MOLECULAR AND CELLULAR BIOLOGY, Aug. 1992, p. 3407–3414
0270-7306/92/083407-08$02.00/0

Vol. 12, No. 8

Microinjection of *smg*/*rap*1/K*rev*-1 p21 into Swiss 3T3 Cells Induces DNA Synthesis and Morphological Changes

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Received 2 January 1992/Accepted 30 April 1992

Microinjection of either Ki-*ras*$^{Val-12}$ p21 or the GDP-bound form of Ki-*ras* p21 plus *smg* GDP dissociation stimulator (GDS), a stimulatory GDP/GTP exchange protein for Ki-*ras* p21, *smg*/*rap*1/K*rev*-1 p21, and *rho* p21, into quiescent Swiss 3T3 cells induced DNA synthesis irrespective of the presence or absence of insulin. The guanosine 5′-(3-*O*-thio)triphosphate (GTPγS)-bound form of *smg* p21B or the GDP-bound form of *smg* p21B plus *smg* GDS also induced DNA synthesis but only in the presence of insulin. Either the GDP-bound form of Ki-*ras* p21 or the same form of *smg* p21B alone was inactive, but *smg* GDS alone was slightly active only in the presence of insulin. The morphology of the cells was analyzed by scanning electron, phase-contrast, and confocal laser scanning microscopies. Ki-*ras*$^{Val-12}$ p21 induced membrane ruffling irrespective of the presence or absence of insulin. The GTPγS-bound form of *smg* p21B showed the same effect only in the presence of insulin. Either the GDP-bound form of Ki-*ras* p21, the same form of *smg* p21B, or *smg* GDS alone was inactive. Upon microinjection of Ki-*ras*$^{Val-12}$ p21, stress fibers markedly decreased and the cells became round and piled up. In contrast, upon microinjection of the GTPγS-bound form of *smg* p21B, stress fibers did not markedly decrease and the cells neither became round nor piled up. These results indicate that both *ras* p21 and *smg* p21 are mitogenic in Swiss 3T3 cells but that their actions are slightly different.

The *smg* p21 family, consisting of two members, A and B, belongs to the *ras* p21-related small GTP-binding protein (G protein) superfamily (for reviews, see references 3 and 41). *smg* p21A is identical to *rap*1A p21 and K*rev*-1 p21, and *smg* p21B is identical to *rap*1B p21 (22, 24, 28, 35, 36). Among many small G proteins, *smg* p21 has the same amino acid sequence as does the effector region of *ras* p21 (3, 22, 24, 28, 35, 36, 41). This structural property suggests that *smg* p21 can share the effector(s) with *ras* p21 and exert actions similar or antagonistic to those of *ras* p21. Consistently, K*rev*-1 p21 has been shown to suppress the transforming activity of v-Ki-*ras* p21 in NIH 3T3 cells (24). *smg* p21B and *rap*1A p21 inhibit the *ras* p21 GTPase-activating protein (GAP) activity in a manner competitive with *ras* p21 in a cell-free system (10, 13). *ras* p21 GAP has been shown to interact with the effector domain of *ras* p21 and to stimulate its GTPase activity (for a review, see reference 29). Moreover, our recent studies have revealed that overexpression of *smg* p21 in NIH 3T3 cells inhibits the *ras* p21-, platelet-derived growth factor (PDGF)-, and 12-*O*-tetradecanoylphorbol-13-acetate-induced activation of the c-*fos* promoter/enhancer element but does not inhibit the c-*raf*-1-induced activation of this element (39). There are several lines of evidence that *ras* p21 is a downstream molecule of the PDGF receptor and protein kinase C and that the c-*raf*-1 protein kinase mediates at least a part of the actions of *ras* p21 (6, 7, 16, 18, 25, 32, 40). These results indicate that *smg* p21 may antagonize *ras* p21 actions presumably by competing for the proteins interacting with the effector domain of *ras* p21. Although *ras* p21 GAP interacts with the effector domain of *ras* p21, there is increasing evidence that this protein is a negative regulatory protein which converts *ras* p21 from the GTP-bound active form to the GDP-bound inactive form (29, 33, 43, 45). At present, the effector protein of *ras* p21 in mammalian cells is unknown.

