MOLECULAR AND CELLULAR BIOLOGY, Aug. 1994, p. 5495–5500
0270-7306/94/$04.00+0

Vol. 14, No. 8

CRK Protein Binds to Two Guanine Nucleotide-Releasing Proteins for the Ras Family and Modulates Nerve Growth Factor-Induced Activation of Ras in PC12 Cells

MICHIYUKI MATSUDA,[1,2]* YUKO HASHIMOTO,[3] KENKOH MUROYA,[3] HIDEKI HASEGAWA,[1] TAKESHI KURATA,[1] SHINYA TANAKA,[4] SHUN NAKAMURA,[3] AND SEISUKE HATTORI[3]

Department of Pathology, National Institute of Health, Shinjuku-ku, Tokyo 162,[1] PRESTO, Research Development Corporation of Japan, Kyoto 619-02,[2] Division of Biochemistry and Cellular Biology, National Institute of Neuroscience, NCNP, Kodaira-shi, Tokyo 187,[3] and Department of Pathology, Hokkaido University School of Medicine, Kita-ku, Sapporo 060,[4] Japan

Received 28 February 1994/Returned for modification 5 April 1994/Accepted 24 May 1994

It has been reported that growth factors activate Ras through a complex of an adaptor type SH2-containing molecule, Grb2, and a Ras guanine nucleotide-releasing protein (GNRP), mSos. We report on the involvement of another adaptor molecule, CRK, in the activation of Ras. Overexpression of wild-type CRK proteins CRK-I and CRK-II enhanced the nerve growth factor (NGF)-induced activation of Ras in PC12 cells, although the basal level of GTP-bound active Ras was not altered. In contrast, mutants with a single amino acid substitution in either the SH2 or SH3 domain of the CRK-I protein inhibited the NGF-induced activation of Ras. Two GNRPs for the Ras family, mSos and C3G, were coimmunoprecipitated with the endogenous Crk proteins in PC12 cells. The association between C3G and the CRK mutants was dependent upon the presence of intact SH3. The SH2 domain of CRK bound to the SHC protein phosphorylated on tyrosine residues by NGF stimulation. The results demonstrate that, in addition to Grb2, CRK participates in signaling from the NGF receptor and that two GNRPs appear to transmit signals from these adaptor molecules to Ras.

Tyrosine kinases play critical roles in the regulation of cell growth and differentiation (1, 40). The signals generated by tyrosine kinases are perceived by Src homology 2 (SH2) domains identified on a wide range of proteins (17, 23, 28). The diversity of SH2-containing molecules has raised a number of questions, particularly with respect to which SH2-containing molecules transmit the most vital signals. Recent data have demonstrated that activation of Ras is a prerequisite for tyrosine kinase-dependent cell proliferation and differentiation in various cells (7, 33). Activation of Ras triggers a serine/threonine kinase cascade including Raf and mitogen-activated protein (MAP) kinase, eventually mobilizing transcription factors such as c-Jun.

The Ras protein is regulated by three groups of proteins (4). First, there are proteins which accelerate the intrinsic GTPase activity of Ras, converting active GTP-bound Ras to the inactive GDP-bound state. One of these proteins, Ras GTPase-activating protein, contains two SH2 domains and is phosphorylated on tyrosine residues after stimulation by various growth factors (25). In PC12 cells, the GTPase-activating protein is activated after treatment with nerve growth factor (NGF) (18). However, activation of Ras does not always correlate with tyrosine phosphorylation of the GTPase-activating protein in hematopoietic cells (9), fibroblasts (25), and epithelial cells (12, 29). The second group of proteins, guanine nucleotide-releasing proteins (GNRPs) for the Ras family, activate Ras by converting GDP-Ras to the GTP-bound state. It has been demonstrated that GDP-GTP exchange-stimulatory activity increases in PC12 cells treated with NGF (18). The third group of proteins, guanine nucleotide dissociation inhibitors, blocks the exchange reaction catalyzed by Ras GNRPs (4).

