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Both the SH2 and SH3 Domains of Human CRK Protein Are Required for Neuronal Differentiation of PC12 Cells

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Human CRK protein is a homolog of the chicken v-*crk* oncogene product and consists mostly of *src* homology region 2 (SH2) and SH3, which are shared by many proteins, in particular those involved in signal transduction. SH2 has been shown to bind specifically to phosphotyrosine-containing peptides. We report here that both SH2 and SH3 are required for signaling from CRK protein. Microinjection of the CRK protein induced neurite formation of rat pheochromocytoma cell line PC12. This activity was abolished by mutation of the CRK protein in either SH2 or SH3. The neuronal differentiation induced by the CRK protein was blocked by an excess amount of peptides containing CRK SH3. Moreover, we identified three proteins, of 118, 125, and 136 kDa, which bound specifically to CRK SH3. The CRK-induced neuronal differentiation was also suppressed by monoclonal antibodies against either CRK SH2 or p21^{ras}. These results suggest that both SH2 and SH3 of the CRK protein mediate specific protein-protein binding and that the resulting multimolecular complex generates a signal for neurite differentiation through activation of p21^{ras}.

The v-*crk* oncogene product shares homologous amino acid sequences, designated *src* homology region 2 (SH2) and SH3, with many molecules involved in signal transduction (24, 45). In addition to its transforming activity, v-*crk* has two particular features. (i) Cells transformed by v-*crk* show an elevated level of phosphotyrosine, despite a lack of tyrosine kinase activity in v-Crk protein (35, 36). (ii) v-Crk associates with a broad range of phosphotyrosine-containing proteins (29, 37). Both of these properties have been attributed to the SH2 of v-Crk, although some enhancing effect of SH3 on these activities has been suggested (30, 32, 38).

The cDNAs of proto-oncogene c-*crk* have been isolated from chicken (48) and human (33) cells. c-*crk* represents a newly emerging class of genes, such as NCK (26) and GRB2/ASH (28, 34), which encode proteins that consist mostly of SH2 and SH3. The homolog of the GRB2/ASH-encoding gene, *sem*-5 of *Caenorhabditis elegans* (10), has been genetically mapped upstream of the *let-60* Ras-like protein-encoding gene. Recent data have suggested that these proteins function as adaptors to connect components in signal transduction pathways (8, 27, 39, 44, 49).

SH2 has been shown to bind to phosphotyrosine-containing peptides (29, 30, 37). SH3 has been identified not only in SH2-containing proteins but also in many molecules related to the cytoskeleton (13, 20, 59). Drubin et al. have suggested that SH3 is the actin-binding site (13). Mutations of the SH3s of p60$^{c\text{-}src}$ and p133$^{c\text{-}abl}$ activate their tyrosine kinase activities (15, 17, 19, 21, 47, 51). Morphological changes induced by p60$^{v\text{-}src}$ are modulated by SH3, suggesting that SH3 associates with cellular factors (1, 2, 23). Recently, a protein which binds to the SH3 of c-Abl has been identified and found to have homology to GTPase-activating protein for rho (9).

Rat pheochromocytoma cell line PC12 has been widely used for studies of signal transduction by growth factors, particularly by the nerve growth factor (NGF) (17). Upon treatment with NGF, PC12 cells stop dividing, develop an excitable membrane, increase the amount of phosphotyrosine-containing protein, grow significant neuronal processes, and acquire a sympathetic neuronal phenotype (6, 11, 12, 46). In this NGF-triggered signaling pathway of PC12 cells, p21^{ras} has been shown to play a pivotal role. Introduction or expression of p21$^{v\text{-}ras}$ protein in PC12 cells induced neurite outgrowth of the cells (4, 43, 50), and microinjection of a neutralizing anti-p21^{ras} antibody (18, 25) or expression of dominant inhibitory mutant forms of p21^{ras} (56) suppressed the neuronal differentiation of PC12 cells induced by NGF or fibroblast growth factor. We have shown that NGF activates p21^{ras} by increasing the level of the p21^{ras}-GTP complex (40). Recently, Rozakis-Adcock et al. reported that the neuronal differentiation of PC12 cells induced by Shc, an SH2-containing protein, was also inhibited by the dominant-negative mutant form of p21^{ras} (49).

Here, we report that two human homologs of the v-Crk protein, designated CRK-I and CRK-II, induce neuronal differentiation of PC12 cells through activation of p21^{ras} and that intermolecular associations mediated by both SH2 and SH3 may be required for this signaling.

