Identification of Rap1 as a Target for the Crk SH3 Domain-Binding Guanine Nucleotide-Releasing Factor C3G

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C3G, which was identified as a Crk SH3 domain-binding guanine nucleotide-releasing factor, shows sequence similarity to CDC25 and Sos family proteins (S. Tanaka, T. Morishita, Y. Hashimoto, S. Hattori, S. Nakamura, M. Shibuya, K. Matuoka, T. Takenawa, T. Kurata, K. Nagashima, and M. Matsuda, Proc. Natl. Acad. Sci. USA 91:3443–3447, 1994). The substrate specificity of C3G was examined by in vitro and in vivo experiments. C3G markedly stimulated dissociation of bound GDP from Rap1B but marginally affected the same reaction of other Ras family proteins (Ha-Ras, N-Ras, and RalA). C3G also stimulated binding of GTP-γS [guanosine 5′-3-O-(thio)triphosphate] to Rap1B. When C3G and Rap1A were expressed in COS7 cells, marked accumulation of the active GTP-bound form of Rap1A was observed, while Sos was not effective in the activation of Rap1A. These results clearly show that C3G is an activator for Rap1. Furthermore, expression of C3G with a membrane localization signal in a v-Ki-ras transformant, DT, induced a reversion of the cells to the flat form, possibly through the activation of endogenous Rap1.

Protein-protein interaction plays important roles in transducing signals elicited from receptors on the cell surface to the nucleus. The Src homology 2 (SH2) and SH3 domains have been shown to bind to tyrosine-phosphorylated proteins (33) and proline-rich motifs (54), respectively (reviewed in references 4, 27, 51, and 61). There are a number of signaling molecules involved in the tyrosine kinase cascade which have either the SH2 domain or the SH3 domain or both, including the GTPase-activating protein for Ras (Ras GAP), phospholipase C-γ, the p85 subunit of PI-3 kinase, and Src and related kinases. While these molecules have enzymatic activities, there is another group of molecules that consist mostly of SH domains without any enzymatic domains. Crk (34, 38), Grb2/Ash (30, 37), Shc (52), and Nck (28) belong to the latter group, the so-called adapter molecules. These multivalent adapter molecules may connect signaling molecules on them. Besides the SH2 and SH3 domains, recently the pleckstrin homology domain also has been identified in a number of signaling molecules (40).

The stimulation of cells with various growth factors or cytokines activates their cognate receptor tyrosine kinases or nonreceptor tyrosine kinases associating with the receptors, which results in the tyrosine phosphorylation of various signaling molecules (60). Each tyrosine-phosphorylated molecule is recognized and bound by a specific SH2 domain (64, 65). Ras

GAP, phospholipase C-γ, and the p85 subunit of PI-3 kinase have been shown to become tyrosine phosphorylated and bind to activated receptors through their SH2 domains (4, 27, 51, 61). The interaction between SH3 domains and proline-rich motifs seems to be constitutive and not to depend on stimulation of the cells (54). These interactions by the SH2 and SH3 domains are responsible for the formation of multimolecular signaling complexes, some of which translocate to the plasma membrane.

We have focused on the function of Crk. v-crk was identified as an oncogene of CT10 avian sarcoma virus (38). Since v-crk did not seem to have any enzymatic domain, the mechanism by which v-crk transforms fibroblastic cells was not known. Matsuda et al. discovered that the SH2 domain of Crk binds to tyrosine-phosphorylated proteins (33). Since this finding, extensive investigations on the function of SH domains have been carried out. We have shown that microinjection of the Crk protein into PC12 cells induces neurite outgrowth of the cells in a Ras-dependent manner, suggesting that Crk binds to some Ras guanine nucleotide-releasing factor (66). By screening a human placenta cDNA expression library with the Crk SH3 domain as a probe, we obtained C3G (for Crk SH3 domain-binding guanine nucleotide-releasing factor) (67).

C3G has multiple proline-rich regions that bind to the Crk SH3 domain in the middle of the molecule (26, 67). C3G has sequence similarity to the catalytic domains of CDC25 and Sos family proteins (5, 6, 10, 31, 62, 63). Drosophila Sos activates Ras in the signaling of photoreceptor cell formation (63), and its mammalian counterpart, mSos, activates Ras in vitro (12). It has been shown that the Grb2-mSos complex binds to activated epidermal growth factor receptor recruiting mSos from cytosol to the membrane, where the substrate of mSos, Ras, is located (8, 12, 15, 29, 55). Recently, however, genetic evidence has suggested that the pleckstrin homology domain of Sos is im-

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portant for signaling from the *sevenless* receptor (20). Genetic and biochemical studies revealed that both CDC25 and CDC25^{Mm}, its mammalian counterpart, activate Ras (7, 10, 19, 62). However, the biochemical properties of C3G have not yet been fully characterized.

In this study we have examined the substrate specificity of C3G. Both in vitro and in vivo experiments show that C3G is an activator for Rap1/*smg* p21/Krev-1 (22, 25, 53), which is closely related to Ras in structure. Furthermore, expression of C3G with a membrane localization signal in a v-Ki-*ras*-transformed cell line, DT (50), increased the proportion of morphologically flat cells, possibly through the activation of Rap1/*smg* p21/Krev-1.

