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Requirement for C3G-dependent Rap1 activation for cell adhesion and embryogenesis

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C3G is a guanine nucleotide exchange factor (GEF) for Rap1, and is activated via Crk adaptor protein. To understand the physiological role of C3G, we generated *C3G* knockout mice. *C3G*$^{-/-}$ homozygous mice died before embryonic day 7.5. The lethality was rescued by the expression of the human *C3G* transgene, which could be excised upon the expression of Cre recombinase. From the embryo of this mouse, we prepared fibroblast cell lines, MEF-hC3G. Expression of Cre abolished the expression of C3G in MEF-hC3G and inhibited cell adhesion-induced activation of Rap1. The Cre-expressing MEF-hC3G showed impaired cell adhesion, delayed cell spreading and accelerated cell migration. The accelerated cell migration was suppressed by the expression of active Rap1, Rap2 and R-Ras. Expression of Epac and CalDAG-GEFI, GEFs for Rap1, also suppressed the accelerated migration of the C3G-deficient cells. This observation indicated that Rap1 activation was sufficient to complement the C3G deficiency. In conclusion, C3G-dependent activation of Rap1 is required for adhesion and spreading of embryonic fibroblasts and for the early embryogenesis of the mouse.

Keywords: C3G/cell adhesion/cell migration/knockout mouse/Rap1

Introduction

Ras-family G proteins function as molecular switches in growth, differentiation, survival and adhesion of eukaryotic cells (Bos, 1997; Campbell *et al*., 1998). They cycle between GDP-bound inactive and GTP-bound active forms. This cycling is mediated by guanine nucleotide exchange factor (GEF), the activator, and GTPase-activating proteins (GAPs), the inactivator (Bourne *et al*., 1990; Downward, 1992). A variety of signals, external or internal, control the on/off status of the Ras-family G proteins via the activation or inactivation of GEFs and GAPs (Overbeck *et al*., 1995; Bos, 1997).

C3G was identified as one of the two major proteins bound to the Src homology 3 (SH3) domain of the *Crk* oncogene product (Knudsen *et al*., 1994; Tanaka *et al*., 1994). The C-terminus of C3G consists of the CDC25 homology domain, which is a catalytic domain of the Ras-family GEF. C3G contains three Crk SH3-binding sequences and one p130^{Cas} SH3-binding sequence in the central region (Kirsch *et al*., 1998). The N-terminal region of C3G negatively regulates its GEF activity (Ichiba *et al*., 1999). Many types of stimulation induce binding of the Crk–C3G complex to phosphotyrosine-containing proteins, including receptor-type tyrosine kinases, p130^{Cas} and paxillin (reviewed by Kiyokawa *et al*., 1997). Following translocation from the cytosol to the cell membrane, C3G becomes phosphorylated on Tyr504, and the negative regulation by the N-terminal region is repressed to increase the GEF activity (Ichiba *et al*., 1999).

C3G promotes the guanine nucleotide exchange reaction of Rap1 and Rap2, and, to a lesser extent, it also stimulates R-Ras and TC21 (Gotoh *et al*., 1995, 1997; Ohba *et al*., 2000a,b). In contrast to Ras, the function of which has been studied extensively, the physiological role of the substrates of C3G remains elusive. A function of Rap1 is to antagonize Ras (Kitayama *et al*., 1989; Yatani *et al*., 1991; Sakoda *et al*., 1992; Boussiotis *et al*., 1997; Okada *et al*., 1998), probably by inhibiting the Ras-dependent activation of mitogen-activated protein kinase (MAPK) (Cook *et al*., 1993; Hu *et al*., 1997). However, in some cell types, Rap1 activates the MAPK cascade, as does Ras (Yoshida *et al*., 1992; Vossler *et al*., 1997; Altschuler and Ribeiro-Neto, 1998; York *et al*., 1998). Moreover, it is reported that growth factor-induced activation of Rap1 does not correlate with the repression of Ras-dependent MAPK activation (Zwartkruis *et al*., 1998). Recently, it has been shown that Rap1 contributes integrin-mediated cell adhesion (Posern *et al*., 1998; Tsukamoto *et al*., 1999; Katagiri *et al*., 2000; Reedquist *et al*., 2000), although the mechanism underlying this phenomenon is yet to be analyzed.

Rap2, the amino acid sequence of which shows 60% identity with Rap1, is regulated by the same set of GEFs and GAPs as is Rap1 (Ohba *et al*., 2000a). Rap2 binds to a set of effectors very similar to those of Rap1 (Janoueix-Lerosey *et al*., 1998; Nancy *et al*., 1999), and it inhibits Ras-dependent MAPK activation, as does Rap1 (Ohba *et al*., 2000a). A unique feature of Rap2 is its low

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sensitivity to GAPs, which permits more than half of Rap2 to remain in a GTP-bound active state in adherent cells.

R-Ras and TC21 are the two members of the R-Ras subfamily and are regulated by the same GEFs and GAPs (Ohba *et al*., 2000b). R-Ras and TC21 activate MAPK, as does the classical Ras (Cox *et al*., 1994; Graham *et al*., 1999; Movilla *et al*., 1999; Rosario *et al*., 1999); however, they may have unique functions, such as inhibition of apoptosis (Suzuki *et al*., 1997) and stimulation of cell adhesion (Zhang *et al*., 1996).

The C3G–Rap1 signaling cascade has been studied genetically in *Drosophila melanogaster*. Overexpression of wild-type C3G (*DC3G*) does not cause any detectable abnormality in the developing eye; however, expression of membrane-targeted active C3G leads to an adult rough-eye phenotype, as does the expression of active Ras or active Rap1 (Hariharan *et al*., 1991; Ishimaru *et al*., 1999). This rough-eye phenotype due to the membrane-targeted C3G is suppressed by reduction of the gene dose of *Ras1*, *ksr*, *rolled* (MAPK) or *Rap1*, indicating that the effect of *DC3G* overactivation is mediated by the RAS–MAPK pathway and RAP1. Overexpression of *Rapgap*, the inactivator, also induces a rough-eye phenotype, which is exacerbated by reduction of the *Rap1* gene dosage (Chen *et al*., 1997). Removal of maternal *Rap1* inhibits ventral furrow closure and head involution of the embryo (Hariharan *et al*., 1991), and it also perturbs the migration of pole cells and mesodermal cells (Asha *et al*., 1999). Rap1 is required for imaginal disc development, but not for adult survival (Asha *et al*., 1999). Thus, the C3G–Rap1 pathway in *Drosophila* has at least two roles: as a regulator of morphogenesis in the adult stage and as a mediator of cell proliferation and cell fate specification in the developmental stage.