MATERIALS AND METHODS

Cell culture. PC12 rat pheochromocytoma cells were cultured on 35-mm-diameter dishes precoated with 10 μg of poly-D-lysine per ml in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and 5% horse serum at 37°C in a humidified atmosphere of 5% CO_2.

Preparation of antibodies for microinjection. Two anti-CRK(SH2) mouse monoclonal antibodies (MAbs), AD2 and

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3A8, were produced and characterized in our laboratory (31). Both MAbs were found to be specific to the SH2 of the CRK protein; however, only AD2 blocked the binding of SH2 to phosphotyrosine-containing proteins. To avoid the possible effect of the constant region of immunoglobulin, fragments of the variable region of antibodies were prepared by papain digestion. Anti-p21^{ras} rat MAb Y13-259 (54) and an anti-vesicular stomatitis virus mouse MAb (42) were also used.

Reagents. NGF (Takara Shuzo, Kyoto, Japan) was used at 50 ng/ml to induce neurite outgrowth of PC12 cells.

Construction of expression plasmids. Molecular cloning of two alternatively spliced cDNAs of the *CRK* gene, *CRK*-I and *CRK*-II, has been described previously (33). *CRK*-II has an extra SH3 at the carboxyl terminus, which was designated SH3(C) to distinguish it from that of the amino terminus, designated SH3(N). pGEX-CRK-I and pGEX-CRK-II were derived from pGEX2T (53), contained the *CRK*-I and *CRK*-II coding regions downstream of the glutathione *S*-transferase (GST) gene, and were used to make fusion proteins.

Expression plasmids for mutant CRK proteins were constructed by a combination of restriction enzyme cleavage, fragment preparation, and ligation together with site-directed mutagenesis. All of the mutant proteins were expressed as GST fusion proteins. The nucleotide sequences and deduced amino acid sequences of wild-type CRK-I (amino acids [aa] 1 to 204) and CRK-II (aa 1 to 304) have already been described (33). The amino acid sequences present in the mutant CRK proteins are as follows: CRK-II-dSH3(N), aa 1 to 121 and 230 to 304; CRK-I-SH2, aa 1 to 120; CRK-I-SH3(N), aa 121 to 204; CRK-II-SH3(C), aa 230 to 304; CRK-I-PST, aa 1 to 28 and 124 to 204; CRK-I-KPN, aa 1 to 177; CRK-I-SAL, aa 1 to 159; CRK-I-BGL, aa 1 to 150. Four mutants were directed to substitute four of the most conserved amino acids among the SH2s and SH3s. In CRK-I-V38, Arg-38 was changed to Val; in CRK-I-L94, Phe-94 was changed to Leu; in CRK-I-K150, Asp-150 was changed to Lys; in CRK-I-L169, Trp-169 was changed to Leu. In the other mutant proteins, clusters of basic amino acids in SH3 were substituted to abolish possible ionic interaction with other molecules. These substitutions are as follows: in CRK-I-MVL, Arg-120 to Met, Ser-121 to Val, and Arg-122 to Leu; in CRK-I-TQ, Lys-154 to Thr and Lys-155 to Gln; in CRK-I-QVQ, Arg-160 to Gln, Arg-162 to Val, and Lys-164 to Gln; in CRK-I-TT, Lys-178 to Thr and Arg-179 to Thr.

The SH3s of the mutant CRK proteins (aa 102 to 204) were also expressed as GST fusion proteins. We have previously described the v-Crk chimeras containing the SH3s of v-Src and phosphatidylinositol-specific phospholipase C-γ1 (PLC-γ1) (32). The SH3s of these mutant proteins (aa 102 to the carboxyl-terminal end) were similarly expressed as fusion proteins. The expression vector that encodes the SH3 of the p85 subunit of phosphatidylinositol 3-kinase (p85-SH3) has been previously described (57).

Purification of CRK peptides. Expression and purification of GST-CRK fusion proteins have been described previously (33). Briefly, CRK proteins were expressed in *Escherichia coli* DH5α, solubilized in phosphate-buffered saline (PBS) containing 1% Triton X-100, purified by glutathione-Sepharose column chromatography (Pharmacia), and dialyzed against PBS.

Microinjection. Microinjection was performed with an automated injection system (Zeiss). PC12 cells were injected with 0.5 pl of a protein solution as described previously (3).