Recently, many groups have reported that a complex of an adaptor protein, Grb2/Sem-5/Drk, and a Ras GNRP, Sos, transmits signals to Ras from tyrosine kinases (2, 5, 8, 10, 13, 19, 27, 31, 34). The SH2 domain of Grb2 binds to phosphotyrosine-containing peptides on the growth factor receptors or other tyrosine kinase substrates. The other domain on Grb2, SH3, binds to proline-rich sequences on mSos. Thus, Grb2 functions as an adaptor of mSos to tyrosine kinases.

The v-Crk protein was originally identified as an oncoprotein of a chicken retrovirus, CT10 (24). The homologs of v-Crk, chicken c-Crk and human CRK, consist mostly of the SH2 and SH3 domains (22, 30) which, together with Grb2 and Nck, belong to the adaptor type of protein. We have found that the CRK protein induces neuronal differentiation of PC12 cells and that this CRK-induced differentiation was inhibited by an anti-Ras neutralizing antibody, suggesting that the CRK protein also transmits a signal to Ras (38). Moreover, in the course of identifying the CRK SH3-binding proteins, we isolated the cDNA of a new GNRP for the Ras family, designated C3G (CRK SH3-associated GNRP) (39). We now report that the CRK protein enhances NGF-induced activation of Ras in PC12 cells and that two Ras GNRPs, mSos and C3G, are bound to the SH3 domain of the CRK protein.

MATERIALS AND METHODS

Cells and viruses. A rabbit kidney epithelial cell line, RK-13, and a thymidine kinase-negative human cell line, 143TK$^-$, were grown in Eagle's minimum essential medium supplemented with 5% heat-inactivated fetal calf serum. PC12 rat pheochromocytoma cells were maintained in Dulbecco's modified minimum essential medium supplemented with 10% fetal calf serum and 5% horse serum. The vaccinia virus LC 16mO

* Corresponding author. Mailing address: Department of Pathology, National Institute of Health, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162, Japan. Phone: 81-3-5285-1111, ext. 2625. Fax: 81-3-5285-1150. Electronic mail address: mmatsuda@nih.go.jp.

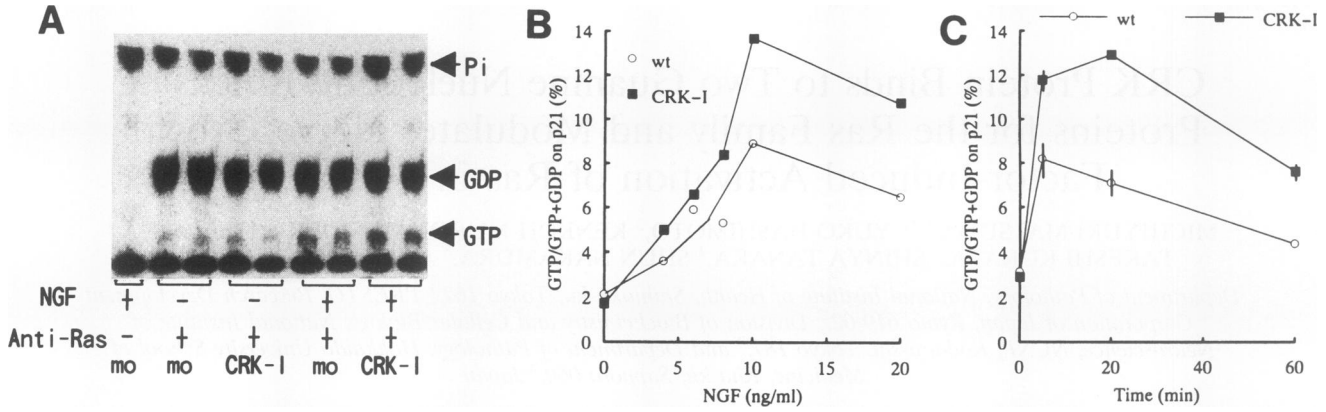

FIG. 1. NGF-induced activation of Ras is enhanced by CRK-I. (A) PC12 cells infected with the wild-type vaccinia virus LC 16mO strain (mo) or the recombinant vaccinia virus encoding CRK-I (CRK-I) were labeled with $^{32}P_i$. Cells stimulated with 10 ng of NGF per ml for 5 min or without any treatment were lysed, and Ras was immunoprecipitated with anti-Ras MAb 259. Guanine nucleotides bound to Ras were separated by thin-layer chromatography. (B) PC12 cells were treated with various amounts of NGF for 5 min. The relative amount of GTP as a percentage of $p21^{Ras}$-associated guanine nucleotides was calculated as previously described (26). (C) Time course of Ras activation. Cells were prepared as described for panel A and treated with 10 ng of NGF for the times indicated. wt, wild type.

strain and its recombinants were propagated in RK-13 cells (16).

