drosophila homolog of Nck, DOCK, is located in photoreceptor growth cones and is involved in axonal guidance (30). Use of dominant inhibitory SH domain mutants has also implicated Nck in dorso-ventral axis development during Xenopus embryogenesis (31). The nck gene is located at a chromosomal break point associated with a variety of cancers (32), and Nck has been reported to weakly transform rodent fibroblast cell lines (5, 33), also implicating it in oncogenesis.

In 1992, a partial mouse cDNA that encoded for an SH2 domain and part of an SH3 domain was isolated from a bacterial expression library and designated Grb4 (34). The predicted amino acid sequence shared 74% identity with human Nck. It was not clear from this original study whether the partial grb4 cDNA encoded the mouse ortholog of Nck or a separate protein. Here we demonstrate that grb4 is, indeed, a unique human gene. Isolation of the full-length human grb4 cDNA showed that Grb4 shares the same SH domain composition and is 69% identical to Nck. Like Nck, Grb4 bound to Sos, Pak, and Prk2 and to activated growth factor receptors. Both Nck and Grb4 cooperated with v-Abl, Ha-Ras(WT), and Sos1 to modulate cell morphology and transformation and to induce gene expression via the Elk-1 transcription factor. These findings suggest that Nck and Grb4 may be functionally redundant.

MATERIALS AND METHODS

Library Screen—A human brain Marathon-ready cDNA library (CLONTECH) was used to isolate full-length grb4. The 5'-end of the gene was amplified using polymerase chain reaction with anchor primers provided with the library and nested grb4 primers located within the middle SH3 domain. Due to difficulty obtaining the complete 3'-end of the clone by rapid amplification of cDNA ends, we used further nested grb4 primers to isolate a central portion and specific primers to

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polymerase chain reaction amplify the 3' coding region. The grb4 fragments were subcloned into pCR2.1 (Invitrogen) and sequenced. Full-length grb4 was generated by ligation using existing restriction sites within the three overlapping fragments.

Northern Analysis—Full-length nck and grb4 cDNA (4) or a 266-base pair fragment of nck (40) were radiolabeled with [α-32P]dCTP using a random-priming DNA labeling kit (Boehringer Mannheim) as outlined by the manufacturer. Two human multltissue Northern blots (CLONTECH) were probed overnight with each individual fragment in hybridization solution (5 × SSPE, 10 × Denhardt’s solution, 100 mg/ml denatured sheared salmon sperm DNA, 2.0% SDS plus 50% formamide) at 42 °C. Blots were washed extensively in 2 × SSC, 0.05% SDS at room temperature followed by two 20-min washes in 0.1 × SSC, 0.1% SDS at 50 °C. The blots were exposed at −80 °C with intensifying screens.

Mammalian Cell Culture—Cos, NIH 3T3, and SV40-immortalized mouse embryo fibroblasts were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (HyClone) or, for NIH 3T3 cells, in 10% calf serum (Colorado Serum Company or Life Technologies, Inc.). Transforming focus and soft agar assays were performed as described (35).

Transcriptional Activation Assays—Activation of Elk-1 was determined by co-transfection of cells with both Gal4-Elk-1 and Gal4-Luc constructs. Gal4-Elk-1 encodes a fusion protein containing the Gal4 DNA-binding domain, together with 10% of the activation domain (containing ERK phosphorylation sites). Gal4-Luc encodes the luciferase gene driven by a minimal promoter containing tandem Gal4 DNA binding sites (36). NIH 3T3 cells were cotransfected with 125 ng of Gal4-Elk-1, 2.5 μg of Gal4-Luc, and 1 μg of pSRoMSVtkvcat (v-act) (provided by A. M. Pendergast, Duke University); 2 μg of pZIP (37), 2 μg of pZIP(Nch), 2 μg of pZIP(grb4), or 0.67 μg of pZIP(oss17) as indicated. 24 h posttransfection, cells were serum-starved (0.5%) overnight, lysed, and analyzed for luciferase activity essentially as described (38).

