*Proc. Natl. Acad. Sci. USA*
Vol. 91, pp. 3443–3447, April 1994
Biochemistry

# C3G, a guanine nucleotide-releasing protein expressed ubiquitously, binds to the Src homology 3 domains of CRK and GRB2/ASH proteins

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*Communicated by Hidesaburo Hanafusa, December 23, 1993*

**ABSTRACT** **CRK protein, together with GRB2/ASH and Nck proteins, belongs to the adaptor-type Src homology (SH)2-containing molecules, which transduce signals from tyrosine kinases. Here another guanine nucleotide-releasing protein (GNRP), C3G, has been identified as a CRK SH3-binding protein. The nucleotide sequence of a 4.1-kb C3G cDNA contains a 3.2-kb open reading frame encoding a 121-kDa protein, and antibodies against C3G have been shown to detect a protein of 130–140 kDa. The carboxyl terminus of C3G has a peptide sequence homologous to GNRPs for Ras, and the expression of this carboxyl terminus region suppresses the loss of *CDC25* function in the yeast *Saccharomyces cerevisiae*. The C3G protein expressed in *Escherichia coli* binds to CRK and GRB2/ASH proteins. Mutational analysis of C3G assigns the SH3 binding region to a 50-amino acid region containing a proline-rich sequence. The mRNAs of both the C3G and CRK proteins are expressed ubiquitously in human adult and fetal tissues. The results of these studies suggest that the complex of CRK and C3G, or GRB2/ASH and C3G, may transduce the signals from tyrosine kinases to Ras in a number of different tissues.**

Growth factors elicit various responses through the activation of receptor-type and non-receptor-type tyrosine kinases (1). A group of cytoplasmic enzymes containing common amino acid sequences, designated Src homology (SH)2 and SH3 domains, play a pivotal role in transducing signals from the tyrosine kinases (2, 3). The SH2 domain responds to the signals from tyrosine kinases by binding to the tyrosine-phosphorylated proteins, including the tyrosine kinases themselves. Some of the signals are also transmitted to proteins bound to the SH3 domains, but much less information is available on SH3-mediated signaling.

The v-Crk protein was originally identified as an oncoprotein of a chicken retrovirus, CT10 (4). The protooncogene product v-Crk represents a newly emerging class of proteins consisting mostly of the SH2 and SH3 domains (5, 6). These proteins, now known as adaptor proteins, include Nck, GRB2/ASH, Sem-5, and Drk (2, 7, 8). Sem-5 of *Caenorhabditis elegans* and Drk of *Drosophila melanogaster* appear to be homologues of mammalian GRB2/ASH. All of the adaptor proteins may be involved in the growth of fibroblasts. Overexpression or microinjection of CRK, Nck, and GRB2 induces transformation of rat 3Y1 fibroblasts or DNA replication in mouse 3T3 fibroblasts (6, 9, 10). One common feature of the adaptor proteins may be signal transmission to Ras. The *sem-5* gene of *C. elegans* has been mapped genetically downstream of *let-23* tyrosine kinase and upstream of *let-60* Ras-like protein (11). Similarly, *drk* of *Drosophila* is mapped between sevenless (*sev*) receptor tyrosine kinase and son of sevenless (*sos*), which encodes a guanine nucleotide-releasing protein (GNRP) for Ras1. Recently, the Sos protein of *Drosophila* was shown to bind to the Drk protein, which contains SH2 and SH3 domains (7, 8). Anti-Ras antibody also inhibited neuronal differentiation of PC12 cells induced by the human CRK protein (12).

Neuronal differentiation of PC12 cells induced by CRK is blocked in the presence of excess quantities of peptides covering the CRK SH3 domain (12), suggesting that the SH3 domain of CRK mediates protein–protein interaction. We have identified a number of cytosolic proteins which bind to the SH3 domain of CRK but not to the SH3 domains of phospholipase C-γ, Src, or the p85 subunit of phosphatidylinositol 3-kinase (12). To investigate the hypothesis that these CRK SH3-binding proteins would transduce the signal from CRK to downstream factors, including Ras, we have now isolated and characterized their cDNAs.†† We report here that one of these encodes a newly identified protein with homology to GNRPs for Ras: CDC25, Ste6, and Sos. This has been designated C3G, as it can be clearly demonstrated to be a CRK SH3-binding GNRP.