MATERIALS AND METHODS

Low-molecular-weight GTP-binding proteins. Rap1B and Ha-Ras were purified from lysates of insect cells (Sf9 cells) that had been infected with baculoviruses carrying each cDNA (43). Posttranslationally processed and unprocessed forms were purified from the membrane and cytosolic fractions, respectively. Ha-Ras and RalA were produced in *Escherichia coli* as fusion proteins with glutathione-*S*-transferase (GST) by using pGEX-2T (Pharmacia Biotech). The cDNAs of Ha-Ras with *Bam*HI sites upstream of the initiation codon and downstream of the termination codon were synthesized by PCR. The fragment was cut with *Bam*HI and inserted into the same site of pGEX-2T to yield pGEX-Ha-Ras. pGEX-RalA was constructed similarly except that *Kpn*I sites were added to both ends of the cDNAs. CDC25^{Mm} was produced in and purified from *E. coli* as described previously (46). Fusion proteins were purified by using glutathione–Sepharose 4B (Pharmacia Biotech) and then subjected to digestion with thrombin (Sigma). *E. coli* producing full-length N-Ras was a generous gift from A. Wittinghofer.

Expression of C3G in insect cells. The *Nco*I-*Bam*HI fragment of C3G cDNA covering the entire coding sequence (67) was subcloned into *Nco*I-*Bgl*II-digested pAcSG2, a transfer vector for baculovirus (Invitrogen Inc.), to generate pAcSG2-C3G. Transfer of the C3G cDNA from pAcSG2-C3G to *Autographa californica* nuclear polyhedrosis virus genome DNA and isolation of recombinant virus were performed as described previously (36). H5 insect cells infected with the recombinant baculovirus were collected by centrifugation at 500 × *g* for 10 min. The cells were lysed in a solution containing 20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 0.5% Triton X-100 and centrifuged for 20 min at 10,000 × *g*. The soluble fraction was loaded on a Resource Q anion-exchange column (6 ml; Pharmacia Biotech) preequilibrated with the same buffer. The column was washed with a solution containing 20 mM Tris-HCl (pH 8.0) and 50 mM NaCl and then developed with a linear salt gradient from 0.05 to 0.5 M NaCl. All fractions were analyzed by Coomassie brilliant blue staining and immunoblotting with anti-C3G antibody (67). The recombinant C3G was eluted as a single peak at 250 to 300 mM NaCl and used for the biochemical studies. H5 cells infected with a recombinant baculovirus expressing an unrelated protein, topoisomerase I, were similarly processed and used as a negative control.

Measurement of guanine nucleotide exchange reaction. Each low-molecular-weight GTP-binding protein (15 pmol) was loaded with [³H]GDP by incubation in 10 µl of a solution containing 3.2 µM [³H]GDP (16,000 cpm/pmol; New England Nuclear), 20 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 20 mM EDTA, 100 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol, and 1 mg of bovine serum albumin per ml for 5 min at 30°C. After the incubation, 30 mM MgCl₂ was added to stabilize the binary complex. The nucleotide exchange reaction was carried out in 20 µl of a solution containing 20 mM Tris-HCl (pH 7.5), 3 mM MgCl₂, 50 mM NaCl, 10 mM 2-mercaptoethanol, 5% glycerol, 5 mg of bovine serum albumin per ml, 1.5 mM GTP, 2.0 to 3.0 pmol of GTP-binding protein · [³H]GDP complex, and an appropriate amount of guanine nucleotide-releasing factor. The sample was incubated for 20 min at 30°C. The reaction was stopped by the addition of 3 ml of ice-cold 20 mM Tris-HCl (pH 8.0)–5 mM MgCl₂–50 mM NaCl, and the diluted sample was poured onto a nitrocellulose membrane filter (0.2-µm-pore-size; Schleicher & Schuell, Inc.) that was then washed twice with the same solution. The radioactivity trapped on the filter was counted by using a liquid scintillation counter (model 2200CA; Packard). Binding of [³⁵S]GTP-γS [³⁵S-labeled guanosine 5′-3-*O*-(thio)triphosphate] was monitored under the same conditions except that 1 µM nonradioactive GDP and 3 µM [³⁵S]GTP-γS (2.3 × 10⁵ cpm/pmol) were used in the first and second incubations, respectively.

Construction of expression vectors. The *Hin*dIII-*Bam*HI fragment of C3G cDNA that covers the entire coding region (67) was inserted into the *Xho*I site of an expression vector, pCAGGS (48), which yielded pCAGGS-C3G. The CAAX box of the c-Ki-*ras2* gene (41) was amplified by PCR with primer KRAS CAAX-5 (5′-ATTCGAGGATCCTCTAGAAAGATGAGC-3′), which corresponds to amino acid positions 163 to 171 of the Ki-*ras2* gene product with an artificial *Xba*I site at the 5′ end, and primer KRASCAAX-3 (5′-GAATTCG GATCCGTCGACTTACATAATTAC-3′), which corresponds to amino acid 186 to the termination codon with an artificial *Bam*HI site at the 3′ end. The cDNA

of the carboxy-terminal region of C3G was amplified by PCR with primers C3G-8 (67) and C3G-Cterm (5′-GGATCCTCTAGAGGTCTTCTCTTCCCGGTC-3′). In C3G-Cterm the authentic termination codon of C3G was replaced with an *Xba*I site, to which the CAAX box sequence amplified as described above was fused. An *Sph*I-*Bam*HI fragment of the fused sequence covering the carboxy-terminal region of C3G with the CAAX box was used to replace the corresponding part of pCAGGS-C3G to generate pCAGGS-C3G-F.