Mammalian Protein Expression and GST Fusion Protein Interaction—For transient overexpression of Prk2 and PAK, 100-mm plates of 50% confluent Cos cells were transfected with 6 μg of either pFLAG-CMV2(grb4) (18) or pCMV8pah(WT) (16) using LipofectAMINE (Life Technologies, Inc.) and harvested after 24 h. Cos1 was stably overexpressed from plp-chase(grb4) (39) in NIH 3T3 cells. Confluent plates of cells were lysed in 1 ml lysis buffer consisting of 20 mM Tris, pH 7.4, 50 mM NaCl, 0.5% IGEPA-CA-630, 5 mM EDTA, 10 μg/mL glyceraldehyde-3-phosphate dehydrogenase, and 1 mM phenylmethylsulfon chloride. Cell lysates were cleared by microcentrifugation for 10 min at 4 °C. GST fusion proteins of Nck, Grb4, or a control GST (containing the C-terminal amino acids of Prk2) were expressed in E. coli in the presence of 10% methionine and a C-terminal 6×histidine tag antibody. Proteins were separated by SDS-PAGE and co-precipitated with 3 μg of M2 anti-FLAG antibody. Proteins were separated by SDS-PAGE and co-precipitated Sox1 detected by Western blotting using anti-Sox antibody.

Western Blotting—Proteins separated by SDS-PAGE were transferred onto Immobilon-P polyvinylidene fluoride membrane (Millipore) and were probed with 1% bovine serum albumin in TBS-Tween 20 (Tris-buffered saline with 0.1% Tween 20) for 1 h. The same blot was then probed with 3 μg of anti-Sox antibody or, in the case of phosphorylated tyrosine detection, with 3% bovine serum albumin in TBS-Tween 20. All antibody dilutions and wash steps were performed in TBS-Tween 20. Primary antibody incubations were for 1 h at room temperature with indicated antibodies. Secondary incubations were done for 30 min at room temperature with anti-HRP (Amersham Pharmacia Biotech) and detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Anti-Nck antibody was from Pharmingen, anti-HA tag antibody was from Berkeley Antibody Co., anti-FLAG (M2) was from Sigma, anti-Sox was from Transduction Laboratories, and anti-HA was from Santa Cruz Biotech. Other anti-Nck sources included Transduction Laboratories and Upstate Biotechnology.

Phosphorylation—Cos cells were transiently transfected with pFLAG-CMV2(nck) or grb4 or pCGN HA-tagged grb2. After 24 h, cells were incubated overnight in phosphate-free Dulbecco’s modified Eagle’s medium, supplemented with 0.5% dialyzed fetal bovine serum and 1.25 μCi of [32P]orthophosphate (NEN). Cells were stimulated for 10 min with 50 μM forskolin/0.2 mM isobutylmethylxanthine, Me,SO, 1 μM phorbol myristate acetate, 50 ng/ml EGF (provided by A. M. Pendergast, Duke University), or 30 μM y-27,000 C (Amersham Pharmacia Biotech). Anti-Nck antibody was from Pharmingen, anti-HA tag antibody was from Berkeley Antibody Co., anti-FLAG (M2) was from Sigma, anti-Sox was from Transduction Laboratories, and anti-HA was from Santa Cruz Biotech. Other anti-Nck sources included Transduction Laboratories and Upstate Biotechnology.

RESULTS

Isolation of Full-length Grb4 cDNA—Sequence homology between human nck and a partial mouse grb4 cDNA isolate indicated that grb4 could either be the mouse ortholog of nck or a separate, related gene (34). Human expressed sequence tags, as well as an additional partial cDNA clone, supported the notion that grb4 was a separate gene. Therefore, to obtain the full-length Grb4 cDNA, we screened a human brain library using polymerase chain reaction/rapid amplification of cDNA ends (see under “Materials and Methods”). Like Nck, Grb4 consisted of three N-terminal SH3 domains and a C-terminal SH2 domain with short linker regions. The two proteins share 69% identity, with most divergence occurring in the regions between the SH domains (Fig. 1). An in-frame stop codon was located just 5′ of the presented sequence, indicating that the Grb4 N terminus does not extend beyond that of Nck. Indeed, significant homology between the nck and grb4 5′ untranslated regions supports a common ancestry. Overall Nck has slightly more identity with the Xenopus Nck homolog than does Grb4, whereas the Drosophila protein is somewhat more divergent (Fig. 1).

