C3G is a guanine nucleotide exchange factor for Rap1 and is activated by the expression of Crk adaptor proteins. We found that expression of CrkI in COS cells induced significant tyrosine phosphorylation of C3G. To understand the mechanism by which C3G is phosphorylated and activated by Crk, we constructed a series of deletion mutants. Deletion of the amino terminus of C3G to amino acid 61 did not remarkably affect either tyrosine phosphorylation or Crk-dependent activation of C3G. When C3G was truncated to amino acid 390, C3G was still phosphorylated on tyrosine but was not effectively activated by CrkI. Deletion of the amino terminus of C3G to amino acid 579 significantly reduced the Crk-dependent tyrosine phosphorylation of C3G and increased GTP-bound Rap1 irrespective of the presence of CrkI. We substituted all seven tyrosine residues in this region, amino acids 391–579, for phenylalanine for identification of the phosphorylation site. Among the substitution mutants, the C3G-Y504F mutant, in which tyrosine 504 was substituted by phenylalanine, was remarkably less activated and phosphorylated than the wild type. All the other substitution mutants were activated and tyrosyl-phosphorylated by the expression of CrkI. Thus, CrkI activates C3G by the phosphorylation of tyrosine 504, which represses the cis-acting negative regulatory domain outside the catalytic region.

C3G was originally isolated as one of the two major proteins bound to the SH3 domain of the Crk adaptor protein (1, 2). C3G consists largely of three regions. The carboxyl-terminal region, which shares a homologous sequence with CDC25, catalyzes the guanine-nucleotide exchange reaction for Rap1, a Ras family protein, but not for the other Ras-family G proteins (3). In the central part of C3G, three Crk SH3-binding sequences have been identified (1, 4). Although the function of the amino-terminal region of C3G remains unknown, recently p130Cas, a major Crk SH2-binding protein, has been shown to bind in this region in vitro (5).

The human crk gene is translated into two products, CrkI and CrkII, by alternative splicing (6). CrkII is regulated negatively by tyrosine phosphorylation, which occurs upon various types of stimulation (7, 8). CrkI, by contrast, lacks the tyrosine phosphorylation site and resembles the v-crk oncogene product in its structure (6, 9).

Many kinds of stimulation induce binding of the Crk-C3G complex to a variety of phosphotyrosine-containing proteins, such as receptor tyrosine kinases (reviewed in Ref. 10). However, the biological consequences of the activation of C3G are still poorly understood, mostly because the function of Rap1, the only known effector of C3G, is obscure. Rap1 competitively suppresses Ras-dependent transformation and ERK/MAP kinase activation (11, 12). It has been shown that the sustained activation of Rap1, probably due to the formation of the Cbl-CrkL-C3G complex, causes T-cell anergy by suppressing the Ras-MAP kinase pathway (13). In the central nervous system, however, Rap1, like Ras, activates the ERK/MAP kinase pathway through the activation of B-raf (14–16).

In platelets, an increase in the intracellular calcium concentration activates Rap1, which in turn binds to and activates RalGDS (17). Recently we have isolated a novel guanine nucleotide exchange factor for Rap1, CalDAGI (18). CalDAGI can be activated by Ca2+ and diacylglycerol; therefore, the Ca2+-dependent activation of Rap1 appears to be triggered by CalDAGI, but not C3G.

It has been reported that C3G activates JNK through SEK (19). Mixed-lineage kinases, MLK3 and DLK, appear to be involved in this pathway (20). Because constitutively active Rap1 does not activate JNK and because dominant-negative Rap1 does not inhibit the C3G-dependent activation of JNK, it has been proposed that another G protein may transduce signals to JNK from C3G.

Activation of C3G occurs primarily through membrane recruitment (10, 21). Expression of CrkI in COS cells activates guanine nucleotide exchange activity of C3G, concomitant with its translocation to the cell membrane (22). An SH2 mutant of CrkI, which is not translocated to the membrane, cannot activate C3G; however, addition of a farnesylation signal to this SH2 mutant restored the CrkI-dependent activation of C3G partially. Deletion of the amino-terminal third activates C3G partially to the level of C3G co-expressed with Crk. These observations strongly suggest the presence of a cis-acting negative regulatory domain in the noncatalytic domain of C3G. In the present paper, we delineate the negative regulatory domain and the tyrosine phosphorylation site of C3G.
EXPERIMENTAL PROCEDURES