Another set of expression vectors was constructed to express the catalytic domain of C3G with and without the CAAX box. A *Hinc*II-*Eco*RI fragment of pC3G which contained the catalytic domain (amino acid 776 to the C terminus) was inserted into the *Bam*HI site of pEBG, a eukaryotic vector derived from pEF-BOS (39) for the expression of GST fusion proteins. The resulting plasmid was designated pEBG-C3GHII. pEBG-C3GHII-F is a derivative of pEBG-C3GHII and contains the CAAX box sequence described above. The CAAX box sequence alone was also subcloned into pEBG to generate a control vector, pEBG-F.

Full-length cDNA of mSos1 was kindly provided by D. Bowtell (6). A *Not*I-*Hinc*II fragment (nucleotides 1 to 4368) blunted with Klenow fragment was ligated with pCAGGS which had been cleaved with *Xho*I and blunted with Klenow fragment. The resulting vector was designated pCAGGS-Sos. The CAAX box of the c-Ki-*ras2* gene was also similarly added to the carboxy terminus of mSos to yield pCAGGS-Sos-F.

pEBG-Krev-1, which expresses Krev-1 (Rap1A/*smg* p21A) protein as a GST fusion protein, was constructed by amplifying the entire coding region of pK*rev*-1 (24) by PCR and then inserting the fragment into the *Bam*HI site of pEBG. The sequences of all of the amplified fragments described above were confirmed after subcloning the fragments into pUC18. The Ras-expressing plasmid SRαRas was described previously (14).

Analysis of guanine nucleotides bound to Rap1A and Ras in intact cells. COS7 cells were cultured in Dulbecco's modified Eagle medium (Nissui, Tokyo, Japan) supplemented with 10% fetal calf serum. The cells (1.5 × 10⁵ per 35-mm-diameter dish) were transfected with 0.3 µg of various expression plasmids, alone or in the combinations specified in the figures, by the DEAE-dextran method and were then cultured for 48 h. Labeling of the cells, preparation of the cell lysates, and analysis of guanine nucleotides bound to Ras or Rap1A were carried out as described previously (45). Briefly, cells were labeled with 0.05 mCi ³²P$_i$ (Amersham) per ml for 4 h, and then the cells were lysed in a lysis buffer. Ras was immunoprecipitated with the Y13-259 anti-Ras monoclonal antibody, and GST-Rap1A was recovered by using glutathione-Sepharose. After denaturation of the protein, eluted nucleotides were analyzed by polyethyleneimine thin-layer chromatography. Guanine nucleotides were detected and quantitated with the BAS 2000 system (Fuji Film, Tokyo, Japan).

Reversion assay. We followed the protocol of Kitayama et al. (24, 25) for the reversion assay. A v-Ki-*ras*-transformed NIH 3T3 cell line, DT (50), was cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. Cells were transfected with expression plasmids by the calcium phosphate method and selected in medium containing 1 mg of G418 (Geneticin; GIBCO BRL) per ml. After 10 days, the total number of colonies and the number of morphologically flat colonies were counted.

RESULTS

C3G stimulates the nucleotide exchange reaction of Rap1. Since C3G has sequence similarity to Sos and CDC25, we investigated the effect of C3G on the nucleotide exchange reaction of GTP-binding proteins of the Ras family in vitro. We first measured the effect of C3G on the time course of release of GDP from Rap1B (Fig. 1A). The release of GDP from Rap1B was greatly accelerated by C3G. Under the conditions used, 1 mol of C3G stimulated the release of nearly 4 mol of Rap1B, indicating that C3G functions catalytically. Next, the effect of C3G on the binding of [³⁵S]GTP-γS to Rap1B was examined. Rap1B was first complexed with nonradioactive GDP and then incubated with [³⁵S]GTP-γS in the presence or absence of C3G. As shown in Fig. 1B, C3G accelerated the binding of GTP-γS to Rap1B, as a mirror image to the results shown in Fig. 1A. These results clearly indicate that C3G indeed serves as a guanine nucleotide exchange factor for Rap1B.