Construction of recombinant vaccinia viruses. Molecular cloning of alternately spliced CRK cDNAs *CRK-I* and *CRK-II* was described previously (22). Arg-38, a phosphotyrosine-binding amino acid residue in the SH2 domain of CRK-I, was substituted by Val in an R38V mutant. Trp-169, an amino acid absolutely conserved among various SH3 domains, was substituted by Leu in a W169L mutant (21). *Nco*I fragments of pVCRK-I, pVCRKI-R38V, and pVCRKI-W169L, an *Nco*I-*Hin*dIII fragment of pVCRK-II (21), and an *Nco*I-*Bam*HI fragment of pC3G (39) were ligated with the pAK10 transfer vectors (16) cleaved with the same combinations of restriction enzymes. RK13 cells were transfected with calcium phosphate-precipitated plasmid DNA of pAK10 recombinants as previously described (16). Thymidine kinase-negative viruses were isolated by plaque assay on $143TK^-$ cells in the presence of 5-bromo-2′-deoxyuridine at a concentration of 25 μg/ml.

Analysis of Ras-bound GDP-GTP. PC12 cells (about 10^6) grown in 35-mm-diameter dishes were infected with the recombinant vaccinia viruses at a multiplicity of infection of 5 for 1 h. Cells were washed once and fed fresh medium as described elsewhere (16). Sixteen hours after infection, cells were washed with phosphate-free medium and labeled with 200 μCi of $^{32}P_i$ per ml for 2 h. After stimulation with NGF, guanine nucleotides bound to Ras were analyzed as previously described (26).

Immunoprecipitation, Western blotting (immunoblotting), and far Western blotting. Cells were lysed in lysis buffer (10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 500 μM Na_3VO_4, 50 μM Na_2MoO_4, 10 mM NaF). CRK proteins were immunoprecipitated with anti-CRK monoclonal antibody (MAb) 3A8 covalently bound to protein A-Sepharose (22). Anti-Shc rabbit serum (Upstate Biochem Inc.) and protein A-Sepharose were used to immunoprecipitate the Shc protein. The immune complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane. Proteins on the filter were probed with anti-CRK rabbit serum (22), anti-C3G rabbit serum (39), anti-mSos rabbit serum (Upstate Biochemicals Inc.), or anti-Shc rabbit serum. For far Western blotting, the filters were probed with a mixture of GST–CRK-I protein (1 μg/ml) and an anti-GST MAb as previously described (38). The bound antibodies were detected by peroxidase-labeled anti-rabbit and anti-mouse antibodies (TAGO Inc.) and the ECL chemiluminescence system (Amersham).

RESULTS

Expression of the CRK protein enhances NGF-induced Ras activation. To examine whether the CRK protein is involved in signaling from tyrosine kinases to Ras, the CRK-I protein, one of the two alternative splicing products, was expressed in PC12 cells by a recombinant vaccinia virus. Activation of Ras was monitored by measurement of the level of GTP-bound Ras. Expression of CRK-I did not increase the ratio of GTP-bound Ras without NGF, but it enhanced the NGF-induced net increase of GTP-bound Ras in a wide range of NGF concentrations (Fig. 1A and B). The effect of CRK on NGF-induced activation of Ras was observed just after stimulation, and the level was still higher after 60 min (Fig. 1C).