The conversion of *smg* p21 from the GDP-bound inactive form to the GTP-bound active form is stimulated by a GDP/GTP exchange protein, named *smg* GDP dissociation stimulator (GDS) (17, 44). This *smg* GDS is active not only on *smg* p21 but also on Ki-*ras* p21 and *rho* p21 (31). On the other hand, *smg* p21 is directly phosphorylated by cyclic AMP-dependent protein kinase (protein kinase A) and cyclic GMP-dependent protein kinase (protein kinase G) at the same serine residue (Ser-179), which is located between the polybasic region and the geranylgeranylated cysteine residue in the C-terminal region of the protein (9, 12, 14, 20, 21, 26, 30). This phosphorylation makes *smg* p21 sensitive to the action of *smg* GDS that eventually leads *smg* p21 to the GTP-bound active form (12, 15, 19). It is well known that the protein kinase A and G systems have pleiotropic actions, and it is likely that *smg* p21 mediates at least a part of these actions of both systems (for reviews, see references 41 and 42).

In NIH 3T3 cells, cyclic AMP does not induce DNA synthesis or inhibits DNA synthesis initiated by serum or PDGF plus insulin (for a review, see reference 34). However, this cyclic nucleotide initiates DNA synthesis in the presence of insulin in Swiss 3T3 cells (for a review, see reference 38). Moreover, PDGF is also mitogenic in this cell type and stimulates the arachidonic acid cascade which finally produces prostaglandins (38). These prostaglandins stimulate cyclic AMP production in Swiss 3T3 cells (38). Thus, cyclic AMP plays an important role in initiating DNA synthesis in Swiss 3T3 cells.

In this study, therefore, we have investigated the roles of *smg* p21 in DNA synthesis and morphological changes of Swiss 3T3 cells by microinjecting it into the cells. We report that both *ras* p21 and *smg* p21 are mitogenic in Swiss 3T3 cells but that their actions are slightly different.

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MATERIALS AND METHODS

Materials and chemicals. Swiss 3T3 cells were kindly supplied by E. Rozengurt (Imperial Cancer Research Fund, London, England). The cDNAs of Ki(2B)-*ras* p21 and Ki-*ras*$^{Val-12}$ p21 were kindly provided by R. A. Weinberg (Massachusetts Institute of Technology). Ki-*ras* p21, Ki-*ras*$^{Val-12}$ p21, and *smg* p21B were expressed in *Spodoptera frugiperda* cells as described previously (31). The posttranslationally unprocessed forms of Ki-*ras* p21, Ki-*ras*$^{Val-12}$ p21, and *smg* p21B were purified from the cytosol fraction of these cells (31). The purified Ki-*ras* p21 and *smg* p21 were the GDP-bound form as described previously (6, 10, 40). We did not examine whether the purified Ki-*ras*$^{Val-12}$ p21 was the GDP-bound form or the GTP-bound form. The guanosine 5′-(3-*O*-thio)triphosphate (GTPγS)-bound form of *smg* p21B was made by incubating the protein with 60 μM GTPγS as described previously (2). *smg* GDS was purified from *smg* GDS-overexpressing *Escherichia coli* (17). All proteins used were concentrated in a Centricon-10 (Amicon) to concentrations of 4 to 28 mg/ml. During the concentration, the buffers contained in the sample preparations were replaced by buffer A (20 mM Tris-HCl [pH 7.4] containing 20 mM NaCl, 2 mM $MgCl_2$, 100 μM ATP, 0.1 mM EDTA, and 1 mM 2-mercaptoethanol). The cell proliferation kit and Texas red-labeled anti-mouse immunoglobulin were purchased from Amersham Corp. Recombinant PDGF-BB and insulin were purchased from Intergen Co. and Sigma, respectively.