We next examined the substrate specificity of C3G by using other Ras family proteins (Fig. 2). C3G stimulated the dissociation of GDP from Rap1B in a dose-dependent manner. However, C3G stimulated the dissociation of GDP from Ha-Ras and N-Ras only slightly and was ineffective with RalA. In the control experiment, CDC25^{Mm} stimulated the dissociation of GDP from Ha-Ras and N-Ras but not from Rap1B or RalA.
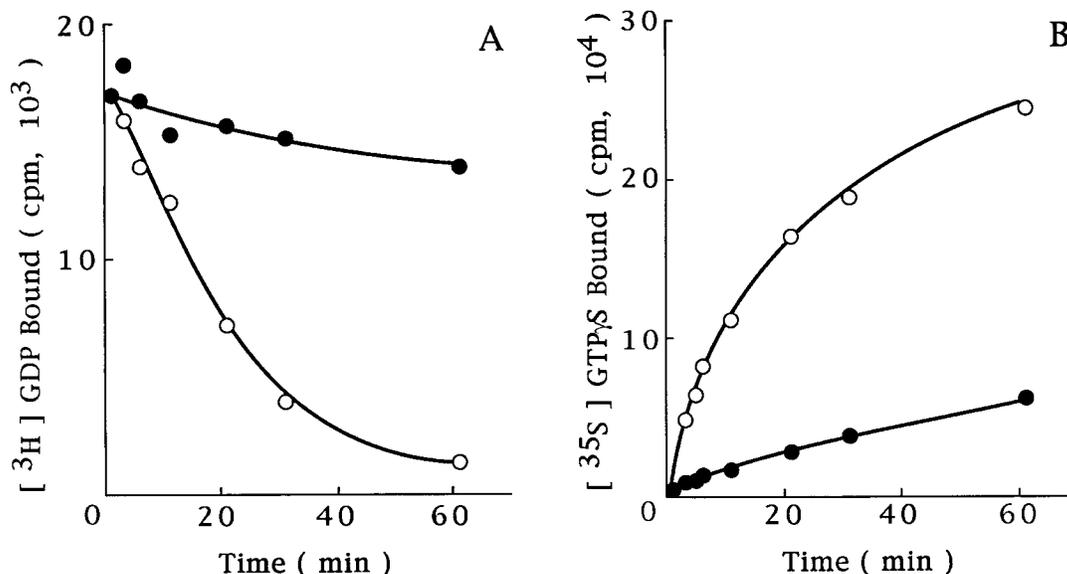
FIG. 1. Effect of C3G on the time course of the nucleotide exchange reaction of Rap1B. (A) The time course of dissociation of [^{3}H]GDP (16,000 cpm/pmol) bound to Rap1B (2.0 pmol; 100 nM) was determined in the presence (open symbols) or absence (closed symbols) of C3G (0.5 pmol; 25 nM) as described in Materials and Methods. (B) The binding of [^{35}S]GTP-γS (2.3 × 10^5 cpm/pmol) to Rap1B · GDP (2.0 pmol) was measured in the presence (open symbols) or absence (closed symbols) of C3G (0.5 pmol).

Ha-Ras produced in insect cells was a somewhat better substrate for CDC25^{Mm} than Ras proteins produced in *E. coli*. C3G did not stimulate release of GDP from Rab3A and RhoA, which are low-molecular-weight GTP-binding proteins from another family (data not shown).

It has been reported that posttranslational processing of Ras is important for the interaction of Ras with its activators (43). Therefore, we measured the dose effect of C3G on the ex-

change reactions of the processed and the unprocessed forms of Rap1B, which were purified from the membrane and cytosol fractions of baculovirus-infected cells, respectively. C3G is twice as effective on the processed form as it is on the unprocessed form (Fig. 3). Thus, the posttranslational modification is also important for Rap1B to interact with C3G.

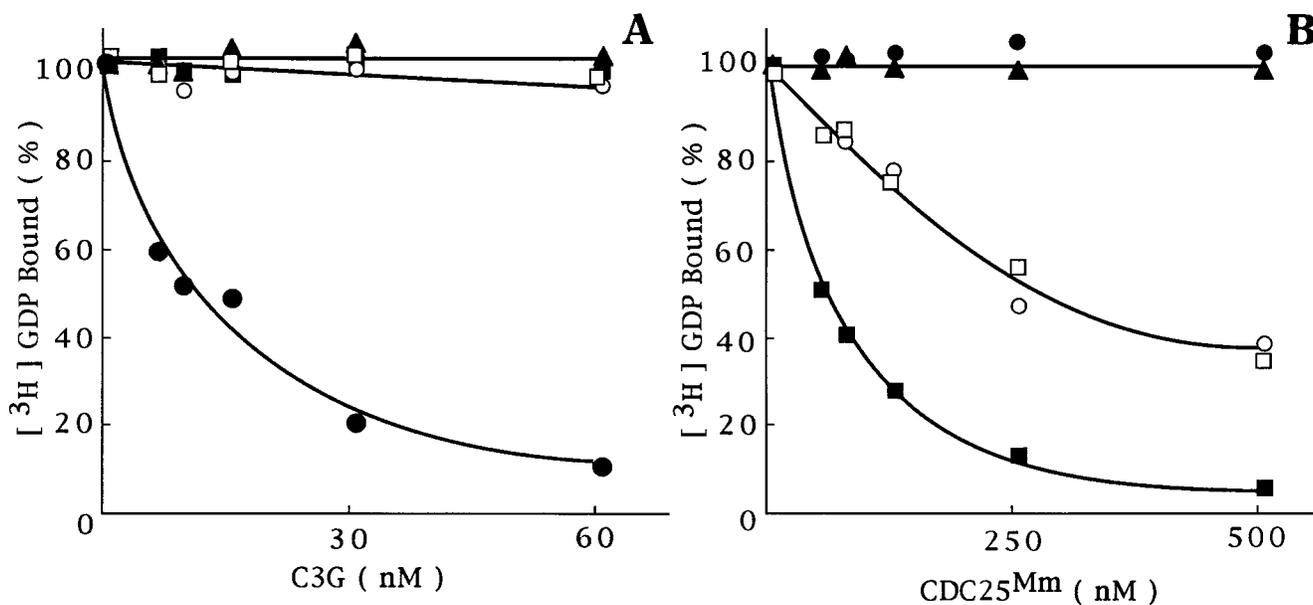**C3G activates Rap1 in intact cells.** We next examined whether C3G activates Rap1A in COS7 cells. Rap1A and