Expression Plasmids—pCAGGS-CrkI encodes the wild-type human CrkI (21). CrkI-R38V and CrkI-W169L are SH2 and SH3 mutants, respectively (21). pCAGGS-C3G and pCAGGS-C3G-F express the wild-type and the farnesylated form of C3G, respectively (3). pEBG-Rap1 and pEBG-C3G encode GST-tagged Rap1 and C3G, respectively (2). Full-length and truncated C3G cDNAs were obtained by polymerase chain reaction and subcloned into the pCAGGS-His expression vector, which contains the polyhistidine-tag sequence derived from pBlueBacHisA (Invitrogen) at the 5' end of the cloning site. pCAGGS-His-C3G-d61 encodes C3G from amino acid 62 to the carboxyl end; -d390 from 391; and -d579 from 580. In other C3G expression vectors, a tyrosine codon(s) was substituted into that of phenylalanine by polymerase chain reaction-based mutagenesis. Substituted tyrosines are indicated by superscripts; the vectors are pCAGGS-C3G-Tyr504, pCAGGS-C3G-Tyr554,561,570, and pCAGGS-C3G-Tyr478,485,579.

Cell Culture and Transfection—COS1 (CCL 1651, ATCC), 3Y1 rat fibroblast (0734, Japan Cell Resource Bank (JCRB)), v-crk-transformed 3Y1 cells, Crk-3Y1 (23), SR-3Y1 v-src-transformed 3Y1 cells (0742, JCRB), and HR-3Y1 v-Ha-Ras-transformed cells (0734, JCRB) were cultured in Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% fetal calf serum. COS1 cells were transfected by the DEAE-dextran method.

Antibody—Antibodies against C3G and Crk were developed in our laboratory (2, 6). Anti-Crk monoclonal antibody and horseradish peroxidase-conjugated anti-phosphotyrosine antibody RC20 were purchased from Transduction Laboratories, Lexington, KY; anti-C3G polyclonal antibody was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Analysis of Guanine Nucleotide Exchange Activity of C3G for Rap1 in COS1 Cells—Guanine nucleotides bound to Rap1 were analyzed essentially as described previously (24). Briefly, 10^7 COS1 cells plated on 35-mm dishes were transfected with 0.2 µg of expression plasmids by the DEAE-dextran method. Forty-eight hours after transfection, cells were labeled for 2 h with 0.05 mCi of ^32P, in 0.5 ml of phosphate-free...
medium. GST-fused Rap1 was collected by glutathione-Sepharose 4B (Amersham Pharmacia Biotech). Guanine nucleotides bound to Rap1 were separated by thin layer chromatography and quantitated with a Molecular Imager (Bio-Rad). For analysis of protein expression, cells were processed similarly without isotopic labeling. Cleared lysates were separated by SDS-PAGE, transferred to polyvinylidene difluoride membrane, probed with antibodies, and detected by an ECL chemiluminescence system (Amersham Pharmacia Biotech).

**Immunoprecipitation**—Cells were lysed in lysis buffer (0.5% Triton X-100, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM MgCl2) and cleared by centrifugation. Equal amounts of lysates were incubated with various antibodies at 4 °C. The immune complexes were collected by protein A-Sepharose FF (Amersham Pharmacia Biotech, Tokyo). In some experiments, SDS was added at 1%, incubated at 95 °C for 3 min, and diluted 10-fold with lysis buffer, followed by immunoprecipitation by the use of anti-C3G antibody and protein A-Sepharose FF. The immune complexes were separated by SDS-PAGE and analyzed by Western blotting as described (21).

**RESULTS**

**Activation of Farnesylated C3G by CrkI**—Previously, we proposed that CrkI activates C3G primarily by recruiting C3G to the membrane fraction (21). We examined whether the translocation to the membrane is sufficient for the activation of C3G by use of a C3G mutant, C3G-F, which localizes mainly in the membrane fraction due to farnesylation (Fig. 1, A and B). The wild-type C3G and C3G-F increased GTP-Rap1 from 8% to 17% and to 16%, respectively. As we discussed previously (21), the protein level of C3G-F is always significantly lower than that of the wild type (Fig. 1C). Considering the protein expression level, the specific activity of the farnesylated C3G was 4.5-fold higher than that of the wild-type C3G (Fig. 1D). Co-expression of CrkI enhanced Rap1 activation by C3G-F as well as the wild-type C3G, suggesting that membrane recruitment is not the sole mechanism of C3G activation by CrkI. In these experiments, we found that the activation of C3G was accompanied by its tyrosine phosphorylation (Fig. 1C). Because the farnesylated C3G also became tyrosine-phosphorylated by CrkI expression, we speculated that tyrosine phosphorylation plays a major role in the activation of C3G.

