Crk is an adaptor protein that consists almost entirely of SH2 and SH3 domains. We have previously demonstrated, by using in vivo and in vitro systems, that C3G, which was identified as a Crk SH3 domain-binding guanine nucleotide exchange factor, specifically activates Rap1. C3G also binds to other adaptor proteins, including CrkL and Grb2. In the present study, we analyzed the effect of Crk, CrkL, and Grb2 on the C3G-Rap1 pathway. Expression of Crk, CrkL, and Grb2 with C3G in Cos1 cells significantly increased the ratio of GDP/GTP bound to Rap1. Both the SH2 and SH3 domains of Crk were required for this activity. However, Crk did not stimulate the guanine nucleotide exchange activity of C3G for Rap1 in vitro, suggesting that Crk does not activate C3G by an allosteric mechanism. The requirement of the SH2 domain of Crk for the enhancement of guanine nucleotide exchange activity for Rap1 could be compensated for by the addition of a farnesylation signal to Crk, indicating that Crk enhanced the guanine nucleotide exchange activity of C3G by membrane recruitment of C3G. These results demonstrate that Crk, CrkL, and Grb2 positively modulate the C3G-Rap1 pathway primarily by recruiting C3G to the cell membrane.

It is now widely accepted that several domains consisting of short consensus motifs play crucial roles in signal transduction. SH2 and SH3 are good examples of such domains (1–3). The SH2 domain binds sequences containing phosphorysorine (4, 5), whereas the SH3 domain forms a complex with polyproline type II helices (6). A group of proteins, including Grb2, Crk, CrkL, and Nck, consist mostly of the SH2 and SH3 domains and are now known as adaptor proteins (7, 8).

c-Crk protein was isolated as a cellular homolog of v-Crk, which was originally identified as an oncoprotein of a chicken retrovirus, CT10 (9). Alternative splicing of the human crk gene generates two Crk proteins, designated as 28-kDa CrkI and 40/42-kDa CrkII (10). CrkII is composed of one SH2 domain and two SH3 domains, whereas CrkI lacks the carboxyl-terminal SH3 domain. The SH2 domain of Crk binds epidermal growth factor receptor and Shc, which are phosphorylated by EGF1 stimulation (5, 11). Integrin stimulation also induces tyrosine phosphorylation of p130 (12) and paxillin, resulting in binding to Crk (12, 13). Cbl, which was originally identified as a cellular counterpart of the v-Cbl oncogene product (14), is tyrosine-phosphorylated upon various extracellular stimulations and binds to Crk (15, 16). Therefore, Crk is implicated in the signal transduction pathway of both growth factor and cell adhesion.

Cellular targets of the SH3 domain of Crk include C3G, Sos, DOC1K180, c-Abl, and EP15 (17–21). C3G was isolated by screening of expression libraries with the amino-terminal SH3 domain of Crk as a probe. The carboxy-terminal region of C3G has homology to the catalytic domain of CDC25M, which is a guanine nucleotide exchange factor for Ras. Using both in vivo and in vitro systems, we demonstrated that the target GTPase of C3G is Rap1 (22).

Rap1, also known as smg p21 or Krev-1, was originally identified as an anti-oncogenic protein which efficiently reverses the morphologic transformation of the v-Ki-ras-expressing NIH 3T3 cell line, DT (23–26). Studies on chimeras of Rap1 and H-Ras have suggested that Rap1 antagonizes Ras through the competition of the effector proteins of Ras (27). This is also supported by the observation that the activation of the c-fos promoter/enhancer from Ras, but not from the activated Raf-1, which is an immediate downstream effector of Ras, is inhibited by Rap1 (28). Furthermore, constitutively activated Rap1 efficiently inhibits Ras-dependent activation of mitogen-activated protein kinase by lysosphosphatidate or EGF (29).

Rap1 is regulated mostly by two groups of proteins. The first one is the GTPase-activating protein, which stimulates the intrinsic GTPase activity of Rap1. Two proteins have been shown to possess GTPase-activating protein activity for Rap1 (30, 31). The second are guanine nucleotide exchange factors for Rap1, which include smg GDS and C3G (22, 32). smg GDS also stimulates the nucleotide dissociation of Ki-Ras, Rho, Rac, and mCdc42 (33). In contrast, C3G specifically stimulates the guanine nucleotide exchange reaction of Rap1 among various Ras-family G proteins (22).