Nck and Grb4 mRNAs Are Ubiquitously Expressed—Because

2 B. K. Kay, personal communication.
Nck and Grb4 shared such a high degree of homology within their functional SH domains, we anticipated that they might serve similar functions in different tissues. We looked for differential expression of nck/grb4 mRNAs in a panel of 16 human tissues using commercially available Northern blots. Surprisingly, both nck and grb4 messages were almost ubiquitously expressed (Fig. 2). This apparent ubiquitous expression pattern was not due to cross-hybridization of the probes because their messages were of distinct sizes (the nck mRNA was slightly smaller). Furthermore, there were slight variations in abundance of the major transcripts in different tissues, most notably pancreas and thymus. The broad tissue distribution of nck was consistent with previously findings (5).

Nck and Grb4 Bind to Common Target Proteins—We next tested the two adapter proteins to see whether the minor deviations in SH domain sequence might impart differential binding properties. Incubation of GST fusions of SH domain adapter proteins with lysates from \[^{35}S\]methionine-labeled NIH 3T3 cells revealed that, although distinct from that of Grb2, the binding profiles of Nck and Grb4 were similar to each other (Fig. 3 A). The SH3 domains of Nck have been shown to bind to a number of target proteins, such as Sos, PAK, and PRK2, all of which contain proline-rich peptide sequences (14–18, 22, 41). We therefore addressed whether GST-Grb4 would also interact with these molecules. Recombinant Grb4 was found to precipitate FLAG-tagged PRK2 and Myc-tagged PAK1 from cell lysates with similar efficacy to Nck (Fig. 3 B). Sos1 could also be co-immunoprecipitated by FLAG-tagged Grb4 or Nck from Cos cell lysates. The SH2 domain of Nck binds to a number of activated growth factor receptors and their substrates (5–13). Grb4 and Nck interacted with a similar pattern of phosphotyrosine-containing proteins. To look more specifically at interaction with these proteins, serum-starved mouse embryo fibroblasts were challenged with EGF or PDGF prior to incubation of lysates with glutathione bead-immobilized full-length adapter proteins or isolated SH2 domains. As shown in Fig. 3C, the SH2 domains of Grb2, Nck, and Grb4 all precipitated acti-

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3 L. E. Braverman and L. A. Quilliam, unpublished observations.
Nck and Grb4 serve as substrates for protein kinases—

Nck has been reported to be phosphorylated on multiple sites (primarily Ser/Thr residues) following agonist stimulation (5, 16, 33, 42–44). Examination of the Nck sequence revealed that most consensus phosphorylation sites are located between the SH domains, where Grb4 and Nck are most divergent. Following 32PO4 labeling of Cos cells transiently overexpressing FLAG-tagged Nck or Grb4, both molecules were heavily phosphorylated, even in the absence of challenge with forskolin/IBMX (Fig. 4), serum, or phorbol myristate acetate. Therefore, although the regions between the SH3 domains share less homology, both proteins are still susceptible to phosphorylation. Indeed, three consensus PKA phosphorylation sites are conserved in the region between the first and second SH3 domains of Nck and Grb4 (see Fig. 1). No such sites were present in the epitope tag.