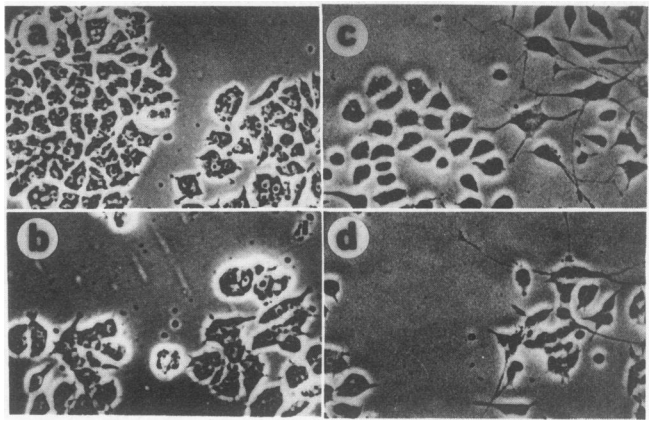

FIG. 1. Neurite outgrowth of PC12 cells induced by CRK-I. PC12 cells were microinjected with PBS (a), GST (b), the CRK-I protein expressed in *E. coli* as a GST fusion protein (c), or p21^{ras} (d). At 24 h after injection, photographs were taken to include both injected cells (left) and noninjected cells (right). Magnification, ×200.

Cells were fixed at 24 h after microinjection and probed with an anti-GST MAb as described below. Positive cells were counted as successfully injected cells, and the number of PC12 cells which extended neurites longer than the diameter of the cell body were counted as differentiated cells.

Immunocytochemistry. Cells were fixed with 4% paraformaldehyde in PBS at 37°C for 15 min, permeabilized with 0.2% Triton X-100 in PBS at room temperature for 3 min, probed with a MAb for 1 h, washed with PBS three times, and incubated with a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin antibody for 1 h. A fluorescein isothiocyanate-conjugated anti-rat immunoglobulin antibody was used for detection of injected anti-p21^{ras} MAb Y13-259. Cells were observed by fluorescence-activated or confocal microscopy (Bio-Rad).

Detection of the SH3-binding protein. Total lysates of PC12 cells were separated on sodium dodecyl sulfate (SDS)-polyacrylamide gels and transferred onto a polyvinylidene difluoride membrane. Nonspecific binding of the filters was blocked by 2% skim milk in PBS containing 0.05% Tween 20 for 12 h at 4°C. The filters were incubated with 2 μg of SH3 peptides per ml in PBS–0.05% Tween 20 for 2 h at 25°C. After being washed in PBS–0.05% Tween 20, bound peptides were detected by successive incubation with an anti-GST MAb, an alkaline phosphatase-conjugated anti-mouse immunoglobulin antibody, and 5-bromo-4-chloro-3-indolylphosphate toluidinium (salt)–nitroblue tetrazolium (BCIP-NBT).

RESULTS

Neurite outgrowth of PC12 cells induced by CRK protein. We microinjected PC12 cells with the CRK-I protein expressed in *E. coli* as a GST fusion protein. The CRK-I protein induced neurite outgrowth of PC12 cells after 16 h, as did p21$^{v\text{-}ras}$ (Fig. 1). To confirm that the differentiated cells contained the CRK-I protein, cells were immunostained with the anti-GST MAb (Fig. 2). All cells with neurite outgrowth were stained positively; however, some positive cells did not fulfill our criteria for neurite outgrowth, probably because of damage during microinjection. Cells positively stained with anti-GST MAb were counted as successfully injected cells. We repeated this experiment 17 times with different batches

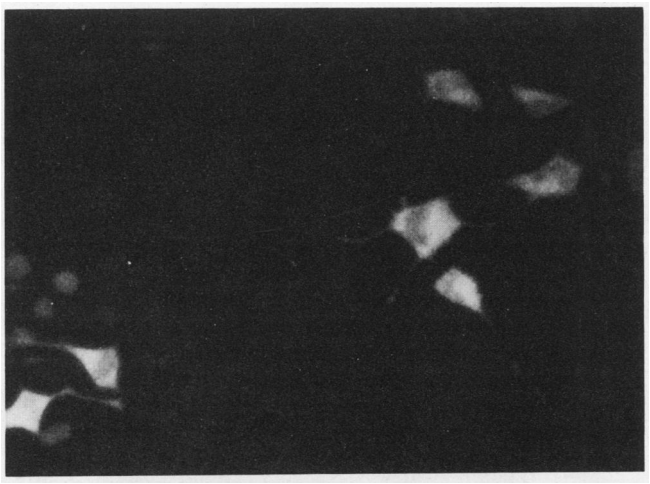

FIG. 2. Immunostaining of PC12 cells microinjected with the CRK-I protein. PC12 cells microinjected with the CRK-I protein were incubated with the anti-GST MAb and then with an anti-mouse antibody conjugated with fluorescein isothiocyanate. The same area is also presented in Fig. 1c.