## MATERIALS AND METHODS

**Probes Used for Far Western Blotting.** Peptides containing the SH3 domains of the CRK-I [amino acids (aa) 121–204] (6), CRK-I-K150 and CRK-I-L169 mutants in which Asp-150 and Trp-169 of CRK-I were replaced by Lys and Leu, respectively, and GRB2/ASH (aa 1–217) (13) were expressed in *Escherichia coli* as fusion proteins of glutathione *S*-transferase (GST). These GST fusion proteins were purified on a glutathione-Sepharose 4B column (Pharmacia) as described (6).

**Screening of the cDNA Library.** A λgt11 cDNA expression library constructed from mRNA isolated from human spleen cells was obtained from Clontech. Recombinant clones expressing SH3-binding proteins were identified by Far Western blotting using GST-CRK SH3 and anti-GST monoclonal antibody as described (12). To isolate the 5′ end of the gene, we rescreened libraries derived from spleen and placenta,

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Abbreviations: GRF, guanine nucleotide-releasing factor; GNRP, guanine nucleotide-releasing protein; SH, Src homology; GST, glutathione *S*-transferase.

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††The sequence reported in this paper has been deposited in the GenBank data base (accession no. D21239).

using $^{32}$P-labeled cDNA isolated from the initial positive phage.

**Sequence Analysis.** The cDNA was sequenced on both strands and across all cloning sites by use of pUC118 and pUC119 vectors. Dideoxynucleotide chain-termination sequencing reactions were carried out with reagents from Pharmacia. Standard 6% polyacrylamide sequencing gels were used, and products were analyzed with a fluorescently tagged primer and an ALF DNA sequencer (Pharmacia).

**Yeast Strains, Media, and Genetic Manipulations.** The *Saccharomyces cerevisiae* strain used in this study was a temperature-sensitive *cdc25* mutant, KMY172-5A (*MATa cdc25-1 trp1 ura3 his3 ade leu2*). The yeast shuttle vector pKT10 (14) was cleaved with *Asp* 718 or *Kpn* I and blunt-ended with T4 DNA polymerase. These vectors were designated pKT10A and pKT10K, respectively. The *Pvu* II/*Xho* I fragment of pST6 (nt 1982–3393) was ligated with pKT10K cleaved with *Pvu* II/*Sal* I to generate pKC3GPv. Similarly, the *Hin*cII/*Xho* I fragment (nt 2452–3393) was ligated with pKT10A to form pKC3GHc. KMY172-5A was transformed with expression vectors pKT10 and YRp7 as negative controls, and with pKC3GPv, pKC3GHc, and pL25/SP, which is a YRp7 derivative containing the wild-type *CDC25* gene (15). The transformants were selected on appropriate selective plates at 25°C. Eight independent transformants from each of these transformations were tested for their ability to grow at a nonpermissive temperature (36°C).

**Construction of Expression Vectors.** *Eco*RI fragments of λgt11 clone ST1 (see Fig. 1*A*) were subcloned into pGEX1 or pGEX2T to generate pGEX-ST1A (aa 285–393) and pGEX-ST1B (aa 393–676). DNA fragments corresponding to aa 502–639, 502–546, and 590–639 were amplified by polymerase chain reaction and subcloned in pGEX2T or pGEX1 to generate pGEX-1B10/9, pGEX-1B10/11, and pGEX-1B8/9, respectively. pGEX-PvuS (aa 617–639) was constructed from pGEX-1B10/9 by restriction enzyme cleavage and ligation. GST fusion proteins under the control of *tac* promoter were induced by isopropyl β-D-thiogalactoside for 3 h, solubilized with SDS sample buffer, and separated by SDS/PAGE. Proteins were detected by Far Western blotting as described (12).