Adapter Proteins Cooperate with v-Abl and Sos to Induce Transcriptional Activation—

Nck has been reported to activate Ras and or Ras-induced phenomena by binding the Ras guanine nucleotide exchange factor, Sos, and replacing the role of Grb2 in mediating growth factor-induced recruitment of Sos to the site of Ras activation (22). Because Sos could co-precipitate with Nck and Grb4, we looked at the ability of these adapters to stimulate transcriptional activation via Elk-1 that is located downstream of the Ras/ERK cascade. In these assays, we co-transfected cells with a plasmid encoding the tyrosine kinase v-Abl to provide an upstream initiating signal (45). As shown in Fig. 5, co-transfection of NIH 3T3 cells with Grb2 augmented the transcriptional activity induced by v-Abl. Nck and Grb4 also enhanced the ability of Abl to induce luciferase expression, and although the effect of these adapter proteins was less than that seen with Grb2, they had similar potency to each other, suggesting a common mode of action. None of the adapter plasmids enhanced luciferase activity when transfected in the absence of v-Abl.

Because PAK has been implicated in Ras-independent ERK activation (14, 46), we next tested whether Nck and Grb4 might signal through PAK to induce gene expression. In our assay system, PAK did not induce transcriptional activation via Elk-1, either alone or in combination with Nck or Grb4. As shown for v-Abl, however, we did find that both Nck and Grb4 could weakly synergize with Sos to induce transcriptional activation (Fig. 5).

Nck and Grb4 Cooperate with Ras and v-Abl to Induce Morphologic Transformation of NIH 3T3 Cells—

Although it has previously been reported that Nck can induce transformation of rodent (NIH 3T3 and 3Y1) fibroblast cell lines (5, 33), in our hands Nck did not affect the morphology, growth rate, or sat-
performed in triplicate. The described (38). Data are representative of five or more experiments and Gal4-luc reporter plasmids. Luciferase activity was detected as foci/
The adapter protein significantly enhanced focus formation. Which v-Abl alone induced the formation of only 1–5 small 3T3 cells. Co-transfection of v-Abl with plasmids encoding ei-
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second SH3 domains) are conserved between Nck and Grb4. Which sites are phosphorylated and whether there is any differential regulation of Nck versus Grb4 by phosphorylation will require further study. Indeed, no physiological role has so far been ascribed to Nck phosphorylation.

Nck has been shown to bind to the Ras guanine nucleotide exchange factor, Sos, in vivo and to activate the c-Fos promoter in a Ras/ERK-dependent fashion (22). In another study, however, Nck dominant inhibitory mutants, unlike Grb2 mutants, did not block the activation of ERKs induced by oncogenic (ΔSH3) c-Abl when transiently overexpressed in 293-T cells (54). Furthermore, overexpression of Nck did not rescue NIH 3T3 cells from the inhibitory effects of the Sos C terminus, implying that Nck did not couple Sos to MAP kinase activation.
Grb7 has also been shown to be up-regulated could contribute to the transformed phenotype. Indeed, the proteins, such as Grb2, are required to transduce these deregulated signals from PTKs to Ras and to Rho protein effectors and to cooperate with signaling molecules to promote cellular transformation. It will be interesting to determine whether Nck or Grb4 overexpression contributes to human malignancies and in what context or at what point in development the body relies on Grb4 expression.