Mutant CRK proteins inhibit NGF-induced Ras activation. To analyze the effect of the CRK protein on the activation of Ras further, we expressed CRK proteins in PC12 cells (Fig. 2A). CRK-I and CRK-II are the wild-type CRK proteins generated by alternative splicing (22). In the R38V mutant, the phosphotyrosine-binding Arg-38 in the SH2 domain is substituted by Val. The W169L mutant contains Leu instead of Trp-169, which is absolutely conserved among various SH3 domains. These wild-type and mutant CRK proteins were expressed from the recombinant vaccinia virus vector to similar extents (Fig. 2B). PC12 cells infected by either nonrecombinant or recombinant vaccinia virus were stimulated with 10 ng of NGF per ml for 5 min, and GTP-bound Ras was measured (Fig. 3). Compared with control PC12 cells infected with nonrecombinant vaccinia virus, in cells expressing CRK-I and CRK-II, NGF-induced Ras activation was enhanced by 154 and 146%, respectively. In contrast, the R38V and W169L mutants suppressed NGF-induced Ras activation to 64 and 51% of that in control cells, respectively.

SH3 mutant W169L does not bind to C3G. To understand the mechanism of the inhibitory effect of CRK mutants on Ras activation, we examined whether the wild-type and mutant CRK proteins bind to the C3G protein, which was identified as

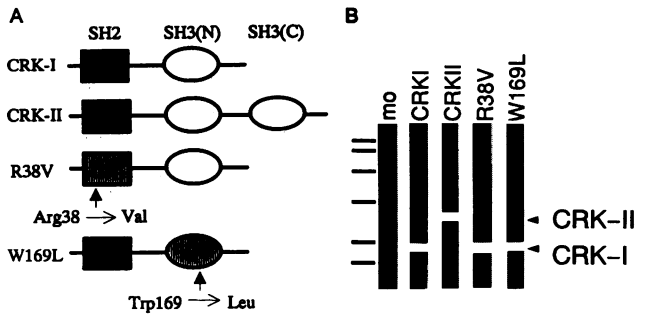

FIG. 2. Expression of CRK proteins by recombinant vaccinia viruses. (A) Schematic representation of wild-type and mutant CRK proteins. CRK-I and CRK-II are the two forms of wild-type CRK proteins generated by alternative splicing. Arg-38 and Trp-169 are replaced by Val in the R38V mutant and by Leu in the W169L mutant. (B) PC12 cells were infected with the recombinant vaccinia viruses at a multiplicity of infection of 5. Cells were lysed 16 h after infection with lysis buffer. A 5-μg sample of protein was separated by SDS-PAGE and analyzed by immunoblotting with anti-CRK serum. Bound antibody was detected by the ECL chemiluminescence system (Amersham) and Polaroid film. mo, vaccinia virus strain LC 16mO.

a CRK SH3-binding GNRP (Fig. 4). C3G and various CRK proteins were coexpressed in PC12 cells, and then CRK proteins were immunoprecipitated. The proteins in the immunoprecipitates were probed with anti-C3G serum. Wild-type CRK proteins CRK-I and CRK-II and SH2 mutant R38V bound to C3G in PC12 cells; however, SH3 mutant W169L did not bind to C3G. This suggests that the W169L mutant inhibits the activation of Ras by sequestering the upstream factor by its intact SH2 domain and that the R38V mutant forms a C3G-CRK complex defective in SH2-mediated signaling.

Binding of the endogenous CRK proteins to Ras-GNRPs. To understand the physiologic role of CRK, we examined the binding of the endogenous CRK proteins to Ras GNRPs. Two

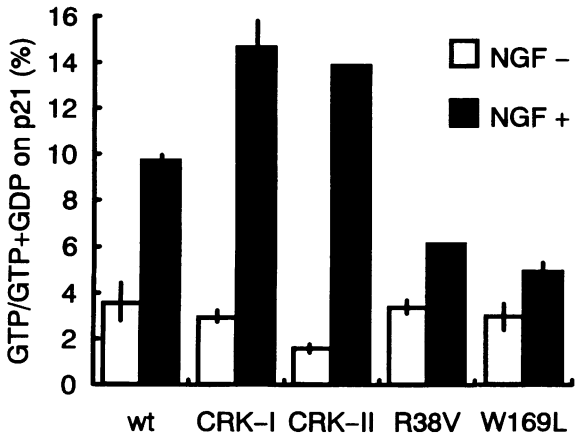

FIG. 3. Inhibition of NGF-induced activation by CRK mutants. PC12 cells were infected with recombinant vaccinia viruses encoding the two authentic CRK proteins, CRK-I and CRK-II, or mutants R38V and W169L. The structures and expression of the CRK proteins are shown in Fig. 2. $^{32}P_i$-labeled cells were stimulated with 10 ng of NGF per ml for 5 min. The guanine nucleotides bound to Ras were analyzed as described in the legend to Fig. 1. The assay was performed in duplicate, and the standard deviations are depicted by the bars. Similar results were obtained in three independent experiments. wt, wild type.