of PC12 cells. On average, 51% ± 15% of CRK-I-injected cells underwent neuronal differentiation.

We microinjected a series of mutant CRK proteins to define the region required for CRK-mediated neuronal differentiation of PC12 cells. Figure 3 shows the schematic structure of the mutant CRK proteins and their abilities to induce neurite outgrowth of PC12 cells. The CRK-II protein, which is another product of alternative splicing and has an extra SH3 at the carboxyl terminus of CRK-I, also induced neurite outgrowth. Deletions or a point mutation in SH2 [SH3(N), SH3(C), PST, V38] which abolished binding to phosphotyrosine-containing proteins completely prevented these proteins from inducing neurite outgrowth of PC12 cells. Another SH2 mutant protein, L94, which showed intermediate binding activity, retained the capacity to induce neurite outgrowth. Deletions or three of five amino acid substitutions in SH3(N) (SH2, KPN, SAL, BGL, K150, TQ, and L169) impaired the ability of CRK-I to induce neurite outgrowth (Fig. 4). These results demonstrate that both SH2 and SH3(N) are required for induction of neurite outgrowth of PC12 cells. MVL, QVQ, and TT were fully active in the induction of neurite outgrowth, suggesting that the amino acids substituted in these mutant proteins were not directly involved in CRK-induced neurite outgrowth.

Competition of CRK-I-induced neurite outgrowth by SH3 peptides. Although some reports have suggested that SH3 binds to cellular proteins (7, 9, 13, 24, 41, 45), there has been no direct evidence that this interaction plays a crucial role in living cells. To evaluate the role of SH3 in CRK-induced signaling, we introduced an excess amount of SH3 peptides into PC12 cells together with CRK-I (Table 1). Prominent inhibition of neurite outgrowth was observed at concentrations of more than 3 mg of SH3(N) per ml, namely, at an SH3(N)/CRK-I molar ratio of greater than 7. Microinjection of SH3(N) also inhibited the neurite outgrowth induced by CRK-II but not that induced by $p21^{v\text{-}ras}$ or NGF (data not shown), indicating that the inhibition was specific to CRK-induced neurite outgrowth. SH3 peptides carrying mutations were similarly comicroinjected with CRK-I (Table 1). Mutations in K150 and TQ abolished the ability of CRK-I to induce neurite outgrowth and impaired the ability of the