**Correlation of C3G Tyrosine Phosphorylation with Its Activation**—To examine further the role of tyrosine phosphorylation of C3G, we used two CrkI mutants, CrkI-R38V and CrkI-W169L. The SH2 mutant CrkI-R38V and the SH3 mutant CrkI-W169L are unable to activate C3G (21). The wild-type and mutant CrkI proteins were expressed to a similar level and did not affect the expression of C3G (Fig. 2A); however, only the wild-type CrkI induced detectable tyrosine phosphorylation of C3G (Fig. 2B). The CrkI-W169L did not bind to CrkI due to its SH3 mutation, and CrkI-R38V could not induce tyrosine phosphorylation of C3G due to its SH2 mutation (Fig. 2C). To confirm that the 135-kDa protein detected by the anti-phosphotyrosine antibody was C3G, we used GST-tagged C3G (Fig. 2C). In the cells expressing GST-C3G, we observed a molecular shift of the tyrosine-phosphorylated protein to the 160-kDa region, as expected from the molecular mass, 28 kDa, of GST.

**Tyrosine Phosphorylation of C3G in Transformed Cells**—We next searched for conditions in which the endogenous C3G is phosphorylated on tyrosine. We examined a series of 3Y1 rat fibroblasts transformed by oncogenes for the tyrosine phosphorylation of C3G. As shown in Fig. 3A, we found a prominent tyrosine-phosphorylated 135-kDa protein in the anti-C3G immune complexes from 3Y1 cells transformed by v-Crk or v-src, but not from parent 3Y1 cells and Ha-Ras-transformed 3Y1 cells. Several proteins of some 130 kDa are known to be phosphorylated on tyrosine in cells transformed by v-Crk or v-Src (25). To remove these proteins from anti-C3G immune complexes, we denatured the cell lysates before immunoprecipitation. Under this condition, most, if not all, phosphorytrosine-binding domains such as SH2 cannot refold (26). Even with this treatment, the 135-kDa phosphotyrosine-containing protein was precipitated with the anti-C3G antibody (Fig. 3B). Thus, we concluded that C3G is phosphorylated on tyrosine in cells transformed by v-Crk or v-Src.

**Delineation of the Phosphotyrosine-containing Region**—We constructed C3G mutants and examined them for their CrkI-dependent tyrosine phosphorylation. Only representative examples are shown in Figs. 4 and 5. C3G-d390 was the shortest among the deletion mutants that were phosphorylated on tyrosine under the presence of CrkI. C3G-d579, deleted to amino acid 579, almost completely lost its tyrosine phosphorylation. C3G-d579 retained its binding to CrkI, as expected from the

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**Fig. 4. Schematic representation of C3G mutants.** Closed boxes represent three Crk-binding sites. The catalytic domain is shown by the hatched box. Tyrosine residues between amino acids 391 and 579 are depicted with circles above the bar. In each mutant, closed circles denote the substitution from tyrosine to phenylalanine.

**Fig. 5. Phosphorylation and binding of the C3G mutants to CrkI.** 293T cells expressing the indicated on the top were lysed and immunoprecipitated with either anti-Crk monoclonal antibody 3A8 or anti-C3G antibody and probed with antibodies as indicated on the bottom. Arrows indicate the positions of the wild-type and C3G-d390 mutant proteins.
presence of the major CrkI-binding site, amino acids 588–617 (4). Our data showed 7 tyrosine residues between amino acids 391 and 579 as candidates for tyrosine phosphorylation site(s). We constantly observed a 120-kDa phosphotyrosine-containing protein co-immunoprecipitated with C3G. It did not react with anti-p130cas antibody and remained unidentified at this moment.

**Guanine Nucleotide Exchange Activity of the C3G Deletion Mutants—**

The activity of the guanine nucleotide exchange reaction for Rap1 was also examined for C3G deletion mutants with or without the expression of CrkI (Fig. 6). The guanine nucleotide exchange activities of C3G-d61 and C3G-d390 were similar to those of the wild-type C3G, and both were activated by the co-expression of CrkI. In contrast, C3G-d579 showed the highest basal guanine nucleotide exchange activity to the level of the wild-type C3G co-expressed with CrkI. As shown in Fig. 5, the expression level of C3G-d579 was similar to that of the wild-type C3G; therefore, C3G-d579 appears to be a catalytically active mutant. The activity of C3G-d579 was enhanced further by the expression of CrkI. These results demonstrate that both the tyrosine phosphorylation site(s) and the negative regulatory region of C3G are between amino acids 391 and 579.