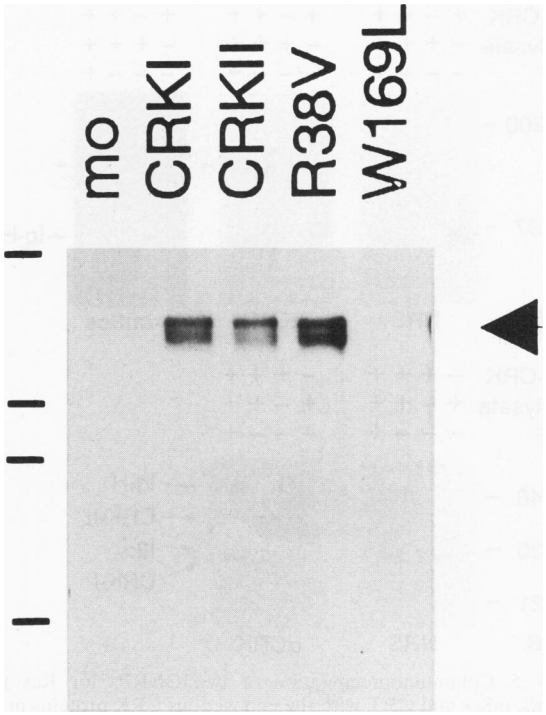

FIG. 4. Binding of CRK proteins to C3G. PC12 cells were coinfected with the C3G-encoding vaccinia virus and either the wild-type LC 16mO strain of vaccinia virus (mo) or the recombinant vaccinia viruses encoding the CRK proteins. Cells were lysed with lysis buffer, and CRK proteins were immunoprecipitated with anti-CRK MAb 3A8. The immune complexes were separated by SDS-PAGE and probed with anti-C3G rabbit serum. The horizontal bars are molecular size markers representing 200, 96, 69, and 45 kDa (from the top). The arrowhead indicates the recombinant C3G protein.

Ras GNRPs, mSos and C3G, have been shown to bind to the SH3 domains of GRB2 and CRK in vitro (39). The endogenous Crk proteins were immunoprecipitated by anti-CRK MAb 3A8 from semiconfluent PC12 cells. The immune complexes were resolved by SDS-PAGE and probed with rabbit serum against CRK, C3G, or mSos (Fig. 5). We detected two forms of the Crk proteins, $p28^{Crk-I}$ and $p40/42^{Crk-II}$, as was reported in rat 3Y1 fibroblasts and HeLa cells (22). The same immune complex contained both mSos and C3G. The antisera used in this experiment were raised against regions unique to mSos and C3G, and we could not detect cross-reactivity of these sera to each other (20). Thus, the endogenous Crk proteins bind to both mSos and C3G in PC12 cells. NGF treatment did not alter the quantity of the Crk-bound GNRPs. We could not detect the mobility shift of mSos and C3G caused by stimulation of NGF.

Binding of CRK to Shc. We have reported that NGF or epidermal growth factor (EGF) induces tyrosine phosphorylation of the Shc protein in PC12 cells (14). The association of the Shc and CRK proteins were examined first by far Western blotting (Fig. 6). The 46- and 52-kDa Shc protein was tyrosine phosphorylated after NGF and EGF stimulation, as demonstrated by binding to the anti-phosphotyrosine antibody. The tyrosine-phosphorylated Shc protein bound to the GST-CRK protein, but the nonphosphorylated Shc protein did not. In a similar experiment, NGF receptor $gp140^{Trk}$ did not bind to the GST-CRK protein (37). Moreover, we found that the 52-kDa Shc protein is associated with the endogenous Crk protein