Our aim in this study was to analyze the effect of adaptor proteins that bind to C3G on the C3G-Rap1 pathway. We found that Crk enhances the guanine nucleotide exchange reaction of

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* The abbreviations used are: EGF, epidermal growth factor; SH2, Src homology 2; SH3, Src homology 3; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
C3G, and that the enhancement occurs mostly by promoting of the translocation of C3G to the membrane.

**EXPERIMENTAL PROCEDURES**

**Expression Plasmids**—cDNAs of CrkI, CrkII, CrkI-R38V, and CrkI-W169L (34) were subcloned into the pCAGGS eukaryotic expression vector, generating pCAGGS-CrkI, pCAGGS-CrkII, pCAGGS-CrkI-R38V, and pCAGGS-CrkI-W169L, respectively (Fig. 1). Similarly, pCAGGS-Grb2 and pCAGGS-CrkI were constructed by ligating cDNA fragments of Grb2 and CrkI. pCAGGS-C3G, encoding the authentic C3G, was described previously (35). The cDNA fragment corresponding to the catalytic domain of C3G (amino acids 619–1077) was amplified by polymerase chain reaction and subcloned into pCAGGS after the addition of the Myc-tag sequence to its 5′ end. The resulting vector was designated as pCAGGS-Myc-C3G-CD. pEBG-Rap1 encodes Rap1 fused to GST (22). For the expression of farnesylated CrkI protein, the cDNA fragment corresponding to the CAAX box of Ki-Ras was fused to the carboxyl-terminal region of CrkI cDNA, as described previously (22). The fused CrkI-CAAX box cDNA was subcloned into pCAGGS, generating pCAGGS-CrkI-F.

**Cell Culture and Transfection**—Cos1 cells and human embryonic kidney 293T cells were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% fetal calf serum. Cos1 cells were transfected by DEAE-dextran method as described previously (22). Briefly, 10^5 Cos1 cells plated on 60-mm dishes were transfected with 0.2 μg of expression plasmid. After 24 h, the transfectants were collected and used for the analysis of protein expression.

**Antibodies**—Anti-Crk antibody was obtained from Transduction Laboratories. Production of the anti-Crk antibodies was described previously (35). Anti-C3G 1A/1B sera was generated against the central region (amino acids 285–676) and the carboxyl terminus region (amino acids 1065–1077) of C3G, respectively. The epitope of anti-C3G monoclonal antibody SN21 was in the region between amino acids 393–676. Anti-GST rabbit antibody was prepared in our laboratory.

**Analysis of Guanine Nucleotide Exchange Activity of C3G for Rap1**—Cos1 cells were transfected with expression vectors encoding GST-tagged Rap1, C3G, and/or adaptor proteins. After 48 h, guanine nucleotides bound to GST-Rap1 were analyzed by thin-layer chromatography (Fig. 2, A and B). As reported previously (22), expression of C3G increased the ratio of GDP/GTP on Rap1. Expression of CrkI or CrkII remarkably enhanced the C3G-dependent increase of GDP-bound Rap1, whereas CrkI or CrkII alone did not affect the ratio of GDP/GTP on Rap1.

**RESULTS**

In Vitro Analysis of Guanine Nucleotide Exchange Activity of C3G for Rap1—The guanine nucleotide exchange activity of C3G for Rap1 was measured as described previously (22). Expression and purification of recombinant C3G, and CrkI were described previously (22, 34). C3G preincubated with GST or GST-CrkI was combined with [3H]GDP-loaded Rap1 and an excess of cold GTP. The radioactivity of [3H]GDP remaining on Rap1 was measured after a 20-min incubation at 30 °C.