**Requirement of Tyrosine 504 for Both Tyrosine Phosphorylation and CrkI-dependent Activation—**

We substituted all of the seven tyrosine residues for phenylalanine to identify the tyrosine residue that is critical for phosphorylation and CrkI-dependent activation (Figs. 4 and 7). Results for three representative mutants, among several mutants tested, are shown in Fig. 7. Two mutants, C3G-Y554F/Y561F/Y570F and C3G-Y478F/Y485F/Y579F, which have three amino acid substitutions, were tyrosine-phosphorylated by the expression of CrkI, as was the wild-type C3G. However, tyrosine phosphorylation of C3G-Y504F was increased significantly by CrkI (Fig. 7A). The activation of the guanine nucleotide exchange for Rap1 by these C3G mutants correlated with the tyrosine phosphorylation (Fig. 7, B and C). Both C3G-Y554F/Y561F/Y570F and C3G-Y478F/Y485F/Y579F were activated by the expression of CrkI, as was the wild-type C3G. In contrast, the activation of the C3G-Y504F mutant by CrkI was marginal. These data demonstrate that tyrosine 504 is the most critical tyrosine residue for the phosphorylation and CrkI-dependent activation of the guanine nucleotide exchange for Rap1.

**DISCUSSION**

We have shown that the increase in the catalytic activity of C3G correlated with its tyrosine phosphorylation. Amino acid substitution of Tyr-504 to Phe renders C3G refractory to CrkI-dependent activation and phosphorylation. Removal of the amino-terminal region, including Tyr-504, generated a C3G mutant that was fully active irrespective of the presence of CrkI; therefore, we concluded that CrkI-induced phosphorylation of Tyr-504 represses the cis-acting negative regulatory domain of C3G.

It should be noted, however, that even the C3G-Y504F mutant was also activated slightly by CrkI. Because Y504F was

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**Fig. 6. Guanine nucleotide exchange activity of C3G truncation mutants.** A, COS1 cells were transfected with pEBG-Rap1 and the C3G mutant expression vectors as indicated at the bottom. After 32P labeling, guanine nucleotides bound to Rap1 were analyzed by thin layer chromatography. B, the radioactivity of each spot was quantitated and is shown as bars. Mean values obtained from three experiments are shown with standard deviation.
Activation of C3G by Tyrosine Phosphorylation

Fig. 7. Phosphorylation and guanine nucleotide exchange activity of C3G amino acid substitution mutants. A, COS1 cells were transfected with pEBG-Rap1 and the expression vectors indicated on the top. After 48 h, the cells were lysed and immunoprecipitated with anti-C3G serum, followed by immunoblotting with anti-C3G serum, anti-Crk monoclonal antibody, or anti-phosphotyrosine antibody. B, in parallel experiments, cells were labeled with 32Pi, and guanine nucleotides bound to Rap1 were analyzed by thin layer chromatography. C, the radioactivity of each spot was quantitated and is shown as bars. Mean values obtained from three samples are shown with standard deviations.

still phosphorylated weakly on tyrosine, tyrosine phosphorylation of other residue(s) may replace the function of Y504F phosphorylation. Alternatively, C3G may be activated by CrkI via a phosphorylation-independent mechanism.

Recently, p130Cas, crk-associated substrate, has been shown to bind directly to C3G (5). Pro-267 and Pro-270 of C3G are critical residues (26). It has also been reported that CDC25Mm/Ras-GRF, the first example of guanine nucleotide exchange factor for the Ras family proteins that can be activated by tyrosine phosphorylation (32). C3G has become the first enzyme that was shown to be activated upon in vitro tyrosine phosphorylation (31). Vav, which is a member of the Dbl family of proteins and promotes the guanine nucleotide exchange of Rac, is recruited to tyrosine kinases and is activated by tyrosine phosphorylation (32). C3G plays a significant role in the oncogenic process (19).

Compared with the number of proteins known to be phosphorylated on tyrosine residues upon various types of stimulation, the number of the enzymes that can be activated by tyrosine phosphorylation are not numerous except for the tyrosine kinases themselves. Phospholipase C-γ appears to be the first enzyme that was shown to be activated upon in vitro tyrosine phosphorylation (31). Vav, which is a member of the Dbl family of proteins and promotes the guanine nucleotide exchange of Rac, is recruited to tyrosine kinases and is activated by tyrosine phosphorylation (32). C3G has become the first example of guanine nucleotide exchange factor for the Ras family proteins that can be activated by tyrosine phosphorylation. While we were revising this manuscript, the cloning of Dro sophila C3G was reported (33). Amino acid sequence of the central part of human C3G, which includes Crk-binding sites and the tyrosine 504, does not show significant homology with that of Dro sophila C3G; while the carboxy-terminal catalytic domain and the amino-terminal region represent high sequence homology. Even so, we have found that the sequence surrounding tyrosine 504, Lys-Pro-Tyr-Ala, is conserved between the human and Dro sophila C3Gs. This finding suggests that the Dro sophila C3G may also be regulated by tyrosine phosphorylation.

Acknowledgments—We thank S. Hattori, T. Gotoh, and J. Miyazaki for materials and technical advice. We also thank Robert Ingham for his critical reading of the manuscript.

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