Here, we demonstrate that the *C3G* gene is essential for mouse embryogenesis and that C3G-dependent Rap1 activation promotes cell adhesion and cell spreading, but represses cell migration.

Results

Structure of the mouse C3G gene

We sequenced the mouse *C3G* genome to determine the exon–intron structure (Figure 1A). We could not obtain a genomic clone that contained the initiation codon of mouse *C3G*. Therefore, an exon, which corresponded to cDNA from nucleotide 281 to 394 of human *C3G* cDNA, was temporarily designated as exon 2. The 23rd exon contained the stop codon.

Generation of C3G knockout mice

To produce a targeted disruption of the mouse *C3G* gene, we constructed a *C3G* disruption vector, p836BL/R, containing 3.5 and 6.0 kb homologous DNA fragments, the PGK-neo cassette replacing exons 15 and 16, and the MC1-tk cassette being at the 5′ end of the targeting vector (Figure 1A). Embryonic stem (ES) cells were transfected with linearized p836BL/R by electroporation, and clones resistant to both G418 and 1-(2-deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodouracil were isolated. Homologous recombinant clones were detected by Southern blot analysis with probe B, a flanking sequence on the 3′ side (Figure 1B), and the neo probe (data not shown). Of 165 clones, two were targeted for the *C3G* gene. Both of the targeted clones were injected into C57BL/6/blastocysts, which gave rise to chimeras. Chimeric mice were crossed with C57BL/6 mice, and mice heterozygous for the mutation in the *C3G* locus were identified among offspring with agouti coat color by Southern blot analysis. Homozygous mutant offspring could not be recovered from crosses between heterozygous mice, suggesting that the mutation causes lethality during embryogenesis.

To determine the stage at which mutant embryos died, we collected embryos from staged matings and genotyped them by using a PCR assay (Table I). When we examined implantation sites at embryonic days 7.5 and 8.5 (E7.5 and E8.5), nearly a quarter of the sites contained only degenerating embryonic tissue within the decidual stroma. In some cases, little DNA for the PCR assay was retrieved from the degenerating tissue and, therefore, these cases

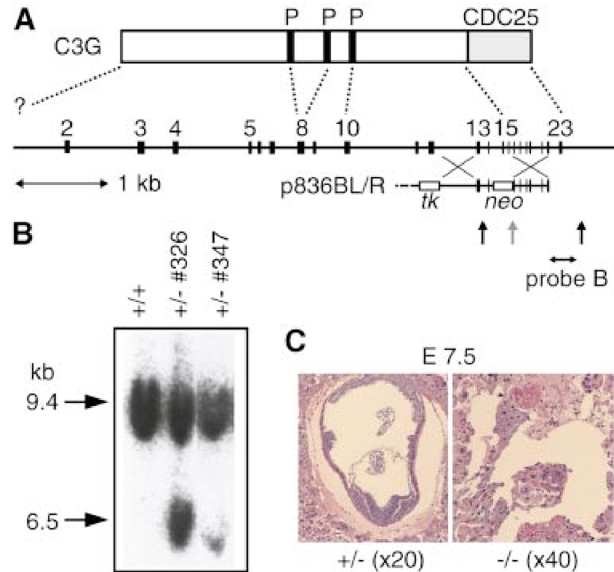

Fig. 1. Generation of *C3G* knockout mice. (**A**) Schematic structure of the mouse *C3G* gene and C3G protein. Coding exons 2–23 are shown at the top. A targeting vector, p836BL/R, consisted of 3.5 and 6.0 kb homologous DNA fragments, the PGK-neo cassette replacing exons 15 and 16, and the MC1-tk cassette at the 5′ end of the targeting vector. The diagnostic probe (probe B) and the *Eco*RV sites (vertical arrows) are indicated at the bottom. P, proline-rich Crk-binding regions; CDC25, CDC25 homology region. (**B**) Southern blot analysis of mouse genomic DNA. *Eco*RV-digested DNAs from ES cells were separated on a 0.5% agarose gel, transferred to a nylon membrane and hybridized with fluorescein isothiocyanate (FITC)-labeled probe B. The mouse genotype was identified by 9.4 and 6.5 kb fragments that were derived from the wild-type and mutant alleles, respectively. (**C**) Horizontal sections of uterus of a $C3G^{+/-}$ mouse crossed with a $C3G^{+/-}$ mouse and examined at E7.5. Embryos of $C3G^{-/-}$ mice did not conform to any histological structure, as shown on the right. A control section from a $C3G^{+/-}$ embryo is shown on the left.

Table I. Genotyping of embryos arising from C3G heterozygous crosses

Gestational age	No. of embryos or postnatal mice			
	Total	+/+	+/–	–/–
E7.5	23	7	14	3
E8.5	19	5	13	1
Postnatal	91	36	55	0

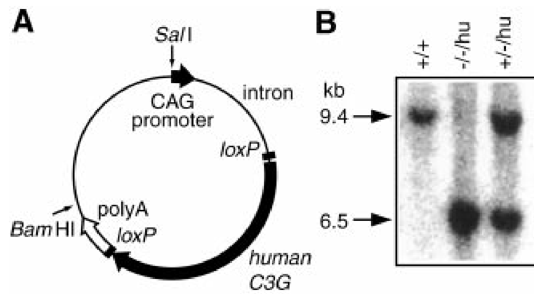

Fig. 2. Complementation of mouse *C3G* deficiency by the human *C3G* transgene. (**A**) Schematic illustration of an expression plasmid of the human *C3G* transgene. The human *C3G* gene, which is sandwiched with two loxP recombination sites, is placed downstream of the chicken β-actin promoter and the CMV immediate early enhancer. *Sal*I and *Bam*HI sites, which were used to isolate the transgene expression unit, are indicated by arrows. (**B**) Southern blot analysis of transgenic mice crossed with *C3G* knockout mice. The genotype of mouse *C3G* and the presence of the human *C3G* transgene are indicated at the top.

were excluded from the data shown in Table I. However, the remaining samples with sufficient amounts of DNA were identified with $C3G^{-/-}$ mice. Histological examination of the implantation sites with these abnormal embryos revealed the absence of typical fetal or placental structures (Figure 1C). These findings indicate that *C3G* was essential for embryogenesis, and the mutant embryos died shortly after implantation (around day 5).