**Preparation of Antibodies.** Two peptides, ST1A (aa 285–393) and ST1B (aa 393–676), were expressed from pGEX-ST1A and pGEX-ST1B and purified with glutathione-Sepharose, and rabbits were immunized as described (6). A peptide corresponding to the carboxyl terminus of C3G (ITRRKT-DREEKT) was synthesized, conjugated with bovine serum albumin, and used to inoculate rabbits as described (16).

***In Vitro* Translation of C3G cDNA.** A *Nco* I/*Bam*HI fragment of C3G cDNA covering the entire coding sequence was subcloned in pBluescript II SK (+), generating pBSC3G. pBSC3G-Bst encoded only the amino-terminal region of the C3G protein (aa 1–588). Transcription and translation were simultaneously performed with an *in vitro* translation and transcription kit (Promega).

**Coimmunoprecipitation of C3G and CRK Proteins.** The preparation of recombinant vaccinia virus will be reported elsewhere (M.M., unpublished work). Rabbit kidney (RK) cells were infected with the recombinant vaccinia viruses and were lysed after 12 h in RIPA buffer (10 mM Tris·HCl, pH 7.5/5 mM EDTA/150 mM NaCl/1% Triton X-100/500 μM $Na_3VO_4$). The CRK and C3G proteins were immunoprecipitated with rabbit antisera and staphylococcal protein A-Sepharose. Proteins bound to the beads were separated by SDS/PAGE and analyzed by Western or Far Western blotting (12).

**Northern Blotting.** The filters blotted with mRNAs from human adult and fetal tissues (Clontech) were incubated with $^{32}$P-labeled probes at 42°C in the presence of 50% (vol/vol) formamide, 6× SSPE (1× SSPE = 150 mM NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 1% SDS, and 5× Denhardt's solution, then washed in 0.1× SSC/0.1% SDS at 50°C. Probes bound on the filter were analyzed by a FUJIX radioanalytic imaging system (Fuji).

## RESULTS

**Isolation of cDNA Clones Encoding a CRK SH3-Binding Protein, C3G.** We have previously shown that the binding of the CRK SH3 domain to several cytoplasmic proteins correlates with the activity of CRK in inducing neurite formation of PC12 cells by the activation of Ras (12). To obtain the cDNAs of these CRK SH3-binding proteins, we employed Far Western blotting using bacterially expressed SH3 peptides fused with GST in combination with anti-GST monoclonal antibody. We screened $1.5 \times 10^6$ plaque-forming units (pfu) from a human spleen λgt11 library and identified four positive plaques. Two of these clones, ST1 and ST6, contained overlapping fragments and were characterized further.

To isolate the full-length cDNA, we rescreened the cDNA libraries derived from human spleen and placenta by using the cDNA fragments of ST1 and ST6 as probes. We sequenced the cDNA inserts of three positive clones, MM63, MM91, and PL91, which extended toward the 5′ end. The combined nucleotide sequence of these five clones was 4070 bp in length and contained a 3231-bp open reading frame encoding 1077 amino acids (Fig. 1*A*). As the protein could be shown to have a region homologous to GNRP for Ras as described below, we designated this protein as C3G, for CRK SH3-binding GNRP. The first ATG codon of C3G meets Kozak's translation initiation criteria (17), and three in-frame stop codons precede this ATG. The predicted molecular mass of the C3G protein is 121 kDa.

A computer-assisted sequence homology search with the GenBank data base revealed that the carboxyl terminus of C3G is homologous to the GNRPs for Ras (Fig. 1*A*). Alignment of C3G with rat guanine nucleotide-releasing factor (GRF), *S. cerevisiae* CDC25, *Schizosaccharomyces pombe* Ste6, and mouse Sos1 showed that they share 30–35% identity in a 250-amino acid region (Fig. 1*B*). Moreover, in the amino terminus of this GNRP domain, we identified the region which has been reported to discriminate GNRP for Ras from GNRP for the other Ras-like proteins (18) (Fig. 1*C*).