FIG. 2. Effect of C3G and CDC25^{Mm} on the release of GDP from Rap1B and other Ras family proteins. The effect of C3G (A) and CDC25^{Mm} (B) on the nucleotide exchange reaction was examined as described in Materials and Methods by using Rap1B · [^{3}H]GDP (100 nM) (closed circles), Ha-Ras · [^{3}H]GDP (100 nM) (closed squares), Ha-Ras · [^{3}H]GDP (150 nM) (open circles), N-Ras · [^{3}H]GDP (150 nM) (open squares), or RalA · [^{3}H]GDP (150 nM) (closed triangles) as the substrate. The first two proteins were produced in insect cells by using the baculovirus system, and the last three were produced in *E. coli*. Samples with the indicated amounts of C3G or CDC25^{Mm} were incubated at 30°C for 20 min. Values obtained with samples without exchange factors were taken as 100%, and the results are shown as relative percentages.
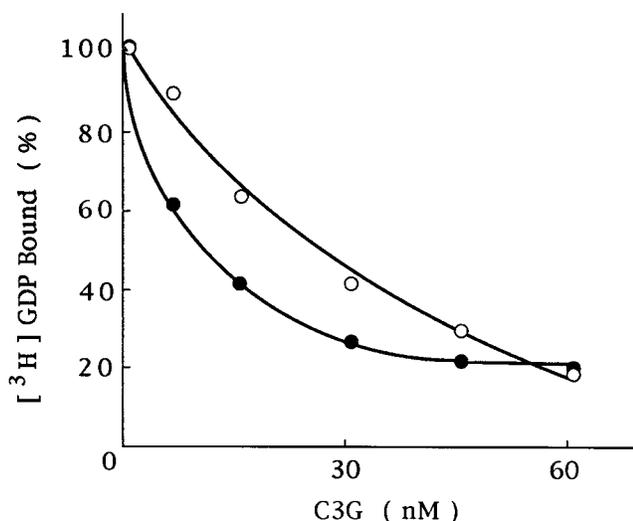
FIG. 3. Effect of carboxy-terminal processing of Rap1B on the stimulation of GDP release by C3G. Release of [³H]GDP from Rap1B (2.0 pmol) with (closed symbols) or without (open symbols) posttranslational modification at the carboxy terminus was measured in the presence of the indicated amounts of C3G.

Rap1B have 97% sequence identity (22, 35). A mammalian GST-fusion vector for Rap1A was introduced into COS7 cells, and guanine nucleotides bound to Rap1A were examined (Fig. 4). When Rap1A alone was expressed, the amount of the GTP-bound form was 5.5% of the total amount of Rap1A. We then examined the effect of C3G or mSos with or without a farnesylation signal on the activation of Rap1A. Cointroduction of pCAGGS-C3G, which directs the expression of C3G without the farnesylation signal of c-Ki-*ras2* (CAAX box), caused a distinct increase in the GTP-bound form of Rap1A, up to 33%. C3G with the farnesylation signal was less active in the activation of Rap1. This result may be due to the fact that

the GST-Rap1A fusion protein was mainly in the soluble fraction, whereas most of the farnesylated C3G was in the membrane fraction (data not shown). Under the same conditions, expression of mSos with or without the CAAX box marginally increased the proportion of the GTP-bound form of Rap1A (Fig. 4).

The effect of C3G expression on the activation of Ras was also examined (Fig. 5). The basal level of the GTP-bound form in the cells transfected with the c-Ha-Ras expression system (SRαRas [14]) alone was less than 2%. The introduction of C3G or its farnesylated form together with Ha-Ras activated Ha-Ras slightly; C3G with the CAAX box was slightly more active. However, the increase observed with C3G was much less than that observed with the expression of mSos, an authentic Ras exchange factor ($P < 0.05$). These results obtained from in vitro and in vivo experiments convincingly indicate that C3G is an exchange factor for Rap1.