Complementation of mouse C3G by the human C3G gene

To confirm that the embryonic lethality of *C3G* homozygous mice resulted from the C3G deficiency, we expressed human C3G to rescue the viability of $C3G^{-/-}$ mice. The human *C3G* cDNA was placed under the regulation of the chicken β-actin promoter and the cytomegalovirus (CMV) immediate early enhancer (Figure 2A). A 5.7 kb *Sal*I–*Bam*HI fragment was microinjected into the pronuclei of fertilized eggs. Of 36 offspring, seven founders (F_0 mice) were identified by PCR screening at the age of 4 weeks. All founders were fertile and were crossed with $C3G^{+/-}$ mice. In F_1 mice, we could not observe any effect of the expression of the human *C3G* gene. $C3G^{+/-}$ mice carrying the human *C3G* transgene, indicated hereafter as $C3G^{+/-/hu}$, were then crossed. From two transgenic lines, we obtained mice deficient in mouse *C3G* and expressing human *C3G*, $C3G^{-/-/hu}$ (Figure 2B). However, in both mouse lines, the numbers of $C3G^{-/-/hu}$ mice were lower than expected (Table II), indicating that expression of human *C3G* did not completely rescue the mouse *C3G* deficiency. We used a CAG promoter, which shows potent activity in a wide range of cells (Niwa *et al*., 1991), to express the human *C3G* gene; however, in some tissues and at some developmental stages, it may not provide sufficient C3G. We never obtained *C3G*-deficient mice without the human *C3G* transgene, confirming that the *C3G* gene was essential for embryogenesis and that the human *C3G* gene complemented the *C3G* deficiency.

Isolation of embryonic fibroblasts from $C3G^{-/-/hu}$ mice

To study the role of C3G further, we obtained mouse embryonic fibroblasts (MEFs) from $C3G^{-/-/hu}$ embryos at E12.5. In these cell lines, which were named MEF-hC3G, the integrated human *C3G* transgene could be excised by infection with the Cre-expressing retrovirus, MX-pie-Cre, or adenovirus, AxCANCre. Equal amounts of genomic DNA of MEF-hC3G and irrelevant mouse genomic DNA mixed with standard *C3G* plasmid were digested with *Xho*I and analyzed by Southern blotting (Figure 3A). MEF-hC3G cells were estimated to carry 20 copies of the human *C3G* transgene per cell. By Cre expression, the quantity of integrated *C3G* was decreased to less than one copy per cell. We further confirmed by immunoblotting that removal of the human *C3G* transgene abolished the expression of C3G protein in MEF-hC3G cells (Figure 3B). We did not detect any truncated C3G protein that might be generated by homologous recombination by use of antibodies raised against three different regions of C3G (Figure 3C). However, contrary to our expectation, the loss of C3G in MEF-hC3G did not decrease the basal level amounts of GTP-bound forms of Rap1, Rap2 and R-Ras, which are the substrates of C3G (Figure 3D).

Table II. Genotyping of living mice from $C3G^{+/-/hu}$ crosses

Parents	No. of mice				
	+/+/hu	+/+/–	+/–/hu	+/–/–	–/–/hu
+/–/huA × +/–/–	9	6	8	17	3
–/–/huA × +/–/–	0	0	11	16	9
+/–/huB × +/–/–	10	17	10	29	3
–/–/huB × +/–/–	0	0	14	15	2

A and B indicate transgenic mouse lines A and B, respectively.

Impaired cell attachment and cell adhesion of C3G-deficient cells

Although the morphology of Cre-expressing MEF-hC3G cells was indistinguishable from that of the parent MEF-hC3G cells, cell attachment and cell spreading after replating were significantly impaired by C3G deficiency (Figure 4A). The replating efficiency was quantitated by labeling of cells with the fluorescent dye 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF, AM). As shown in Figure 4B, C3G enhanced cell attachment to dishes coated with collagen type I or fibronectin (FN), but not those coated with poly-L-lysine. Cell spreading was quantitated by measurement of cell sizes 1 and 3 h after replating. Again, C3G induced cell spreading on the dishes coated with collagen type I, but not with poly-L-lysine (Figure 4C). These results suggested that C3G was required for the cell attachment and cell spreading that were mediated by specific interaction between the extracellular matrices and integrin.

Cell attachment-induced Rap1 activation in C3G-deficient cells

To understand the mechanism by which C3G modulated cell attachment and cell spreading, we examined the activation of Rap1 after replating (Figure 5A). Rap1 was activated rapidly upon cell attachment in the untreated MEF-hC3G cells, whereas Rap1 activation was significantly attenuated in the MEF-hC3G cells infected with MX-pie-Cre. C3G activates the Rap and R-Ras