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REFERENCES

1. Pawson, T. (1995) Nature 373, 573–580
2. Schlessinger, J. (1993) Trends Biochem. Sci. 18, 273–275
3. Egan, S. E., and Weinberg, R. A. (1993) Nature 365, 781–783
4. Lehman, J. M., Lambertullier, G., and Johnson, J. P. (1990) Nucleic Acids Research 18, 1048
5. Li, W., Hu, P., Skolnik, E. Y., Ulrich, A., and Schlessinger, J. (1992) Mol. Cell. Biol. 12, 5824–5833
6. Nishimura, R., Li, W., Kashishian, A., Mondino, A., Zhou, M., Cooper, J., and Schlessinger, J. (1993) Mol. Cell. Biol. 13, 6898–6906
7. Guo, J., Qiu, J., Song, H. Y., Warren, R. S., and Donner, D. B. (1995) J. Biol. Chem. 270, 6729–6733
8. Kochhar, K. S., and Iyer, A. P. (1996) Cancer Lett. 104, 163–169
9. Lee, C. H., Li, W., Nishimura, R., Zhou, M., Batzer, A. G., Myers, M., Jr., M. F., Schlessinger, J., and Skolnik, E. Y. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 11713–11717
10. Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yanopoulous, G. D., and Pawson, T. (1997) EMBO J. 16, 3877–3888
11. Stein, E., Huynh-Do, U., Lane, A. A., Cerretti, D. P., and Daniel, T. O. (1998) J. Biol. Chem. 273, 13003–13008
12. Tang, J., Feng, G. S., and Li, W. (1997) Oncogene 15, 1823–1832
13. Schlaepfer, D. D., Broome, M. A., and Hunter, T. (1997) Mol. Cell. Biol. 17, 1702–1713
14. Lu, W., Katz, S., Gupta, R., and Mayer, B. J. (1997) Curr. Biol. 7, 85–94
15. Bagrodia, S., Taylor, S. J., Creasy, C. L., Chernoff, J., and Cerione, R. A. (1995) J. Biol. Chem. 270, 22731–22737
16. Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A., and Knaus, U. G. (1996) J. Biol. Chem. 271, 25746–25749
17. Galistone, M. L., Dikic, I., Batzer, A. G., Langdon, W. Y., and Schlessinger, J. (1995) J. Biol. Chem. 270, 20242–20245
18. Quilliam, L. A., Lambert, Q. T., Mickelson-Young, L. A., Westwick, J. K., Sparks, A. B., Kay, B. K., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and Der, C. J. (1996) J. Biol. Chem. 271, 28772–28776
19. Lussier, G., and Larose, L. (1997) J. Biol. Chem. 272, 2688–2694
20. Su, Y. C., Han, J., Xu, S., Cobb, M., and Skolnik, E. Y. (1997) EMBO J. 16, 1279–1290
21. Ren, R., Ye, Z. S., and Baltimore, D. (1994) Genes Dev. 8, 783–786
22. Hu, Q., Milloy, D., and Williams, L. T. (1995) Mol. Cell. Biol. 15, 1169–1174
23. Rivero-Lezcano, O. M., Marcilla, A., Sameshima, J. H., and Robbins, K. C. (1995) Mol. Cell. Biol. 15, 5725–5731
24. Cotterman, J. C., Urasulich, Z., Clemens, K. K., Price, J. V., and Dixon, J. E. (1994) J. Biol. Chem. 269, 17002–17005
25. Rivero-Lezcano, O. M., Sameshima, J. H., Marcilla, A., and Robbins, K. C. (1994) J. Biol. Chem. 269, 17383–17386
26. Lawe, D. C., Hahn, C., and Wong, A. J. (1997) Oncogene 14, 223–231
27. Symons, M., Derry, J. M., Jr., Kajuk, B., Jiang, S., Lemahieu, V., McCormick, F., Francke, U., and Abo, A. (1996) Cell 84, 723–734
28. Lu, L., Manser, E., Leung, T., and Hall, C. (1996) Eur. J. Biochem. 242, 171–185
29. Hall, A. (1998) Science 279, 509–514
30. Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T., and Zipursky, S. L. (1996) Cell 85, 639–650
31. Tanaka, M., Lu, W., Gupta, R., and Mayer, B. J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4398–4403
32. Hubscher, K., Kastury, K., Druck, T., Salcini, A. E., Lanfrancone, L., Pelici, G., Lowenstein, E., Li, W., Park, S. H., Cazzaniga, L., Pelici, P. G., and Schlessinger, J. (1994) Genomics 22, 281–287
33. Chow, M. L., Fajardo, J. E., and Hanafusa, H. (1992) Mol. Cell. Biol. 12, 5834–42
34. Margolis, B., Silvoninen, O., Comoglio, F., Roosprapunt, C., Skolnik, E., Ulrich, A., and Schlessinger, J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8894–8898
35. Clark, G. J., Cox, A. D., Graham, S. M., and Der, C. J. (1995) Methods Enzymol. 255, 395–412
36. Marais, R., Wynne, J., and Treisman, R. (1993) Cell 73, 381–393
37. Cepko, C. L., Roberts, B. E., and Mulligan, R. C. (1984) Cell 37, 1055–1062
38. Hauser, C. A., Westwick, J. K., and Quilliam, L. A. (1995) Methods Enzymol. 255, 412–426
39. Aronheim, A., Engelberg, D., Li, N., al-Alawi, N., Schlessinger, J., and Karin, M. (1994) Cell 78, 949–961
40. Shi, Z. Q., Lu, W., and Feng, G. S. (1998) J. Biol. Chem. 273, 4904–4908
41. Byrne, J. L., Paterson, H. F., and Marshall, C. J. (1996) Oncogene 13, 5793–5801