Morphological reversion of *ras*-transformed cells by expression of C3G. DT, a v-Ki-*ras*-transformed NIH 3T3 cell line, has been successfully used to isolate and characterize several transformation suppressor genes, including K*rev*-1 (Rap1A gene) (24, 25, 49). Upon overexpression of K*rev*-1, a significant fraction of these cells become flat, a phenotype of revertant cells. We have observed that C3G activates Rap1 by stimulating the release of bound GDP, as shown above. Kitayama et al. (24) reported that activated forms of Rap1A are more potent in this reversion assay. Therefore, it is expected that the expression of C3G would cause a similar potentiation of reversion through the activation of endogenous Rap1. We expressed C3G and its farnesylated form in DT cells. As shown in Fig. 6, some of the colonies derived from cells transfected with pCAGGS-C3G-F (with a farnesylation signal) consisted of flat cells. The results obtained from four independent experiments are summarized in Table 1. Expression of C3G with the farnesylation signal alone clearly induced reversion of DT cells to the flat form. Expression of the unfarnesylated form of C3G or introduction of the control vector had littler effect on the frequency of the
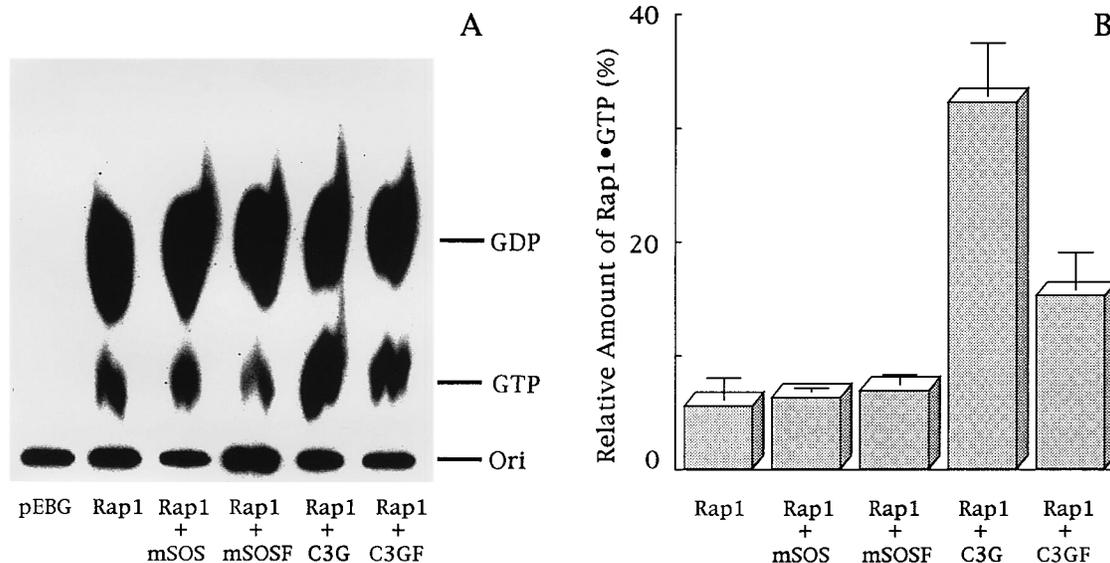

FIG. 4. Activation of Rap1A by C3G in intact COS7 cells. (A) COS7 cells were transfected with the mammalian expression vector for GST-Rap1A, pEBG (a control vector for GST-Rap1A), mSos, mSos with a farnesylation signal (mSosF), C3G, C3G with a farnesylation signal (C3GF), or combinations of these. Analysis of guanine nucleotides bound to Rap1A was carried out as described in Materials and Methods. Ori, origin of chromatography. (B) The radioactivity in each spot was quantitated by using the Fuji BAS2000 system, and the ratio of Rap1A · GTP to total Rap1A is shown. Mean values obtained from three independent experiments are shown with standard deviations.
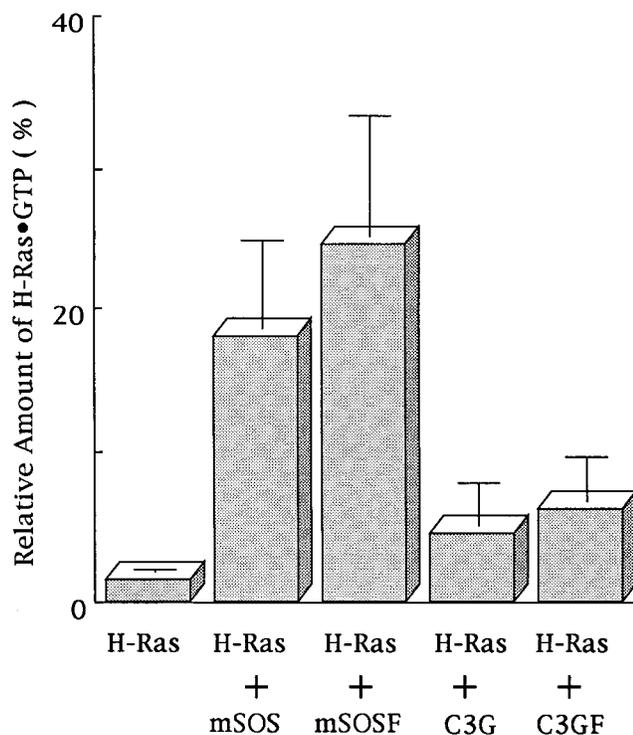
FIG. 5. Effect of mSos and C3G on the activation of Ras in intact cells. The activation of Ha-Ras in intact COS7 cells by either mSos, mSosF, C3G, or C3GF was measured as described for Fig. 4. Values represent averages of results from four independent experiments with standard deviations.

reversion. Transfection of pKrev-1, in addition to pCAGGS-C3G or pCAGGS-C3G-F, slightly increased the reversion frequency.