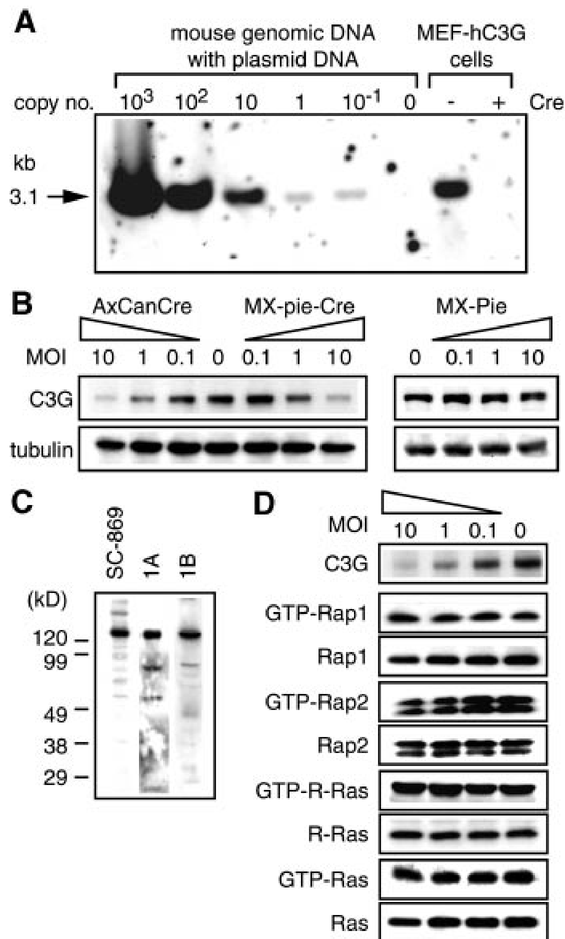

Fig. 3. Cre-dependent disruption of the *C3G* gene. (**A**) MEF-hC3G cells were infected with a recombinant retrovirus, MX-pie (indicated as Cre –) or MX-pie-Cre (Cre +), and selected in DMEM containing 2 µg/ml puromycin for 48 h. A 5 µg aliquot of DNA from the cells and from an irrelevant mouse tail biopsy sample containing the indicated copy numbers of the human *C3G* genes were digested with *Xho*I and analyzed by Southern blotting. (**B**) MEF-hC3G cells were infected with Cre-carrying adenovirus (AxCanCre), Cre-carrying retrovirus (MX-pie-Cre) and control virus (MX-pie) at the multiplicity of infection (MOI) indicated at the top of panels. Forty-eight hours after infection, cells were lysed in lysis buffer and separated by SDS–PAGE, followed by immunoblotting by use of anti-C3G antibody. The filter was reprobed with anti-tubulin monoclonal antibody to confirm that similar amounts of lysates were analyzed (lower panels). (**C**) Cell lysates of MEF-hC3G were separated by SDS–PAGE, followed by western blotting by use of anti-C3G polyclonal antibody, sc-869, anti-C3G serum 1A or 1B. (**D**) MEF-hC3G cells were infected with Cre-carrying retrovirus. After 48 h, cells were lysed in lysis buffer and GTP-bound G proteins were collected by use of either GST–Raf-RBD (for Ras and R-Ras) or GST–RalGDS-RBD (for Rap1 and Rap2). The resulting complexes were precipitated by glutathione–Sepharose beads and analyzed by SDS–PAGE and western blotting by use of antibodies. Small aliquots of lysates were analyzed by immunoblotting to confirm a similar level of expression of G proteins. For the detection of GTP-bound R-Ras, MEF-hC3G cells were infected with MSCV-R-Ras retrovirus and maintained in DMEM containing 2 µg/ml puromycin for 48 h. The cells were analyzed as described, except that anti-FLAG antibody was used to detect the expressed R-Ras.

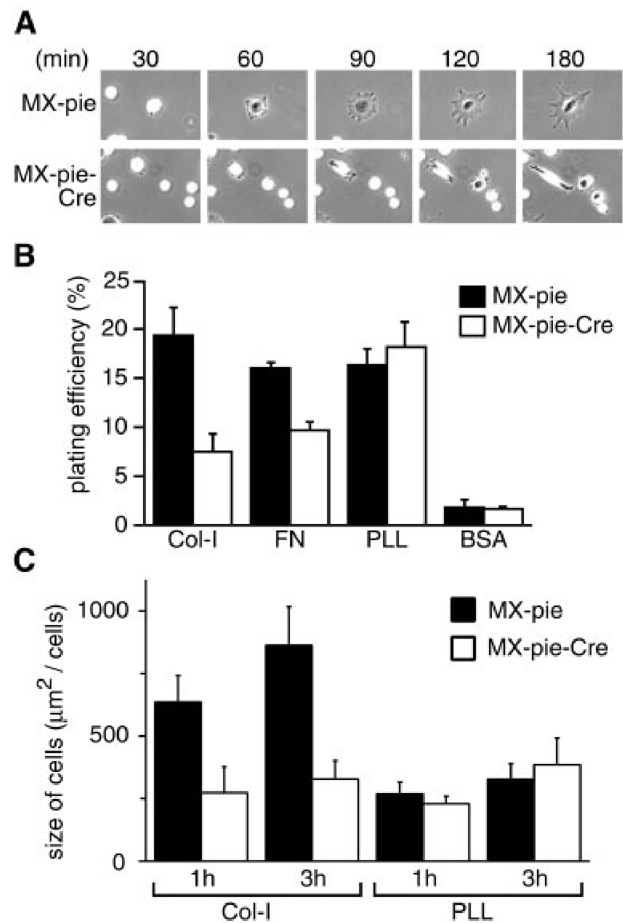

Fig. 4. Requirement for C3G in cell adhesion and cell spreading. (**A**) MEF-hC3G cells were infected with MX-pie-Cre or MX-pie. After 48 h, cells were trypsinized, kept in suspension for 1 h and plated on dishes coated with collagen type I. The cells were observed by time-lapse microscopy. We show representative photographs at the indicated time points. (**B**) MEF-hC3G cells were infected with MX-pie or MX-pie-Cre. After 48 h, cells were trypsinized and labeled with BCECF, AM. The cells were plated on 96-well black-colored plates coated with the reagents indicated at the bottom and incubated for 1 h at 37°C in a CO_2 incubator. Cells were washed three times with HBSS, and the fluorescence intensity was measured at excitation and emission wavelengths of 488 and 530 nm, respectively. Plating efficiency is shown as the average for three wells, with the SE. (**C**) MEF-hC3G cells infected with MX-pie or MX-pie-Cre were plated to dishes coated with collagen (Col-I) or poly-L-lysine (PLL). Twenty EGFP-positive cells were photographed after 1 and 3 h and measured for size. Average and SE are shown.

subfamilies *in vitro* and in 293T cells (Ohba *et al.*, 2000a,b). Therefore, we next proceeded to examine the effect of C3G deficiency on Rap2 and R-Ras (Figure 5B). The results are summarized as follows: (i) Rap1, Rap2 and R-Ras were all activated upon attachment of MEF-hC3G cells to the culture dishes; (ii) C3G deficiency inhibited the activation of Rap1 upon cell attachment, but not that of Rap2: neither of these phenomena was affected by the type of extracellular matrix; (iii) C3G deficiency inhibited R-Ras activation partially, suggesting the involvement of other GEF(s) in cell attachment-induced R-Ras activation. These results indicated that lack of Rap1 activation caused a decrease in cell attachment and cell spreading of C3G-deficient cells.