A. D. Cox and L. A. Quilliam, unpublished observations.
42. Chou, M. M., and Hanafusa, H. (1995) J. Biol. Chem. 270, 7359–7364
43. Park, D., and Rhee, S. G. (1992) Mol. Cell. Biol. 12, 5816–5823
44. Meisenhelder, J., and Hunter, T. (1992) Mol. Cell. Biol. 12, 5843–5856
45. Gibbs, J. B., Marshall, M. S., Scolnick, E. M., Dixon, R. A., and Vogel, U. S. (1996) J. Biol. Chem. 271, 20437–20442
46. Frost, J. A., Steen, H., Shapiro, P., Lewis, T., Ahn, N., Shaw, P. E., and Cobb, M. H. (1997) EMBO J. 16, 6426–6438
47. Suen, K. L., Bustelo, X. R., Pawson, T., and Barbacid, M. (1993) Mol. Cell. Biol. 13, 5500–5512
48. Fath, I., Schweighoffer, F., Rey, I., Multon, M. C., Boiziaz, J., Duchesne, M., and Tocque, B. (1994) Science 264, 971–974
49. Feng, G. S., Ouyang, Y. B., Hu, D. F., Shi, Z. Q., Gentz, R., and Ni, J. (1996) J. Biol. Chem. 271, 12129–12132
50. Matsuda, M., and Kurata, T. (1996) Cell. Signalling 8, 335–340
51. Chen, M., She, H., Davis, E. M., Spicer, C. M., Kim, L., Ren, R., Le Beau, M. M., and Li, W. (1998) J. Biol. Chem. 273, 25171–2518
52. Johnson, L., Greenbaum, D., Cichowski, K., Mercer, K., Murphy, E., Schmitt, E., Bronson, R. T., Umanoff, H., Edelman, W., Kucherlapati, R., and Jacks, T. (1997) Genes Dev. 11, 2468–2481
53. Koera, K., Nakamura, K., Nakas, K., Miyoshi, J., Toyoshima, K., Hatta, T., Otani, H., Aiba, A., and Katsuki, M. (1997) Oncogene 15, 1151–1159
54. Tanaka, M., Gupta, R., and Mayer, B. J. (1995) Mol. Cell. Biol. 15, 6829–6837
55. Khosravi-Far, R., Solski, P. A., Clark, G. J., Kinch, M. S., and Der, C. J. (1995) Mol. Cell. Biol. 15, 6443–6453
56. Renshaw, M. W., Kipreos, E. T., Albrecht, M. R., and Wang, J. Y. (1992) EMBO J. 11, 3941–3951
57. Pendergast, A. M., Quilliam, L. A., Cripe, L. D., Bassing, C. H., Dai, Z., Li, N., Batzer, A., Rabun, K. M., Der, C. J., Schlessinger, J., and Gishizky, M. L. (1993) Cell 75, 175–185
58. Stein, D., Wu, J. J., Fuqua, S. A., Roonrapunt, C., Yajnik, V., D'Eustachio, P., Moskow, J. J., Buchberg, A. M., Osborne, C. K., and Margolis, B. (1994) EMBO J. 13, 1331–1340
59. Tu, Y., Li, F., and Wu, C. (1998) Mol. Biol. Cell 9, 3367–3382