**Complementation of Temperature-Sensitive *cdc25* by the Truncated C3G Protein.** To examine whether the 3′ region of the C3G gene can substitute for the CDC25 function, we expressed this region in *S. cerevisiae*. We cloned the regions of aa 619–1078 and 776–1078 of the C3G gene in the yeast expression vector pKT10 (19), to produce pKC3GPv and pKC3GHc, respectively (Fig. 2*A*). A temperature-sensitive *cdc25* mutant, KMY172-5A, was transformed with expression vectors. The result with respect to the two independent transformants is shown in Fig. 2*B*. The pKC3GHc transformants grew at 36°C, as did the cells transformed by pL25/SP, a YRp7-derived plasmid containing the wild-type *CDC25* gene. The cells transformed by pKC3GPv or expression vectors YRp7 and pKT10 could not grow at 36°C. The result demonstrates that the C3G gene can complement the loss of *CDC25* function in *S. cerevisiae* and that appropriate truncation of the C3G gene may be necessary for this activity. The plasmid pKC3GHc did not suppress the temperature-sensitive mutations of other genes of the RAS–cAMP pathway, *cyr1-230* and *ras1 ras2-125* (20) (data not shown), indicating that the complementation of the *cdc25* defect by C3G was due to actual replacement of the *CDC25* function and not to activation of a downstream element of the RAS–cAMP pathway.

**Detection of a 130- to 140-kDa Protein(s) by Anti-C3G Sera.** We immunized rabbits with two polypeptides covering different regions of C3G, designated ST1A (aa 285–393) and

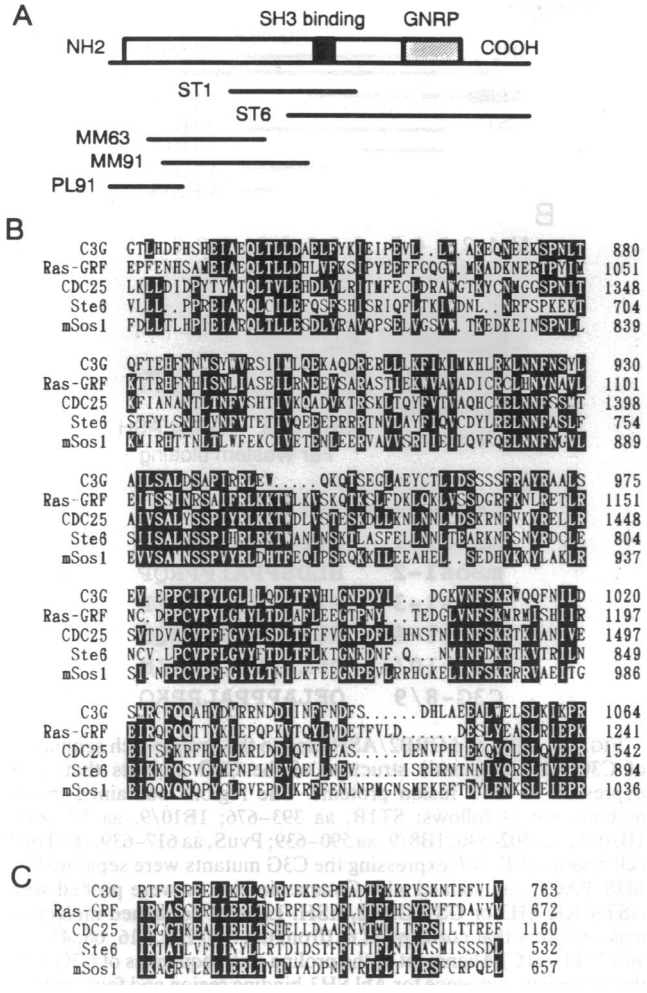


FIG. 1. Comparison of the C3G protein with GNRP for Ras. (*A*) Structure of the C3G protein. The bars indicate cDNAs encoded by each recombinant λgt11-derived phage. The rectangular box indicates the open reading frame. (*B*) Alignment of the C3G protein with GNRPs: rat Ras-GRF, CDC25 of *S. cerevisiae*, Ste6 of *Schizosaccharomyces pombe*, and mouse Sos1. Residue numbers are shown to the right of each line. The following groups of amino acids were considered as conservative substitutions: L, I, V, and M; R, K, and H; F, Y, and W; E and D; A and G; S and T; Q and N. Residue pairs conserved in three or more proteins are shown in black boxes. (*C*) A similar comparison of the amino acid sequences shared only among Ras GNRPs.