Next, only the C-terminal catalytic domain of C3G was expressed as a GST-fusion protein to exclude any possible side effect derived from another portion of C3G. The introduction of pEBG-C3GHII-F, which encodes the C3G catalytic domain with the farnesylation signal, significantly increased the frequency of reversion to the flat form in DT cells, while neither
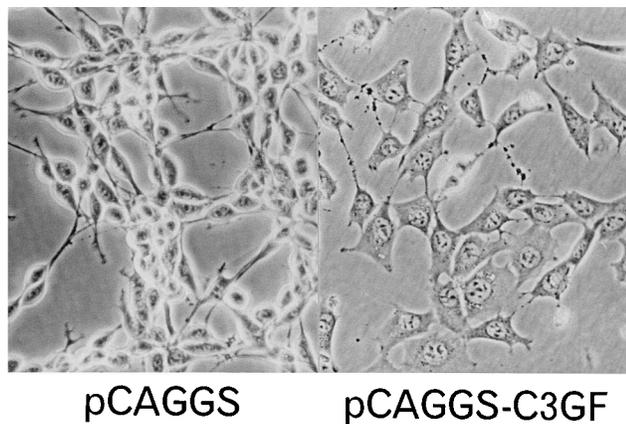

pCAGGS   pCAGGS-C3GF

FIG. 6. Reversion of DT cells by expression of C3G. DT cells were transfected with the expression plasmids and selected with G418 as described in Materials and Methods. The morphology of a representative flat colony that appeared after transfection with pCAGGS-C3G-F (right) is compared with that of a transformed colony transfected with pCAGGS (left).

TABLE 1. Morphological reversion of DT cells by expression of C3G[a]

Transfected DNAs	No. of colonies with flat cells/total no. of colonies in expt:				Ratio (%)[b]
	1	2	3	4	
pSV2neo, pCAGGS	0/240	0/180	0/76	0/60	0
pSV2neo, pCAGGS-C3G	0/210	1/256	0/144	0/86	0.1
pSV2neo, pCAGGS-C3G-F	12/439	23/358	8/124	7/115	5.4
pKrev-1, pCAGGS	2/170	0/223	0/87	1/93	0.6
pKrev-1, pCAGGS-C3G	0/68	1/108	2/85	1/74	1.2
pKrev-1, pCAGGS-C3G-F	6/129	2/109	13/111	12/188	6.1

[a] DT cells were cotransfected with 2 μg of either pSV2neo or pKrev-1 and 5 μg of one of the pCAGGS-derived expression vectors. Transfectants were selected in medium containing G418 (1 mg/ml) for 10 days.
[b] Average of the frequencies of flat-cell colonies in the four independent experiments.

GST alone (pEBG), farnesylated GST (pEBG-F), nor the GST-C3G catalytic domain (pEBG-C3GHII) without the farnesylation signal caused any significant increase in the frequency of reversion (Table 2).

DISCUSSION

In this study we have shown that C3G stimulated the nucleotide exchange reaction of Rap1B in vitro. The stimulation was marginal with other Ras family low-molecular-weight GTP-binding proteins, including Ha-Ras, N-Ras, and RalA. C3G also activated Rap1A when coexpressed in COS7 cells. These results indicate that C3G is an activator for Rap1. Furthermore, expression of C3G with a farnesylation signal in a v-Ki-ras-transformed cell line, DT, resulted in an induction of the reversion to the flat form similar to that observed upon overexpression of Rap1A (24, 25).

Since C3G has sequence similarity to CDC25 and Sos (67), we first expected that C3G was an activator for Ras. Indeed, overexpression of the C3G catalytic domain in *Saccharomyces cerevisiae* carrying *CDC25ts* suppressed the growth defect at 36°C, suggesting that C3G could activate Ras (67). In accordance with this result, overexpression of C3G with the farnesylation signal slightly increased the accumulation of the GTP-bound form of Ras in COS7 cells. However, the increase in the active form of Ras observed with C3G was far less than that observed with mSos, an authentic activator for Ras. Residues on the L2, L4, and helix α2 regions of Ras which are critical for the stimulation by CDC25 or SCD25 are well conserved between Ras and Rap1 (42, 44, 68), which may be the reason for Ras being stimulated by C3G. Activation of Rap1 by C3G might also contribute indirectly to the activation of Ras by

TABLE 2. Morphological reversion of DT cells by expression of the catalytic domain of C3G[a]

Transfected DNAs	No. of colonies with flat cells/total no. of colonies[b] in expt:	
	1	2
pSV2neo, pEBG	0/120 (0.0)	0/155 (0.0)
pSV2neo, pEBG-F	0/125 (0.0)	0/195 (0.0)
pSV2neo, pEBG-C3GHII	0/99 (0.0)	ND[c]
pSV2neo, pEBG-C3GHII-F	3/123 (2.5)	2/246 (0.8)

[a] DT cells were cotransfected with 2 μg of pSV2neo and 5 μg of one of the pEBG-derived expression vectors as described for Table 1.
[b] The frequency of morphological reversion is shown in parentheses.
[c] ND, not determined.