**Cell Fractionation**—Cell fractionation was performed as described previously (36). Briefly, 10^6 Cos1 cells transfected with expression plasmids were washed twice with phosphate-buffered saline, disrupted by freeze-thawing in liquid nitrogen, suspended with 150 μl of detergent-free W buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 20 mM MgCl2). The soluble and the insoluble fraction were separated by centrifugation at 15,000 × g for 10 min. Proteins in the insoluble fraction were solubilized with 37.5 μl of lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2). Extracts were centrifuged at 15,000 × g for 10 min, and the supernatants were used as a partially fractionated 2 μl of soluble and 4 μl of particulate fraction were subjected to SDS-PAGE and immunoblotting using anti-C3G 1A/1B sera.

EGF Stimulation—293T cells were plated at a density of 2.0 × 10^4 cells in a 35-mm diameter dish and transfected with expression plasmids. Thirty-six hours after transfection, cells were starved for 14 h in Dulbecco's modified Eagle's medium containing 0.5% bovine serum albumin and stimulated with 100 ng/ml EGF (Takara Biochemicals, Kyoto) at 37 °C for 3 min. Cells were washed twice with TBS-V buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1 mM Na3VO4) and lysed in lysis buffer (10 mM Tris (pH 7.5), 5 mM MgCl2, 150 mM NaCl, 0.5% Triton X-100, 1 mM Na3VO4). Cell lysates were cleared by centrifugation and immunoprecipitated with anti-EGF receptor polyclonal antibody (Amersham, Little Chalfont, UK). The immune complex was analyzed by SDS-PAGE and immunoblotting using anti-EGF receptor monoclonal antibody (MBL, Nagoya, Japan) or anti-C3G 1A/1B sera.
activates C3G through an allosteric mechanism, we measured the guanine nucleotide exchange activity of C3G in vitro in the presence or absence of CrkI. As shown in Fig. 5A, addition of GST-fused CrkI did not enhance the catalytic activity of C3G for Rap1. The binding of the recombinant CrkI to C3G in vitro was confirmed by immunoblotting (Fig. 5B). We concluded that binding of Crk to C3G is not sufficient to enhance the catalytic activity of C3G in vitro.

Crk Recruited C3G to the Particulate Fraction—The SH2 domain of Crk binds to tyrosine-phosphorylated proteins such as EGF receptor (5, 11), p130cas (37), Shc (17), and paxillin (13). These proteins are localized to the plasma membrane either constitutively or transiently after various stimulations. It is also known that overexpression of v-Crk or CrkI induces tyrosine phosphorylation of these Crk SH2-binding proteins either by the activation of tyrosine kinases or by the competitive inhibition of protein-tyrosine phosphatases (9–11). Thus, it has been postulated that at least one of the functions of the SH2 domain of Crk is to recruit the SH3-binding proteins to the plasma membrane. To test this possibility, we analyzed the subcellular distribution of C3G in the presence or absence of CrkI. As shown in Fig. 6, co-expression of CrkI increased the amount of C3G in the particulate fraction.

Membrane Targeting of CrkI Complemented the SH2 Mutation of CrkI—To examine whether membrane translocation of C3G can account for the activation of C3G by Crk, we constructed plasmids encoding CrkI-F and CrkI-R38V-F that have the Ki-Ras-derived CAAX box at the carboxyl termini of CrkI and CrkI-R38V, respectively. The CAAX box was shown to be farnesylated and required for the membrane-targeting of Ki-Ras protein. As shown in Fig. 7, CrkI-R38V-F en-
hanced the activation of Rap1 by C3G to a level similar to that by CrkI-F. The expression level of C3G was not influenced by the expression of the wild-type and mutant CrkI proteins. This result clearly demonstrated that the SH2 domain of CrkI was not required when CrkI was localized to the plasma membrane by the farnesylation. Enhancement of the activation of C3G by CrkI-F was always less efficient than that by the wild-type CrkI, although CrkI-F was expressed more abundantly than the wild-type CrkI (Fig. 7C). Expression of these wild-type and mutant CrkI did not alter the amount of C3G expressed in Cos1 cells (Fig. 7C). We also confirmed that the addition of the farnesylation signal did not alter the amount of Crk bound to C3G in immunoprecipitation experiments (data not shown).