ST1B (aa 393–676). By immunoblotting with these two sera, proteins with apparent molecular masses of 130–140 kDa were detected (Fig. 3*A*). In addition to the 130- to 140-kDa protein, proteins of 150, 160, and 180 kDa were weakly detected, suggesting the presence of posttranslational modification, products of alternative splicing, and/or closely related genes. Furthermore, a polyclonal antibody against the carboxyl terminus polypeptide (aa 1065–1077) was prepared and used for immunoprecipitation of the C3G protein, which was then probed with the wild-type GST-CRK SH3 and the K150 mutant (Fig. 3*B*). Again, proteins of 130–140 kDa were detected only when anti-C3G serum was used. Treatment with nerve growth factor did not shift this 130- to 140-kDa band. When the serum was preincubated with the peptide, the 130- to 140-kDa proteins were not detectable (data not shown). Translation *in vitro* of the C3G cDNA yielded 130- and 140-kDa proteins (Fig. 3*C*, lane pBSC3G). C3G cDNA truncated at the carboxyl terminus yielded proteins of 80 and 90 kDa, suggesting alternative initiation of translation (Fig. 3*C*, lane pBSC3G-Bst).

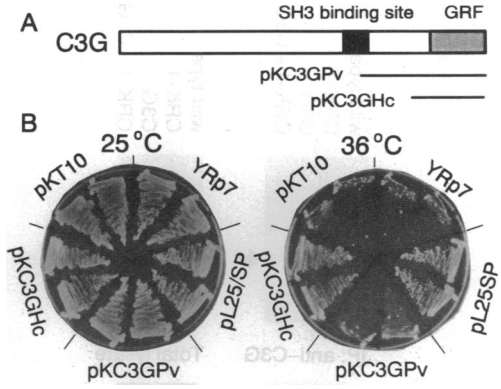


FIG. 2. Complementation of temperature-sensitive *cdc25* by truncated C3G. (*A*) Schematic representation of the C3G expression vectors used in the experiment. (*B*) Temperature-dependent growth of the *cdc25* strain KMY172-5A was examined after transformation with the plasmids indicated in *A*. Transformants for each plasmid were streaked on YPD (yeast extract/peptone/D-glucose) plates, and the plates were incubated at 25°C (left) or 36°C (right) for 3 days before being photographed.

**Association of CRK and C3G Proteins in Living Cells.** Both CRK and C3G proteins were expressed using vaccinia virus vectors, to determine whether these proteins associate *in vivo* (Fig. 4). RK cells were infected with the recombinant vaccinia viruses moCRK-I and moC3G, which encode $p28^{CRK-I}$ and the C3G protein, respectively. Double infection with moC3G and moCRK-I resulted in C3G-CRK complex, as demonstrated by the coimmunoprecipitation of these two proteins by anti-C3G or anti-CRK antibodies.

**Amino Acid Sequence Essential for Binding to CRK SH3.** We constructed a series of C3G deletion mutants to delineate the CRK SH3 binding site (Fig. 5*A*). Mutant proteins were expressed as fusion proteins with GST and were probed with GST-CRK SH3 and GST-GRB2/ASH (Fig. 5*B*). The anti-GST monoclonal antibody used in this experiment recognizes only the native conformation (data not shown); it detects GST fusion proteins only when they are used as probes, not