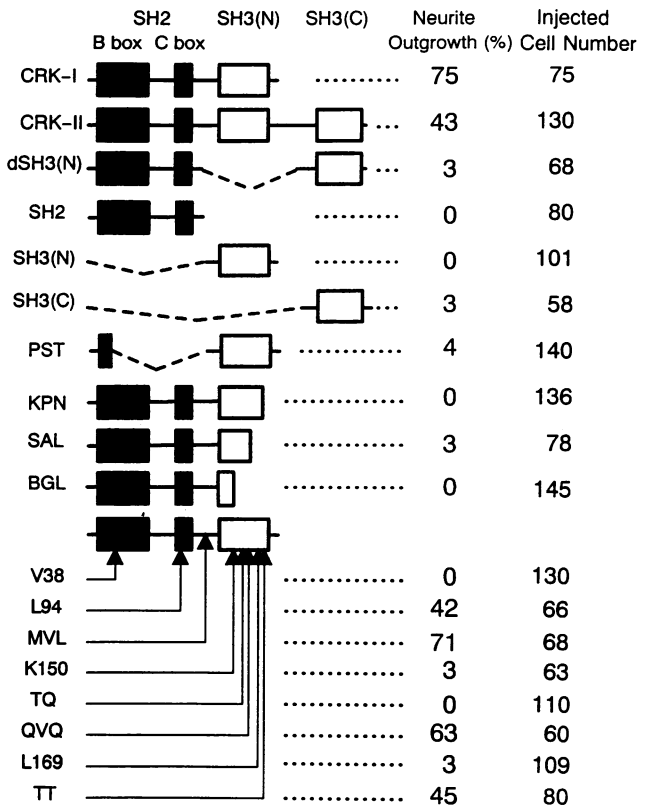

FIG. 3. Schematic structure of the CRK proteins and their abilities to differentiate PC12 cells. Closed boxes represent the B and C boxes of SH2. Open boxes show the amino- and the carboxyl-terminal SH3s, designated SH3(N) and SH3(C), respectively. The names of the mutant proteins listed here omit the prefix GST-CRK-I or GST-CRK-II, indicating that the mutants are derived from CRK-I or CRK-II and expressed as fusion proteins of GST. The precise deletions and the amino acid substitutions of the mutant proteins are described in the text. Briefly, the proteins are divided into four categories: wild type, CRK-I, and CRK-II; deletion of all of SH2 or SH3 from dSH3(N) to SH3(C); partial deletion of SH2 or SH3 from *Pst* to *Bgl*; amino acid substitutions in SH2 or SH3 from V38 to TT. Each mutant was microinjected into PC12 cells at a concentration of 1 mg/ml. The percentages of cells extending neurites among successfully injected cells are shown on the right. The data represent typical results of three independent experiments.

corresponding SH3(N) peptides to compete with CRK-I. In contrast, a mutation in TT affected neither its ability to induce neurite outgrowth nor the capacity of the corresponding SH3(N) to compete with CRK-I. These results strongly suggest that binding of SH3(N) to other cellular factors was required for neuronal differentiation of PC12 cells by CRK-I. We examined the specificity of the interaction mediated by SH3(N) by introducing SH3(C) and the SH3s of the other proteins: v-Src, PLC-γ1, and the p85 subunit of phosphatidylinositol 3-kinase (Table 1). The SH3 peptides of SH3(C), v-Src, and p85 did not decrease the ratio of the differentiated cells to the same extent as did the SH3(N) of CRK. We could not concentrate the SH3 peptide of PLC-γ1 to more than 3 mg/ml; therefore, a clear result was not obtained with this peptide.

Binding of SH3 peptides to cellular proteins. We searched for the targets of CRK SH3 by performing a filter-binding assay. Proteins from PC12 cells were probed with the

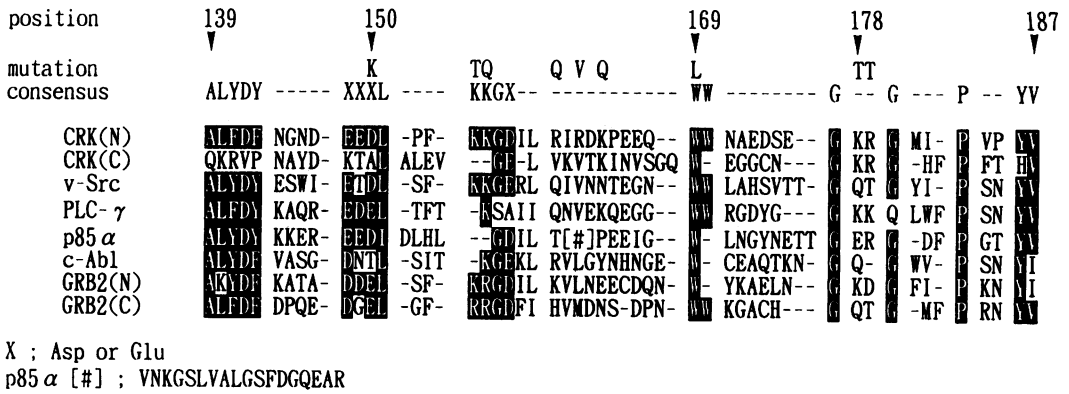

FIG. 4. Amino acid substitutions in SH3(N) of mutant CRK-I proteins. Amino acid sequences of the SH3s of CRK proteins were aligned with those of v-Src (22), PLC-γ (55), the p85α subunit of phosphatidylinositol 3-kinase (52), c-Abl (5), and GRB2 (28). Residues that are conserved within at least half of the SH3s are designated consensus residues and are shown as black boxes in each sequence. Two of the most conserved amino acids (Asp-150 and Trp-169) are substituted in two mutant proteins (K150 and L169). Clusters of basic amino acids in SH3 are substituted as follows in the other three mutant proteins: TQ, Lys-154 to Thr and Lys-155 to Gln; QVQ, Arg-160 to Gln, Arg-162 to Val, and Lys-164 to Gln; TT, Lys-178 to Thr and Arg-179 to Thr. The mutations are indicated above the consensus sequence.