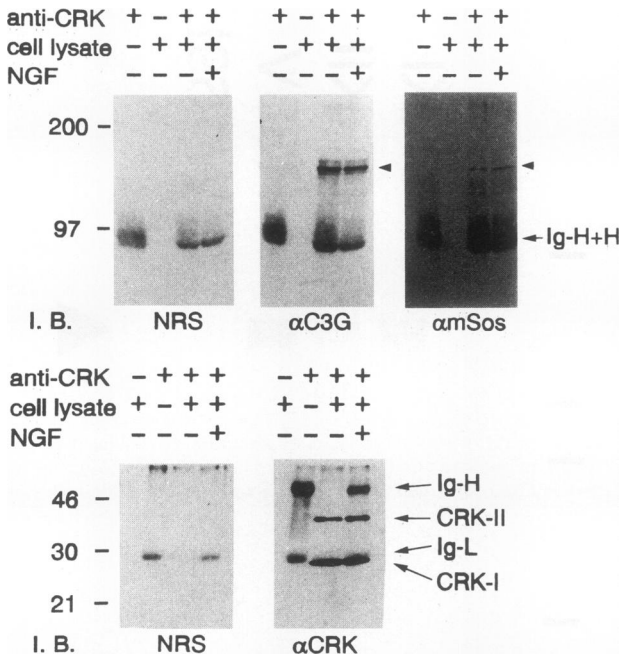

FIG. 5. Coimmunoprecipitation of two GNRPs for Ras family proteins, mSos and C3G, with the endogenous CRK proteins in PC12 cells. Semiconfluent PC12 cells (10^7) were stimulated with 10 ng of NGF per ml for 5 min. Cells with and without stimulation were lysed and incubated with 5 μg of anti-CRK MAb 3A8 covalently bound to protein A-Sepharose. As a negative control, a cell lysate was incubated with protein A-Sepharose alone or lysis buffer was incubated with 3A8-protein A-Sepharose. The immune complexes were separated by SDS-PAGE and subjected to immunoblotting with normal rabbit serum (NRS), rabbit serum against C3G (αC3G), mSos (αmSos), or CRK (αCRK). The arrowheads show the C3G and mSos proteins. Two CRK proteins are also indicated. Ig-H+H, immunoglobulin heavy chains dimerized during antibody coupling. Ig-H and Ig-L, immunoglobulin heavy and light chains, respectively. I. B., antibodies used for immunoblotting. The numbers on the left are molecular sizes in kilodaltons.

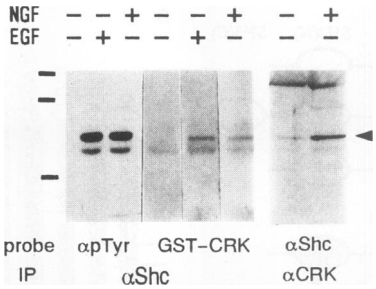

FIG. 6. Binding of CRK to Shc. PC12 cells were stimulated with NGF and EGF. The Shc protein was immunoprecipitated (IP), separated by SDS-PAGE, and transferred to a polyvinylidene difluoride membrane. After blocking, the Shc protein was detected by Western blotting with anti-phosphotyrosine antibody or by far Western blotting with GST-CRK protein and an anti-GST MAb. The bars are molecular mass markers representing 97, 69, and 46 kDa (from the top). The arrowhead indicates the Shc protein.

immunoprecipitated from NGF-stimulated PC12 cell lysates (Fig. 6). The 52-kDa Shc protein was also coimmunoprecipitated with Crk from unstimulated cell lysates but at a level significantly lower than that found in the immune complex from NGF-stimulated cells.

DISCUSSION

The neuronal differentiation of PC12 cells induced by the CRK protein is inhibited by an anti-Ras MAb (38), suggesting that CRK is an upstream factor of Ras. In this study, we have demonstrated that expression of CRK proteins modulates the activity of Ras in PC12 cells.