Increase in cell motility by C3G deficiency

Another phenotype of the C3G-deficient cells that we noticed was an increase in cell motility. To quantitate this observation, we recorded phase-contrast and fluorescence images of MEF-hC3G cells infected with MX-pie or

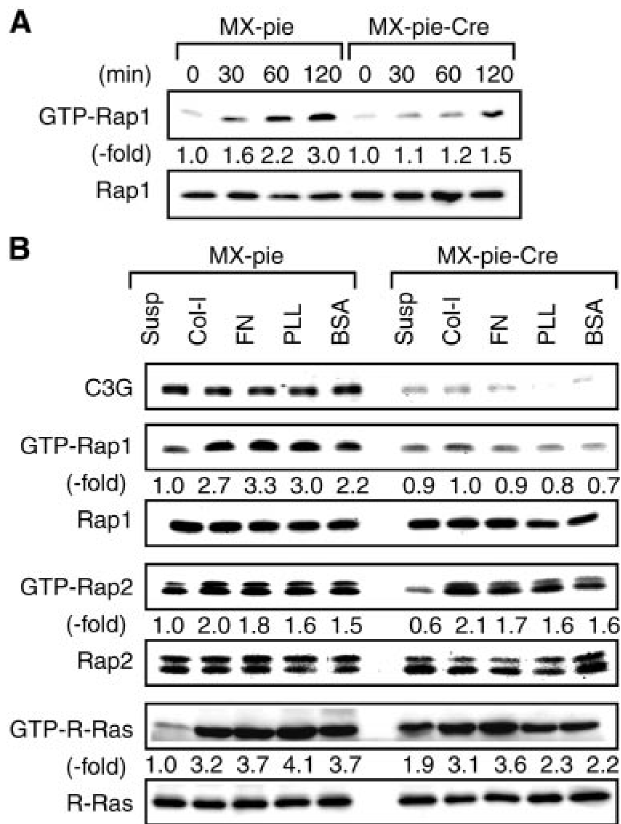

Fig. 5. Rap1 activation induced by cell adhesion. (**A**) MEF-hC3G cells were infected with MX-pie-Cre or MX-pie. After 48 h, cells were trypsinized, kept in suspension for 1 h and plated on dishes coated with collagen type I. Cells were lysed at the indicated times. After normalizing on the protein quantity, the level of GTP-Rap1 was analyzed by Bos's method. (**B**) MEF-hC3G cells were infected with MX-pie or MX-pie-Cre, maintained for 48 h, trypsinized, kept in suspension for 1 h and plated on dishes pre-coated as noted at the top. After 1 h, we harvested cells and examined the levels of GTP-bound Rap1, Rap2 and R-Ras. For the detection of GTP-R-Ras, we used MEF-hC3G cells infected with MSCV-R-Ras retrovirus as described in Figure 3C.

MX-pie-Cre for 8 h with a time-lapse fluorescence microscope and obtained the cell paths with a cell-tracking program (Figure 6A). The velocity of C3G-deficient MEF-hC3G cells was higher than that of the control MEF-hC3G cells on collagen-coated dishes (Figure 6B). The difference was statistically significant by *t*-test and Welch's test (P <0.01). When we used poly-L-lysine-coated dishes, the velocity of MEF-hC3G cells was not affected by the expression of Cre (data not shown). Similar results were obtained by using different isolates of MEF-hC3G cells. In another cell line derived from C3G$^{+/-/-}$ embryos, we did not find any effect of Cre on the cell motility (data not shown).

We next examined whether the expression of other GEFs for Ras-family G proteins could reduce the cell motility of C3G-deficient MEF-hC3G cells (Figure 6B). As expected, re-introduction of C3G into C3G-deficient MEF-hC3G cells reduced the cell motility to the level of the parent MEF-hC3G cells. Expression of Epac, a cAMP-responsive GEF for Rap1 and Rap2, reduced the cell motility to the level of the parent MEF-hC3G cells only in the presence of a cAMP analog, Sp-cAMPS. CalDAG-GEFI, which is another GEF for Rap1, Rap2 and R-Ras