SH3(N) peptides of CRK-I and its mutant forms (Fig. 5A and B). By comparing the proteins detected with GST alone and with peptides containing CRK SH3(N), we found that three proteins, of 118, 125, and 136 kDa, bound specifically to CRK SH3(N) (Fig. 5A, lanes 3, 4, and 6). These proteins were in the cytoplasmic fraction after subcellular fractionation (data not shown).

The binding of mutant SH3s to these three proteins correlated well with the capacity of corresponding CRK-I proteins to induce neurite outgrowth in PC12 cells. The SH3s of QVQ (Fig. 5A, lane 10) and TT (lane 12) bound to these three peptides, whereas those of K150 (lane 8), TQ (lane 9), and L169 (lane 11) did not. This strongly suggested that binding of SH3(N) to the 118-, 125-, and 136-kDa proteins was specific and that this binding was required for signal transduction from the CRK protein. We did not detect any specific binding of proteins to the SH3s of SH3(C) and the p85 subunit of phosphatidylinositol 3-kinase (lanes 7 and 15). The

TABLE 1. Competition for CRK-I-induced neurite outgrowth by SH3 peptides[a]

Competitor[b]	Concn (mg/ml)	Neurite outgrowth (%)	No. of injected cells
GST	5.5	45	138
SH3(N)	0	36	120
	0.6	32	121
	3.0	8	78
	5.0	4	66
	10.0	3	108
K150	6.0	30	115
TQ	4.0	20	60
TT	3.0	9	88
SH3(C)	4.0	31	104
PLC-γ	3.0	18	95
v-Src	6.0	20	60
p85	5.0	20	99

[a] CRK-I (0.6 mg/ml) was microinjected into PC12 cells with the SH3s of CRK, its mutant forms shown in Fig. 1, and other signal-transducing molecules.

[b] Mutant peptides corresponding to SH3(N) were expressed and purified as GST fusion proteins. The peptides containing SH3 are from v-Src Ala-80 to Pro-139 (32), PLC-γ Arg-790 to Glu-848 (32), and phosphatidylinositol 3-kinase p85 Met-1 to Gln-111 (52).