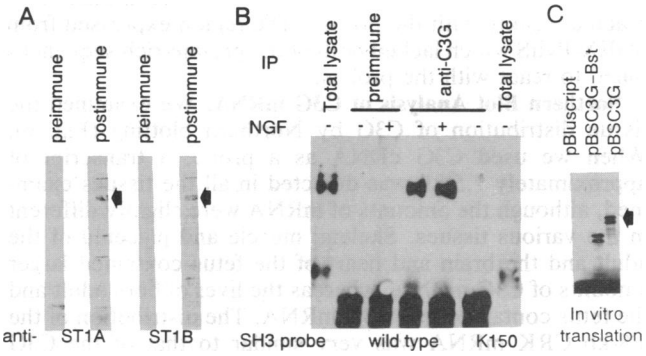


FIG. 3. Detection of the C3G protein. (*A*) Two peptides, ST1A (aa 285–393 of C3G) and ST1B (aa 393–676), were expressed as GST fusion proteins and used for immunization of rabbits. Total lysates of A431 cells were separated by SDS/PAGE and probed with antisera. Arrows indicate proteins of 130–140 kDa, detected by both anti-ST1A and anti-ST1B. The bars on the left are molecular mass markers that correspond to (from the top) 200, 97, 69, and 46 kDa. (*B*) PC12 cells with or without nerve growth factor (NGF) treatment were lysed and immunoprecipitated (IP) with antisera to the carboxyl terminus of C3G (aa 1066–1077), followed by Far Western blotting with GST-CRK SH3 or its mutant, K150. The arrow indicates the 130- to 140-kDa C3G protein. (*C*) C3G cDNA was translated *in vitro* in the presence of [$^{35}$S]methionine. Products were separated by SDS/PAGE. pBSC3G is a pBluescript-derived plasmid containing the C3G cDNA. pBSC3G-Bst lacks the 3′ region of C3G cDNA from pBSC3G. The arrow indicates the 130- to 140-kDa C3G protein.

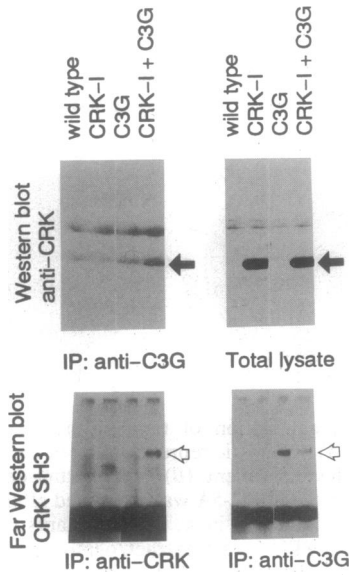


FIG. 4. Binding of C3G to CRK in living cells. RK cells were inoculated with recombinant vaccinia viruses encoding CRK-I and C3G as shown on the top. Either total lysates or immunoprecipitates (IP) with rabbit sera against C3G or CRK were analyzed by Western blotting with anti-CRK monoclonal antibody 3A8 or by Far Western blotting with GST-CRK SH3. Closed and open arrows indicate $p28^{CRK-I}$ and the C3G protein, respectively. The bands appearing in all four lanes are light and heavy chains of immunoglobulin used for immunoprecipitation.