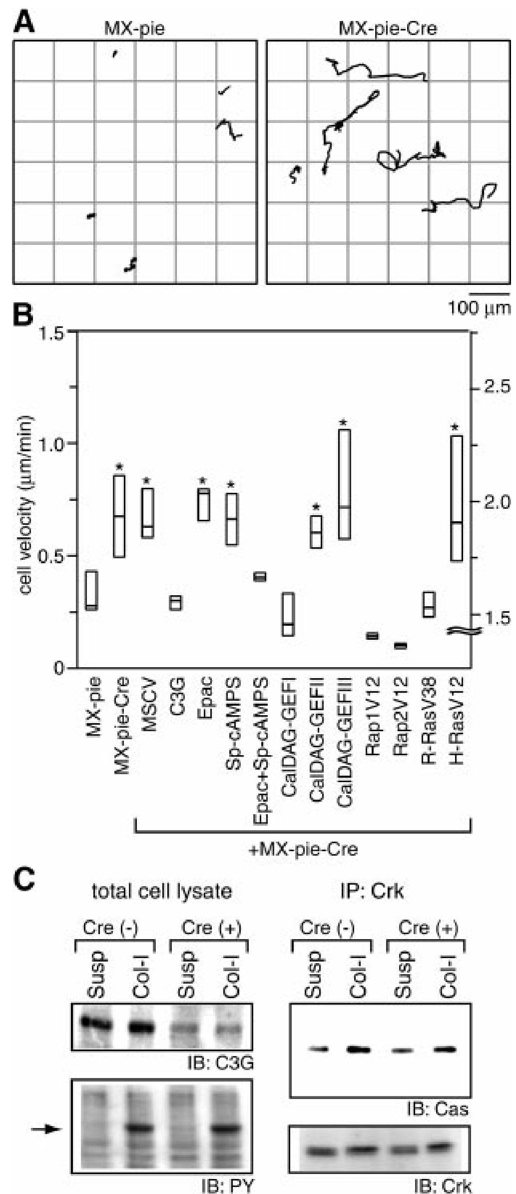

Fig. 6. Increased cell motility of C3G-deficient cells. (**A**) MEF-hC3G cells infected with MX-pie or MX-pie-Cre were trypsinized, kept in suspension for 1 h and plated on collagen-coated dishes. Starting after 1 h, cell images were collected every 3 min under time-lapse fluorescence microscopy equipped with a cooled CCD camera. Paths of the center of EGFP-positive cells during 8 h recording time were traced with MetaMorph2 software. (**B**) MEF-hC3G cells or MEF-hC3G cells expressing the proteins listed at the bottom were infected with MX-pie-Cre as indicated. We analyzed the cells as in (A) and obtained the mean velocities of 20 cells for each sample. Mid-line, top and bottom of each box indicate median, 75th quartile and 25th quartile, respectively. Cells that show a significant difference from the control, MX-pie infected cells, by *t*-test and Welch test (P <0.01) are marked with an asterisk at the top of the box. Note that scales shown on the right are for the cells expressing H-RasV12 used. (**C**) MEF-hC3G cells prepared as described in (A) were lysed and immunoprecipitated with anti-Crk monoclonal antibody and a mixture of protein G– and protein A–Sepharose. Total cell lysates and immunoprecipitated proteins were separated by SDS–PAGE, followed by immunoblotting with anti-C3G anbibody, anti-phosphotyrosine antibody (PY), anti-p130Cas antibody (Cas) or anti-Crk antibody.

and is constitutively active in many cell types (Yamashita *et al*., 2000), also reduced the cell motility. In contrast, CalDAG-GEFII, a GEF for the Ras and R-Ras subfamilies,

and CalDAG-GEFIII, a pan-Ras GEF, did not reduce the cell motility of C3G-deficient MEF-hC3G cells. We further tested the effect of GTPase-deficient mutants of Ras-family G proteins. Rap1 and Rap2 reduced the cell motility most strongly, and R-Ras did so moderately. GTPase-deficient H-Ras, in contrast, remarkably increased the cell motility. Thus, the effect of C3G deficiency in cell migration was antagonized by the activation of its substrates, Rap1, Rap2 and R-Ras. Finally, because Crk and $p130^{Cas}$ are postulated to function upstream to C3G and to increase cell motility (Ohashi *et al.*, 1999; Uemura and Griffin, 1999; Cho and Klemke, 2000; Yano *et al.*, 2000), we confirmed that the C3G deficiency did not affect cell adhesion-induced phosphorylation of $p130^{Cas}$ or Crk binding to $p130^{Cas}$ (Figure 6C).

Discussion

Requirement for C3G in early embryogenesis

At least eight GEFs have been reported to activate Rap1 in mammalian cells (reviewed by Zwartkruis and Bos, 1999). The mortality of *C3G*-deficient embryos showed that none of these GEFs could complement the loss of C3G during development. This is not surprising because, except for C3G, Rap1 GEFs are expressed in a more or less tissue-specific manner. Moreover, it should be noted that C3G is the only Rap1 GEF that has been linked definitively to the tyrosine kinase signaling pathway. The defect in the very early stage of embryogenesis also suggested that C3G functions in a signal transduction cascade that is universal and essential to most animal cells. In concordance with this view, C3G is expressed ubiquitously in mammalian cells, and the orthologs of C3G have been identified in organisms from *D.melanogaster* to man (Ishimaru *et al.*, 1999).

Substrates of C3G

We have shown previously that C3G promotes the guanine nucleotide exchange reaction of Rap1, Rap2 and R-Ras *in vitro* and in 293T cells (Gotoh *et al.*, 1995, 1997; Ohba *et al.*, 2000a). Although Rap1, Rap2 and R-Ras were all activated upon cell attachment to the substratum, only Rap1 activation was abolished by C3G deficiency. This observation suggests the activation of other GEFs, which stimulate Rap2 and/or R-Ras upon cell adhesion. A plausible candidate for such a molecule is PDZ-GEF1, which may interact with cell adhesion molecules via its PDZ domain and which stimulates Rap2 more efficiently than Rap1 (de Rooij *et al.*, 1999; Liao *et al.*, 1999; Ohtsuka *et al.*, 1999). Alternatively, Rap2 may not require any GEF to restore its basal GTP level after cell attachment because of its low sensitivity to GAP (Ohba *et al.*, 2000a). It is of note that our finding does not negate the possibility that C3G activates Rap2 or R-Ras under other conditions. For example, we have found that the v-*crk* oncogene product activates R-Ras in a manner dependent on C3G (Mochizuki *et al.*, 2000). Compared with Crk-II, v-Crk protein possesses viral Gag at its N-terminus and lacks the SH3 domain at the C-terminus. This difference may be responsible for the recruitment of the v-Crk–C3G complex to a different subcellular localization, where R-Ras is a preferable substrate for C3G.

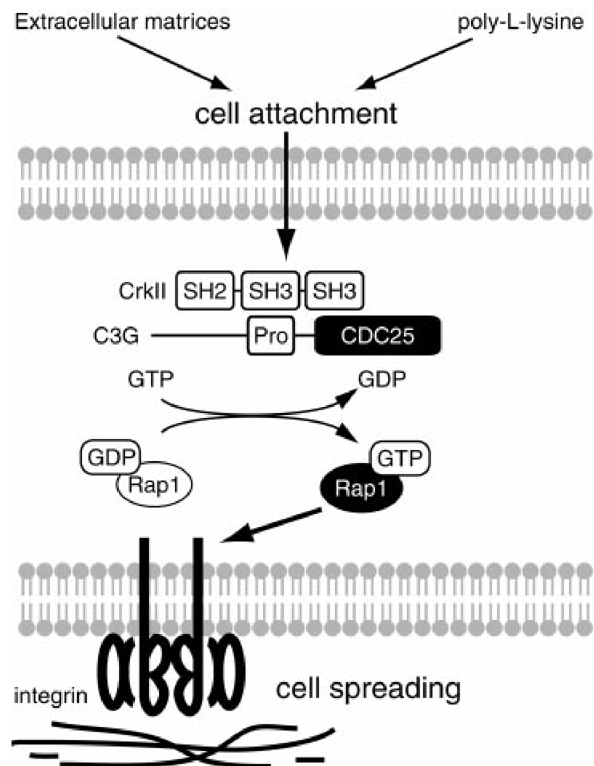

Fig. 7. A model for the role of C3G–Rap1 signaling pathway in cell attachment and cell spreading. In mouse embryonic fibroblasts, Rap1 is activated by C3G upon cell attachment, irrespective of the substratum. The activated Rap1 triggers an inside-out signal of integrin to induce cell adhesion, which requires specific interaction between integrin and extracellular matrices. SH2, SH3, Pro and CDC25 indicate Src homology 2, Src homology 3, proline-rich and yeast CDC25 homology domains, respectively.