C3G, since Rap1, when activated, binds to and inhibits Ras GAP (13, 17).

smg GDS (GDP dissociation stimulator) has been reported to stimulate the dissociation of guanine nucleotides bound to Ki-Ras, Rap1, Rho, and Rac (18, 43). *smg* GDS differs from C3G in that the substrate specificity of *smg* GDS is broader than that of C3G. *smg* GDS binds to these proteins, which results in the translocation of these proteins from the membrane to the cytoplasm (21). Although the exact functional difference between C3G and *smg* GDS is yet to be clarified, our results suggest that multiple signaling pathways converge on Rap1 protein.

Rap1 becomes phosphorylated by cyclic AMP (cAMP)-dependent protein kinase at a seryl residue located at the carboxy-terminal basic region. This phosphorylation stimulates the nucleotide dissociation of Rap1 by *smg* GDS (16). Since the intracellular concentration of cAMP is regulated by the system in which the heterotrimeric G proteins are involved, this phenomenon is important as the intracellular cross-talk of a pathway derived from heterotrimeric G-protein with one that involves Rap1. The effect of phosphorylation of Rap1 on C3G activity is under examination in our laboratory.

Since the fact that C3G stimulates the nucleotide exchange of Rap1 was established, we were able to examine whether the expression of C3G could revert the morphologically transformed phenotype of DT, an NIH 3T3 cell line transformed by v-Ki-*ras*. In DT cells the expression of Rap1A causes a reversion to the flat form. This effect becomes more prominent when the activated forms of Rap1A are expressed (24). Expression of C3G with the farnesylation signal induced the morphological reversion of DT cells. This reversion-inducing effect of C3G may be brought about through the activation of endogenous Rap1, since the expression of the catalytic domain of C3G also caused a similar effect. Overexpression of Rap1A together with C3G did not increase the reversion frequency much over that with C3G alone. Endogenous Rap1, when activated, may be enough for the reversion of DT cells to the flat form. Rap1 is located mainly in the Golgi complex in Rat-1 cells (1), whereas a significant population of Rap1 associates with plasma membrane of synapse (23). Although we did not analyze the localization of Rap1 in DT cells, the result that the unfarnesylated form of C3G had little effect on the frequency of reversion to the flat form suggests that the membrane localization of C3G is crucial for the activation of endogenous Rap1. A chimeric protein of Rap1A and Ha-Ras, which is expected to be located at plasma membrane, also has transformation-suppressing activity (70), suggesting that subcellular localization of Rap1 may not be so important for the transformation-suppressing activity of Rap1.

It has also been shown that Rap1 interferes with the Ras signaling pathway in several other systems. Rap1 inhibits the stimulation of GTPase of Ras by GAP (13, 17). It also inhibits the stimulation of expression of AP-1-driven genes by Ras but not that by activated c-Raf-1 (57). Rap1 blocks Ras-induced germinal breakdown of *Xenopus* oocytes (9) and Ras-dependent activation of mitogen-activated protein kinase (11). These inhibitory effects of Rap1 seem to be mediated by direct binding of Rap1 to Raf-1 kinase (47). Consistent with this model, expression of C3G with a farnesylation signal in NIH 3T3 cells transformed by activated Raf-1 with amino-terminal truncation (57) did not induce any morphological reversion of the cells (no flat-cell colonies among 232 colonies). Besides interfering with the Ras signaling pathway, Rap1 may have its own pathway. Yoshida et al. demonstrated that microinjection of Rap1 protein into Swiss 3T3 cells stimulates thymidine incorporation by the cells when they are treated with insulin (69).

We have previously shown that Crk binds to both C3G and mSos and that Grb2 binds to C3G in addition to mSos, both in a signal-independent manner (32, 67). Thus, these adapter proteins could potentially link their upstream stimuli to both the Ras and Rap1 signaling pathways. The amount of C3G recovered in anti-Crk immunoprecipitates is higher than that in anti-Grb2 immunoprecipitates, suggesting that the main adapter protein for C3G is Crk (data not shown). However, when *crk* is overexpressed, a significant population of mSos may be bound by Crk, which may result in the transformation of the cells through the activation of the Sos-Ras pathway. Since C3G could weakly activate Ras, activation of Ras by Crk-C3G might also contribute to the transforming activity of *crk*, depending on the cell types. We have reported that microinjection of Crk protein into PC12 cells induces neurite outgrowth of the cells in a Ras-dependent manner (66). Either mechanism or both may work in this system.

Although the upstream signal of Crk is not yet fully characterized, recent observations indicate that Crk binds to several molecules, including the epidermal growth factor receptor, Shc, paxillin, p120 in T cells, and p130^{CAS} (2, 3, 32, 56, 58, 59). It has been shown that Ras is activated by various extracellular signals (60). Thus, the intracellular balance between the Ras and Rap1 signaling pathways may be regulated by complex extracellular signals and by the relative amounts of the adapter proteins and the exchange factors. This balance may be responsible for the control of cell growth and differentiation.

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