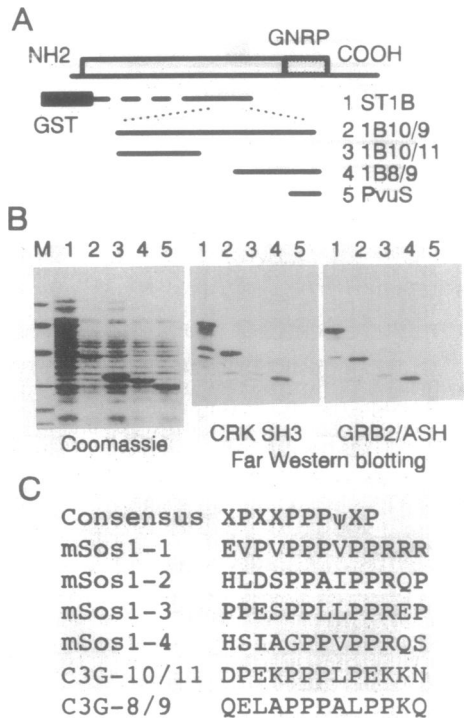


FIG. 5. CRK and GRB2/ASH bind to the proline-rich sequences of C3G. (*A*) Schematic structure of the C3G mutants that were expressed as GST fusion proteins. The regions contained in the mutants are as follows: ST1B, aa 393–676; 1B10/9, aa 502–639; 1B10/11, aa 502–546; 1B8/9, aa 590–639; PvuS, aa 617–639. (*B*) Total cell lysates of *E. coli* expressing the C3G mutants were separated by SDS/PAGE and stained with Coomassie blue or were probed with GST-CRK SH3 or GST-GRB2/ASH. Lane M contained molecular mass markers that correspond to (from the top) 200, 116, 66, 45, 31, and 21 kDa. (*C*) Alignment of the proline-rich sequences of C3G with the consensus sequence for Abl SH3-binding region and four proline-rich GRB2/ASH-binding sequences of mouse (m)Sos1. The amino acid sequences shown as C3G-10/11 and C3G-8/9 are from aa 536–548 and 603–615, respectively. Ψ and X in the consensus sequence represent hydrophobic and nonconserved amino acid residues, respectively.

when they are separated by SDS/PAGE. The region overlapped by λST1 and λST6 was expressed from pGEX-ST1B and was detected by the probes as expected. In this region, we found two proline-rich sequences which resemble the proposed consensus sequence for Abl SH3 binding (21) and the GRB2/ASH-binding sites on mouseSos1 (8) (Fig. 5*C*). The regions containing both and each of the two proline-rich sequences were expressed from pGEX-1B10/9, pGEX-1B10/11, and pGEX-1B8/9, respectively. The minimal sequence sufficient for the binding was aa 590–639 expressed on the 1B8/9 mutant, although the 1B10/11 mutant also reacted weakly with the probes. The region expressed from pGEX-PvuS which lacked both of the proline-rich sequences failed to react with the probes.

**Northern Blot Analysis of C3G mRNA.** We examined the tissue distribution of C3G by Northern blotting (Fig. 6). When we used C3G cDNA as a probe, a transcript of approximately 7.5 kb was detected in all the tissues examined, although the amounts of mRNA were slightly different in the various tissues. Skeletal muscle and placenta of the adult and the brain and heart of the fetus contained larger amounts of C3G mRNA, whereas the liver of both adult and the fetus contained less C3G mRNA. The distribution of the 4.8-kb CRK mRNA was very similar to that of the C3G mRNA. This result suggests that the C3G protein functions to transduce signals from CRK to Ras in a wide range of tissues and at various stages of the development.

## DISCUSSION

We have shown previously that Ras is required for CRK-induced neuronal differentiation of PC12 cells (12). By use of a monoclonal anti-SH2 antibody and competing SH3 peptides, it could also be demonstrated that both the SH2 and SH3 domains must bind to the cellular proteins for this CRK-mediated signaling (12). These initial findings have now allowed the identification of the protein that binds to the SH3 domain of CRK and activates Ras. By screening an expression library with the SH3 peptides of CRK, we have isolated the cDNA of the protein, designated C3G, which is an additional member of the GNRPs. Binding of C3G to CRK has been demonstrated both *in vivo* and *in vitro*.

As Ras conducts vital signals for both proliferation and differentiation, the identification of the upstream factor, the GNRP for Ras, has been an issue of considerable investigation (22). In *S. cerevisiae*, CDC25 (23) and SCD25 (24) have been proved to be the Ras GNRP *in vitro*. A protein with homology to the yeast CDC25 has been identified in rats, mice, and humans, and it has been designated Ras-GRF or $CDC25^{Mm}$ (25). Genetic approaches in *Drosophila* have identified another protein, Sos, as a Ras GNRP (26). The mouse and human homologues of Sos have also been identified (27, 28) and have been designated as mSos and hSos, respectively. The carboxyl-terminal peptide sequence of the C3G protein reported here shares 31% and 30% identity to Ras-GRF/$CDC25^{Mm}$ and mSos, respectively. The same regions of Ras-GRF/$CDC25^{Mm}$ and mSos have some 27% identity. Thus the C3G protein and the two Ras GNRPs identified formerly, Ras-GRF/$CDC25^{Mm}$ and mSos, are to similar extents distant from each other. Although we have demonstrated that the C3G protein has an amino acid sequence extremely similar to Ras GNRPs and that C3G complements the loss of *CDC25* function in yeast, it is possible that the C3G protein is a GNRP for Ras-related proteins.