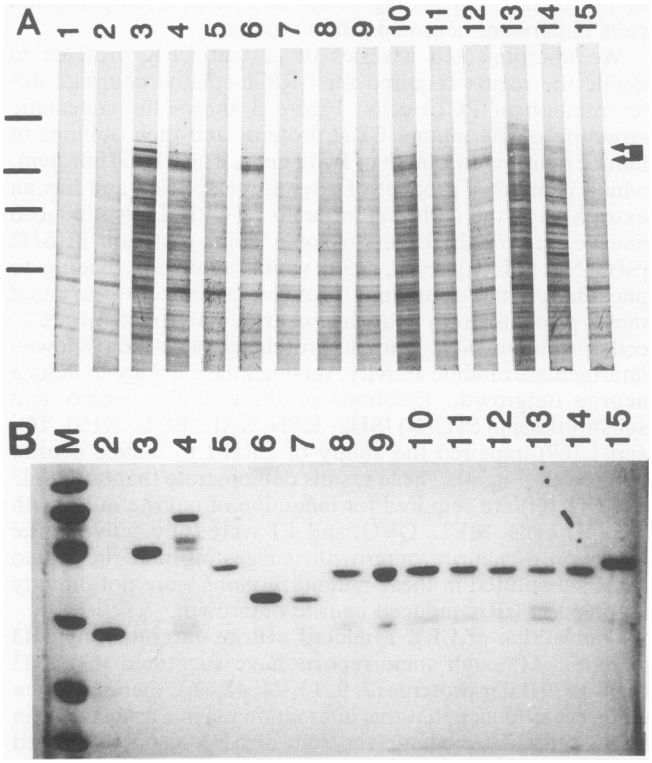

FIG. 5. Binding of SH3 peptides to cellular proteins. (A) Cellular proteins from PC12 cells were separated on SDS-polyacrylamide gels and probed with peptides expressed as GST fusion proteins. Lanes: 1, no peptide; 2, GST alone; 3, CRK-I protein; 4, CRK-II protein; 5, SH2. In the following lanes, only the SH3s of the proteins were used as probes: 6, SH3(N); 7, SH3(C); 8, K150; 9, TQ; 10, QVQ; 11, L169; 12, TT; 13, PLC-γ; 14, v-Src; 15, p85 subunit of phosphatidylinositol 3-kinase. The bars are molecular mass markers that correspond to (from the top) 200, 116, 97, and 66 kDa. The arrows indicate proteins of 118, 125, and 136 kDa. (B) The peptides used as probes were analyzed by SDS-polyacrylamide gel electrophoresis. Lane M contained standard proteins of 106, 80, 50, 33, 28, and 19 kDa.

TABLE 2. Inhibition of the CRK-induced neurite outgrowth of PC12 cells by MAbs[a]

Injected protein	Comicroinjected MAb[b]	Neurite outgrowth (%)	No. of injected cells
CRK-I	None	57	156
	Anti-CRK(SH2) AD2	1	180
	AD2 Fab fragment	0	103
	Anti-CRK(SH2) 3A8	51	109
	Anti-p21^{ras} Y13-259	1	166
	Anti-vesicular stomatitis virus	63	154
p21$^{v\text{-}ras}$	None	66	114
	Anti-p21^{ras}	0	128

[a] CRK-I (0.6 mg/ml) and p21$^{v\text{-}ras}$ (1 mg/ml) were microinjected into PC12 cells with the MAbs indicated at a concentration of 10 mg/ml.

[b] Anti-CRK(SH2) mouse MAbs AD2 and 3A8 were produced and characterized in our laboratory (31). To avoid the possible effect of the constant region of immunoglobulin, fragments of the variable region (Fab) of antibodies were prepared by papain digestion. A neutralizing anti-p21^{ras} rat MAb, Y13-259, and an anti-vesicular stomatitis virus mouse MAb were also used.

SH3s of PLC-γ and v-Src bound to distinct sets of proteins (lane 13 and 14). The results show that each SH3 binds to and transmits a signal to a distinct subset of proteins.

Inhibition of CRK-induced neurite outgrowth by MAbs against CRK(SH2) and p21^{ras}. To examine further the requirement for binding of SH2 to phosphotyrosine-containing proteins during neurite outgrowth, we microinjected anti-CRK(SH2) MAbs together with CRK-I (Table 2). MAb AD2, which was shown to inhibit binding of SH2 to phosphotyrosine-containing proteins, completely abolished induction of neurite outgrowth. Fab fragments of MAb AD2 yielded a similar result, excluding the possible effect of the constant region of the antibody. In contrast, another anti-CRK(SH2) MAb, 3A8, did not inhibit either binding to phosphotyrosine-containing proteins or neurite outgrowth of PC12 cells.

We introduced neutralizing anti-p21^{ras} MAb Y13-259 into PC12 cells together with the CRK-I protein to examine the requirement of p21^{ras} for CRK-induced neurite outgrowth (Table 2). Microinjection of MAb Y13-259 prominently inhibited neurite outgrowth induced by the CRK-I protein, as well as that induced by p21$^{v\text{-}ras}$. An irrelevant MAb against vesicular stomatitis virus did not suppress the neurite outgrowth induced by CRK-I or p21$^{v\text{-}ras}$. These data suggest that CRK protein transduces the signals for neuronal differentiation through activation of the p21^{ras} protein.

DISCUSSION

The CRK protein belongs to a newly emerging class of proteins that are thought to play an important role in signal transduction from tyrosine kinases. These proteins, CRK, Nck (26), and GRB2/ASH/Sem-5 (10, 28, 34), consist mostly of SH2s and SH3s, the number and arrangement of which vary in each protein. A genetic study with *C. elegans* revealed that the *sem*-5-encoded protein acts upstream of a *let*-60-encoded Ras-like protein and downstream of a *let*-23-encoded epidermal growth factor receptor-like protein in vulval formation (10). Moreover, human GRB2 promoted cell growth under overexpression of the H-Ras protein (28) and antisense RNA of mouse ASH inhibited cell replication (34). Our results demonstrate that the CRK protein also transduces signals by activating p21^{ras}. Therefore, this group of proteins appears to have the common property of activating Ras family proteins. Recently, the 3BP-1 protein, which has homology to GTPase-activating protein for rho, was identified as a protein bound to the SH3 of c-Abl, suggesting a direct or indirect link of the SH3-containing protein to the Ras and Ras-like proteins (9).