Requirement for C3G for cell spreading

Accumulating evidence has shown that the Crk–C3G–Rap1 pathway plays a pivotal role in integrin-mediated signaling; however, the precise function of this pathway in cell adhesion remains elusive (Zwartkruis and Bos, 1999; Caron *et al.*, 2000; Katagiri *et al.*, 2000; Reedquist *et al.*, 2000). Rap1 is activated not only by integrin-mediated cell attachment, but also by non-specific attachment to the positively charged substratum (Figure 5B; and Posern *et al.*, 1998). By use of conditional knockout of *C3G*, we found that C3G was required for Rap1 activation in both cases. However, Rap1 activation by C3G was not sufficient for cell spreading, which required specific extracellular matrices for integrin (Figure 4C). This view is supported by several reports that Rap1 activates some types of integrin to increase cell adhesion (Caron *et al.*, 2000; Katagiri *et al.*, 2000; Reedquist *et al.*, 2000). Thus, Rap1 activation by cell adhesion is dependent on C3G, but it is not necessarily mediated by a specific interaction between integrin and the substratum. In contrast, Rap1-dependent cell spreading requires a specific interaction between integrin and the substratum (Figure 7).

Increased cell migration in C3G-deficient fibroblasts

Crk and its related protein, CrkL, are known to enhance migration in various cell types, probably through binding

to p130^{Cas} (Ohashi *et al*., 1999; Uemura and Griffin, 1999; Cho and Klemke, 2000; Yano *et al*., 2000). In Ba/F3 hematopoietic cells, expression of C3G enhances CrkL-dependent cell migration, suggesting a positive role for C3G in cell migration. In clear contrast to these previous reports, we found that C3G deficiency increased cell migration in MEFs. This increase in cell migration was suppressed by the expression of Epac or CalDAG-GEFI, as well as C3G, indicating that Rap1 activation inhibited cell migration. The discrepancy between the role of C3G and Crk in cell migration may be explained by the fact that previous reports relied mostly on the overexpression of Crk, which elevates tyrosine phosphorylation of p130^{Cas} and paxillin (Birge *et al*., 1993; Sakai *et al*., 1994). It has been reported that tyrosine phosphorylation of p130^{Cas} increases cell migration, whereas tyrosine phosphorylation of paxillin suppresses cell migration (Yano *et al*., 2000). Thus, under physiological conditions, the Crk–C3G complex may function downstream of paxillin, whereas, in cells overexpressing Crk, the complex may function downstream of p130^{Cas} to increase cell migration. Alternatively, the difference in the cell types used in each study may explain the discrepancy. For example, upon cell adhesion, Cbl is the major phosphotyrosine-containing protein in Ba/F3 hematopoietic cells (Uemura and Griffin, 1999), whereas p130^{Cas} and paxillin are phosphorylated predominantly on tyrosine in fibroblasts or epithelial cells (Birge *et al*., 1993; Sakai *et al*., 1994).

Downstream of Rap1 and Ras in cell migration

Rap1 inhibits Ras-induced transformation and MAPK activation in some cell types (Kitayama *et al*., 1989; Yatani *et al*., 1991; Sakoda *et al*., 1992; Boussiotis *et al*., 1997; Okada *et al*., 1998), which is, at least partially, due to the inhibition of Ras-induced activation of Raf serine/threonine kinase (Cook *et al*., 1993; Hu *et al*., 1997). We showed that expression of active Ras transformed the sluggish C3G-deficient cells into rapidly migrating cells (Figure 6), providing another example that Rap1 and Ras antagonize each other. However, between the C3G-deficient cells and the parental cells, we did not find any difference in MAPK activation induced by cell adhesion or epidermal growth factor stimulation (data not shown). Thus, a target or targets other than Raf appear to respond to Rap1 and Ras in opposite ways and control the velocity of cell migration. Such a candidate molecule is AF-6/canoe, which binds to both Ras and Rap1 and serves as a peripheral component of tight junctions in epithelial cells (Kuriyama *et al*., 1996; Yamamoto *et al*., 1997; Linnemann *et al*., 1999; Boettner *et al*., 2000).

In conclusion, we have shown that C3G-dependent Rap1 activation promotes cell adhesion and cell spreading, but suppresses cell migration. These functions of the C3G–Rap1 pathway appear to underlie the lethality of *C3G*-deficient mice in the very early stage of development.

Materials and methods

Generation of C3G$^{-/-}$ mice, and creation and identification of transgenic mice

The generation of the knockout mice and transgenic mice is described in the Supplementary data (available at *The EMBO Journal* Online).

Establishment of embryonic fibroblast cell lines

C3G$^{+/-}$ mice expressing the human *C3G* transgene were intercrossed, and E12.5 embryonic fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) as described previously (Brugarolas *et al*., 1998). The genotype of each cell line was determined by Southern blotting and PCR as described in the Supplementary data. The cell lines that lacked the mouse *C3G* and carried the human *C3G* were named MEF-hC3G.

Virus packaging and infection

Coding sequences of C3G, Epac (de Rooij *et al*., 1998; Kawasaki *et al*., 1998a), CalDAG-GEFI, II and III (Kawasaki *et al*., 1998b; Yamashita *et al*., 2000), Rap1V12 (Kitayama *et al*., 1989), Rap2V12 (Ohba *et al*., 2000a), R-RasWT and R-RasV38 (Rey *et al*., 1994) were subcloned into pMSCV-pac-Flag, which was a derivative of pMSCV-pac (Hawley *et al*., 1994) with a Flag tag sequence at the 5′ end of the cloning site. The coding sequence of Cre recombinase was subcloned into another retroviral plasmid, pMX-pie (Onishi *et al*., 1996), which carries enhanced green fluorescent protein (EGFP) downstream of the internal ribosomal entry site (IRES). The resulting plasmid was named pMX-pie-Cre.