Recently, the *Drosophila* homologue of GRB2/ASH, Drk, has been mapped genetically to a position upstream from Sos,

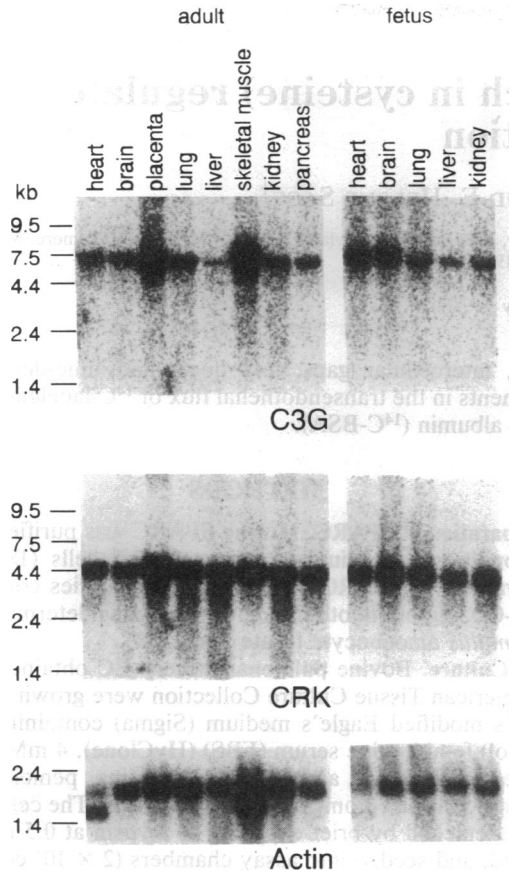


FIG. 6. Expression of C3G mRNA in human adult and fetal tissues. Poly(A)$^+$ RNA extracts were separated by formaldehyde/agarose gel electrophoresis and transferred to a nylon membrane. The filter was hybridized with C3G, CRK, or actin cDNA labeled with [$^{32}$P]dCTP.

and the association of Drk and Sos could be reconstituted *in vitro* (7, 8). Subsequent to this finding, it has also been shown that the GRB2/ASH connects mammalian Sos proteins to the tyrosine kinases, such as epidermal growth factor receptor (29–33) and the insulin receptor (34–36). The carboxyl terminus of Sos, which is responsible for the association with the SH3 domains of GRB2/ASH, contained four proline-rich SH3-binding sequences. We identified similar amino acid sequences in C3G. Deletion mutants containing these sequences bound to GRB2/ASH *in vitro*, strongly suggesting that C3G also serves as a signal transducer to Ras from GRB2/ASH.

In conclusion, we have identified a GNRP, C3G, which binds to CRK and GRB2/ASH. The widespread distribution of C3G in both adult and fetal tissues suggests that this molecule has a fundamental function in the cell. As both CRK and GRB2/ASH are also expressed ubiquitously, they could transmit signals to C3G in a wide range of cells. The SH2 domains of GRB2/ASH and CRK have a different specificity to the phosphotyrosine-containing peptides (37); therefore, binding of C3G to the two adaptor-type molecules would be expected to enlarge the upstream pathway of Ras. GRB2/ASH appears to bind more cellular proteins than CRK as demonstrated by Far Western blotting (data not shown). This suggests that cells may use CRK rather than GRB2/ASH when signals must be transmitted to a limited number of effectors including Ras.

We thank Y. Tachibana for the anti-GST monoclonal antibody, T. Yamamoto for the cDNA library, M. Nakafuku for the yeast strains, and W. W. Hall and H. Hanafusa for useful suggestions. This work was supported in part by grants from the Ministry of Education, Science, and Culture and by grants from the Ministry of Health and Welfare, Japan.

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