An excess amount of the peptide covering SH3(N) inhibited CRK-induced neuronal differentiation of PC12 cells. This implies the presence of a limited amount of the targets of the CRK SH3(N) domain in PC12 cells. Because the neurite formation induced by p21$^{v\text{-}ras}$ or NGF was not inhibited by CRK-SH3(N) (data not shown), the inhibition was specific to CRK-induced neurite formation. Mutations of SH3(N) which impaired the capacity of the CRK protein to induce neuronal cell differentiation also abolished the inhibitory effect of the corresponding SH3(N) peptides on CRK-I-induced neurite formation. These results suggested that SH3 transduces a signal through direct binding to cellular targets. Lack of inhibition of CRK-induced neuronal cell differentiation by SH3(C) and SH3 of v-Src, PLC-γ1, and the p85 subunit of phosphatidylinositol 3-kinase indicated that the intermolecular association mediated by SH3 is specific to each SH3, as is the case with SH2 (14, 24, 30). In agreement with the findings described above, we identified three proteins, of 118, 125, and 136 kDa, in PC12 cells that bound specifically to CRK SH3(N). Another piece of evidence for the specificity of each SH3 has been obtained by using a v-Crk chimera containing the SH3s of v-Src, c-Src, and PLC-γ (32). Only the SH3 of v-Src partially restored the transforming phenotype of the v-Crk protein.

Although the CRK protein induced neuronal differentiation of PC12 cells, as did NGF, the CRK protein did not appear to be an essential component of NGF-triggered signal transduction. The neuronal differentiation of PC12 cells induced by NGF was not inhibited by microinjection of the neutralizing anti-CRK(SH2) MAb or the SH3 mutant proteins which competed with wild-type CRK (data not shown). Because many factors can induce neurite outgrowth of PC12 cells, it is possible that CRK is responsible for signal transduction of factors other than NGF. Alternatively, other CRK-like proteins, such as GRB2/ASH/Sem5 or Nck, might compensate for each other.

Previous studies have strongly suggested that binding of SH2 to phosphotyrosine-containing proteins in vitro represents an essential function in vivo (24). Microinjection of neutralizing anti-CRK(SH2) MAb AD2 has provided direct evidence that the protein-protein interaction mediated by SH2 is an indispensable function of SH2s in vivo.

On the basis of the results presented here, we propose a model for signal transduction from the CRK protein (Fig. 6). Stimulation of cells by growth factors or cytokines leads to tyrosine phosphorylation of proteins localized in and beneath the cytoplasmic membrane (7, 58). By means of the interaction between tyrosine-phosphorylated peptides and SH2, the CRK protein may translocate to the membrane, accompanying the cytosolic SH3-binding proteins, including those of 118, 125, and 136 kDa. Because the SH2 of CRK binds to a broad range of phosphotyrosine-containing proteins (29) and because the SH3 of CRK binds to at least three proteins, various combinations of multimolecular complexes may be formed. These multimolecular complexes, consisting of CRK, SH3-binding molecules, and SH2-binding proteins, may transduce various second signals, one of which is directed toward Ras.

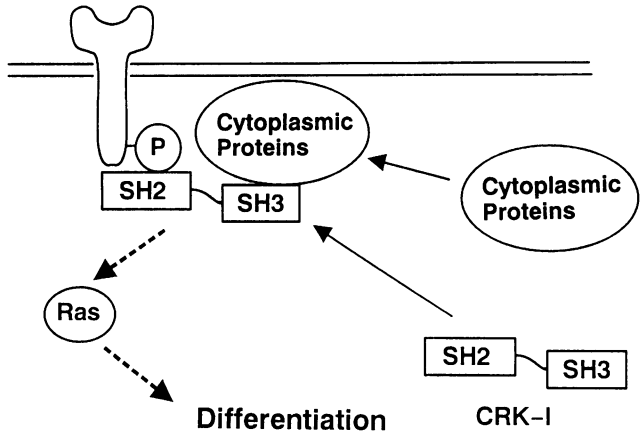

FIG. 6. A model for neuronal differentiation of PC12 cells caused by the CRK-I protein. An increased amount of CRK-I protein facilitates formation of multimolecular aggregates composed of CRK-I, tyrosine-phosphorylated proteins bound to SH2, and cytoplasmic proteins bound to SH3.

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