**DISCUSSION**

In the present study, we have demonstrated that the expression of Crk enhances the guanine nucleotide exchange activity of C3G to Rap1 in vivo. Crk did not enhance the guanine nucleotide exchange activity of C3G in vitro, arguing against the allosteric activation of C3G by Crk. Analysis by use of SH2...
and SH3 mutants of Crk demonstrated that both the SH2 and SH3 domains of Crk are required for the enhancement. In contrast to CrkI-R38V, a CrkI mutant with a non-functional SH2 domain, the mutant that has a membrane localization signal on its carboxyl terminus, CrkI-R38V-F, enhanced the catalytic activity of C3G to a level similar to that of CrkI-F, the wild-type CrkI with a membrane localization signal. The result indicates that the function of the SH2 domain is essentially to recruit Crk to the plasma membrane.

However, we should point out the difference between the membrane recruitment of C3G by CrkI and that by the farne-sylation. The enhancement of C3G activity by CrkI-F was less efficient than that by the wild-type CrkI (Fig. 7). Thus, CrkI-F may not recruit C3G to the place where the optimum activation of C3G occurs. In the cells overexpressing v-Crk or CrkI, we and others observed the increase of several phosphotyrosine-containing proteins including paxillin and p130cas, to which v-Crk and CrkI bind (9, 10). C3G may be activated most efficiently in the close vicinity of these Crk-binding proteins, which is known to localize at the focal adhesions (8).

It is unknown why membrane recruitment of C3G by CrkI enhances guanine nucleotide exchange activity of C3G. It is possible that the membrane targeting of C3G simply facilitates its interaction with the substrate, Rap1. Alternatively, C3G may be modified at the membrane and activated enzymatically. To address this question, we have to measure the specific activity of C3G from the cells with or without the expression of CrkI.

We previously reported that farnesylated C3G activated Rap1 in Cos1 cells less efficiently than did the wild-type C3G (22). This observation appears to argue against our proposal that CrkI enhances guanine nucleotide exchange activity of C3G by membrane recruitment. We have found that the amount of the farnesylated C3G expressed in Cos1 cells is significantly less than that of the wild-type; therefore, we cannot conclude that the farnesylated C3G was not as active as the wild-type C3G. Moreover, only the farnesylated C3G but not the wild-type C3G could revert the transformation of NIH 3T3 cells expressing Ki-Ras (22). This finding supports our proposal that membrane-targeted C3G is more active than the wild-type C3G.

The significance of the translocation of the guanine nucleo-tide exchange factor to the membrane has been suggested for Sos, a guanine nucleotide exchange protein of Ras (38–41). In this case, another adaptor protein, Grb2, recruits Sos to the membrane upon various stimulations, as we have observed for CrkI and C3G. It has also been reported that the binding of Grb2 to Sos does not activate Sos in vitro (42).

Rap1 is phosphorylated by protein kinase A at Ser169, located at the carboxyl-terminal basic region that is critical for the interaction with smgGDS (43). This phosphorylation stimulates the guanine nucleotide dissociation of Rap1 by smgGDS (44). However, we could not observe any difference in the phosphorylation level of Rap1 in the presence or absence of CrkI (data not shown). Therefore, it is unlikely that Crk enhances the guanine nucleotide exchange reaction of C3G through the phosphorylation of Rap1.

Crk and CrkL enhance the guanine nucleotide exchange
activity of C3G more efficiently than does Grb2. It is possible that Grb2 cannot alter C3G localization efficiently because Grb2 does not bind to C3G as strongly as do Crk and CrkL (45). Alternatively, because the SH2 domains of Crk and CrkL bind similar sets of proteins (45, 46), both may recruit C3G to a position closer to Rap1 than does Grb2. Experiments on SH2/SH3 chimeras of Crk/CrkL and Grb2 would be useful.

C3G-CD, which lacks the amino-terminal region of C3G, catalyzed the guanine nucleotide exchange reaction for Rap1 more efficiently than did the authentic C3G. Although the in vitro study neglected the allosteric activation of C3G by Crk, it is still possible that expression of Crk can counteract the neg-
catalyzed the guanine nucleotide exchange reaction for Rap1 signaling pathway.

that it is involved in the down-regulation of the C3G-Rap1 signaling pathway.

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