Recombinant retroviruses were produced as described previously (Pear *et al*., 1993). Bosc23 cells were transfected with retroviral vectors and a replication-incompetent helper vector, pCL-Eco (Naviaux *et al*., 1996), by the calcium phosphate co-precipitation method. After 48 h, the culture medium was cleared by centrifugation and used as virus stock. For virus titration, we developed an NIH-3T3-derived cell line that was transfected stably with loxP-LacZ (Kanegae *et al*., 1995). These NIH3T3-loxP-LacZ cells were infected with serially diluted virus stocks. At day 2, puromycin (Sigma, St Louis, MO) was added to 2 μg/ml. At day 4, the number of cell colonies was counted under a microscope for determination of the virus titer. Expression of EGFP or LacZ in the remaining colonies was confirmed by fluorescence microscopy or by staining with X-gal. By this procedure, we obtained virus stocks with at least 2×10^6 colony-forming units/ml. Cre-expressing adenovirus, AxCANCre (RIKEN Gene Bank, 1748), was propagated in HEK293 cells (Japan Cell Resource Bank) as described previously (Kanegae *et al*., 1995). Cells were infected with AxCANCre at multiplicities of infection of 0.1–10 and were assayed at least 48 h after infection.

Antibodies and reagents

Anti-C3G polyclonal antibody, sc-869 and anti-Rap1 polyclonal antibody, sc-065, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-C3G antisera 1A and 1B have been reported previously (Tanaka *et al*., 1994). Anti-pan-Ras and anti-tubulin monoclonal antibodies were from Calbiochem (San Diego, CA). Anti-Rap2 and anti-FLAG monoclonal antibodies were from BD Transduction Laboratories (Bluegrass Lexington, KY) and Sigma, respectively. Sp-cAMPS triethylamine (Sp-cAMPS) was purchased from Research Biochemical International (Natick, MA).

Detection of GTP-bound Ras-family G proteins

Escherichia coli expression vectors for the GST-fused Ras/Rap1-binding domain (RBD), pGEX-RalGDS-RBD and pGEX-Raf-RBD, were obtained from J.L.Bos (Utrecht University, The Netherlands) and S.Hattori (National Center for Neurology and Psychiatry, Tokyo, Japan), respectively. Detection of GTP-bound Ras-family G proteins and purification of GST fusion proteins were performed by Bos's method with slight modifications (Franke *et al*., 1997; Ohba *et al*., 2000a). Briefly, cells were lysed in lysis buffer (50 mM Tris pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1 mM Na_3VO_4), clarified by centrifugation, and incubated with GST–RBD fusion proteins. We used GST–Raf-RBD for the detection of GTP-Ras and R-Ras, and GST–RalGDS-RBD for Rap1 and Rap2, respectively. The resulting complexes of GTP-bound G proteins and GST–RBD were precipitated by use of glutathione–Sepharose beads, and proteins bound to the beads were separated by SDS–PAGE, followed by immunoblotting with specific antibodies for each G protein. Bound antibodies were detected by an ECL chemiluminescence system (Amersham Pharmacia) and analyzed with an LAS-1000 image analyzer (Fuji-Film). We could not detect endogenous GTP-R-Ras in MEF-hC3G cells; therefore, cells were infected with an R-Ras encoding retrovirus, MSCV-R-RasWT, selected with puromycin and analyzed as described above.

Cell adhesion assay

The cell adhesion assay was performed essentially as described previously (Newton *et al*., 1997). Briefly, 96-well microplates (Greiner Labortechnik GmbH, Frickenhausen, Germany) were coated for 2 h at

room temperature with type-I collagen (Nitta Gelatin, Osaka, Japan), fibronectin or poly-L-lysine (Sigma). Cells were treated with 0.125% trypsin and 2 mM EDTA in phosphate-buffered saline (PBS), resuspended in minimal essential medium (MEM) containing 100 μg/ml trypsin inhibitor (Sigma), labeled with the fluorescent dye BCECF, AM (Molecular Probes, Leiden, The Netherlands) for 30 min at 37°C in Hanks' balanced salt solution (HBSS; 140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 2 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.1% glucose, 0.035% $NaHCO_3$), washed twice in HBSS and resuspended in MEM. The labeled cells were seeded to coated 96-well plates at a density of 5×10^4 cells/well in a volume of 100 μl and allowed to adhere for 1 h at 37°C. Plates were washed three times with pre-warmed HBSS. The fluorescence intensity was measured by FluoroscanII (Labsystems, Helsinki, Finland). The efficiency of cell adhesion was expressed as (fluorescence intensity after wash)/(input fluorescence intensity). In another set of experiments, MEF-hC3G cells were harvested similarly, kept in suspension for 1 h at 37°C, plated to 6-cm-diameter dishes and allowed to adhere for 1 h. The GTP-bound forms of Rap1, Rap2 and R-Ras were quantitated as described.

Time-lapse microscopy and cell-tracking analysis

MEF-hC3G cells infected with MX-pie or MX-pie-Cre were seeded on a collagen-coated 35-mm-diameter glass base dish (Asahi Techno Glass Co., Tokyo). Cells were imaged on a Zeiss Axiovert microscope (Carl Zeiss, Jena, Germany) with a cooled CCD camera (Roper Scientific, Trenton, NJ), controlled by MetaMorph2 software (Universal Image, West Chester, PA) as described previously (Miyawaki *et al*., 1997). Phase-contrast and fluorescence images were recorded every 3 min. We obtained a series of time-lapse images and analyzed the size, paths and velocities of EGFP-positive cells by use of a cell-tracking application handled with MetaMorph2. In other experiments, MEF-hC3G cells were inoculated with MSCV-pac-derived retroviruses and selected in the presence of puromycin for 2 days. The puromycin-resistant cells were inoculated with MX-pie-Cre and analyzed as described.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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