Expression of the Grb2 proteins enhanced and prolonged the EGF-induced activation of Ras in fibroblasts (13, 36). A similar phenomenon has been observed in the NGF-induced activation of Ras in CRK-expressing PC12 cells. Moreover, we demonstrated that the CRK mutants with dysfunctioning SH2 or SH3 inhibited the activation of NGF. This result strongly suggests that these mutants compete with endogenous Crk in the binding to tyrosine-phosphorylated signaling molecules or to the GNRPs for the Ras family, such as mSos and C3G, and thus inhibit the NGF-induced activation of Ras. Indeed, we have detected both the mSos and C3G proteins in Crk immune complexes from PC12 cells.

v-Crk enhances the stimulation of both NGF and EGF (15). However, the association of v-Crk with the activated receptors is demonstrated only in the EGF receptor (15). We have demonstrated the NGF-dependent binding of GST-CRK to Shc, overexpression of which induces neuronal differentiation of PC12 in a Ras-dependent manner (32). The result suggests that Crk accepts the signal from the NGF receptor through Shc. In a similar experiment, however, we found that the GST-CRK protein bound less to Shc proteins than the GST-Grb2 protein did (14). Considering our preliminary finding that the Grb2 protein is more abundant than CRK (20), this result suggests that CRK functions supplementarily to Grb2 in the Trk (NGF high-affinity receptor)-Shc pathway.

In PC12 cells, Grb2 also transmits signals from the NGF receptor to Ras (14, 32). Therefore, there are at least two adaptor molecules which transmit signals from the NGF receptor to Ras in PC12 cells. Consistent with this idea, microinjection of neutralizing antibodies against neither CRK nor Grb2 inhibited NGF-induced neurite formation (37). Although the SH3 domains of CRK and Grb2 can bind to common proteins, including C3G and mSos, the spectrums of the proteins bound by the SH3 domains apparently differ between CRK and Grb2 (38, 39); therefore, utilization of two adaptor molecules, Grb2 and CRK, increases the number of NGF-dependent effectors. In another aspect, the presence of two adaptor molecules upstream of Ras also increases the number of tyrosine kinases which transduce signals to Ras because the SH2 domains also possess divergent target specificities (35).

Expression of the CRK proteins by recombinant vaccinia viruses was not sufficient for activation of Ras. This result is consistent with the finding that overexpression of v-Crk is not sufficient to induce neuronal differentiation of PC12 cells (15); however, this conclusion argues against our previous study showing that microinjection of the CRK protein expressed as a GST fusion protein induces neuronal differentiation of PC12 cells (38). Recent studies have demonstrated that the SH2 domain of the CRK protein fused with GST has a higher capacity to bind to phosphotyrosine-containing proteins than does the wild-type CRK protein (3, 11). Because the SH2 domain of the GST-CRK protein competitively inhibits protein tyrosine phosphatase (3), microinjection of the GST-CRK

protein may increase the cellular phosphotyrosine level and thus mimic the stimulation of NGF.

Use of the genetic approach with *Drosophila melanogaster* has revealed that a complex of Grb2-Drk and Sos transduces signals from receptor type tyrosine kinases to Ras in R7 photoreceptors (27, 34). However, these findings do not exclude the possibility of other pathways connecting tyrosine kinases to Ras. Our results have demonstrated that CRK also transduces signals from tyrosine kinases to Ras in PC12 cells. We have recently identified a new Ras GNRP, C3G, which binds to Grb2 and CRK (39). Although the guanine nucleotide-releasing activity of C3G has been demonstrated only for yeast RAS, the results raise the possibility that signaling from the adaptor molecules to Ras is also divergent. Another Ras GNRP molecule, $CDC25^{Mm}$ (Ras GRF), appears to transduce signals generated by serum (6), suggesting that this molecule is also involved in tyrosine kinase-dependent Ras activation. In conclusion, the current study strongly suggests that tyrosine kinases activate Ras via several combinations of adaptor proteins and Ras GNRPs.

ACKNOWLEDGMENTS

This work was supported in part by grants from the Ministry of Education, Science, and Culture, from the Ministry of Health and Welfare, and from the Human Science Foundation, Japan.

We thank Asato Kojima for recombinant vaccinia virus preparation, Yoichi Tachibana for the anti-GST MAb, and Masato Nakafuku and Kazuo Nagashima for useful discussions.

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