Cell culture. Stock cultures of Swiss 3T3 cells were maintained at 37°C in a humidified atmosphere of 10% CO_2–90% air in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were seeded into 35-mm-grid tissue culture dishes (Nunc Inc.) at a density of 5.7×10^4 cells per dish in 2.5 ml of Dulbecco's modified Eagle's medium containing 10% FCS, refed with the same medium after 2 days, and then used 5 days after the last change of medium. These cells were confluent and quiescent.

Microinjection. Each sample to be tested was microinjected into living Swiss 3T3 cells as described previously (4, 8). Briefly, glass capillaries drawn to a tip diameter of less than 1 μm were used to microinject each sample. About 50 cells in an area surrounded by four grids were usually microinjected within 5 min, and all cells were confirmed to be microinjected by using lucifer yellow. Trypan blue exclusion test showed that more than 90% of the cells survived the microinjection procedure. The cells were returned to the incubator and incubated in the presence or absence of 1.7×10^{-7} M insulin for the indicated periods of time at 37°C.

According to an early report that about 5×10^{-14} liter of sample was microinjected by one injection (8), about 0.2 pg each of the proteins was calculated to be microinjected into a cell when we used Ki-*ras* p21 or *smg* p21B at 4 mg/ml each. The intracellular concentrations of the microinjected Ki-*ras* p21 and *smg* p21B were calculated to be about 28 μM, and this concentration was about 120-fold more than their endogenous levels. The intracellular concentration of the microinjected *smg* GDS was calculated to be about 19 μM when *smg* GDS at 8 mg/ml was microinjected. This concentration was about 150-fold more than its endogenous level.

DNA synthesis assay. Swiss 3T3 cells in 35-mm-grid tissue culture dishes were stimulated with each growth factor to be tested or microinjected with each sample to be tested and then incubated for 18 h at 37°C. 5-Bromo-2′-deoxyuridine (BrdU) incorporation was detected by using a cell proliferation kit according to the manufacturer's manual, with slight modifications. Namely, BrdU was added to a final concentration of 20 μM to each dish after the 18-h incubation, and the cells were further incubated for 12 h. The cells were washed once with 2 ml of phosphate-buffered saline (PBS) and fixed with 2 ml of 5% acetic acid–95% ethanol for 30 min at room temperature. Then, the cells were incubated with an anti-BrdU antibody. BrdU incorporation was detected by horseradish peroxidase-labeled anti-mouse immunoglobulin and a chromogenic substrate, 3,3′-diaminobenzidine tetrahydrochloride. The specimen was observed with a light microscope (model IMT-2; Olympus, Tokyo, Japan) equipped with a 35-mm camera (150× lens).

Scanning electron, phase-contrast, and confocal laser scanning microscopies. Scanning electron microscopy was performed by using a scanning electron microscope (model T-330A; JEOL, Tokyo, Japan), with slight modifications as described previously (4). Briefly, Swiss 3T3 cells were seeded on a coverglass. The cells were microinjected with each sample to be tested and incubated for 12 h. After the incubation, the cells were fixed with 2.5% glutaraldehyde in PBS for 1 h at 4°C, postfixed in 2% OsO_4, dehydrated through graded ethanol, dried in a critical-point drier (model HCP-2; Hitachi, Tokyo, Japan), and gold coated in an ion coater (model 1B-3; Eiko, Ibaragi, Japan). The specimen was observed with the scanning electron microscope (magnification, ×2,000).

Phase-contrast microscopy was performed by using a light microscope (magnification ×400) equipped with a video camera system (model C2400; Hamamatsu Photonics, Hamamatsu, Japan) and a time-lapse video tape recorder (model BR-9000; Victor, Tokyo, Japan). Swiss 3T3 cells were microinjected with each sample to be tested. After the microinjection, 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) was added to the medium to a final concentration of 20 mM, and the medium was covered with 5 ml of mineral oil (E. R. Squibb and Sons, Inc.) to prevent the medium from evaporating. The culture dish was placed on a heat plate at 37°C. The cells were monitored at a tape speed of one frame per 2 s for 6 h.

Confocal laser scanning microscopy was performed by using a confocal laser scanning microscope (model MRC-600; Bio-Rad). Fluorescein isothiocyanate (FITC)-labeled phalloidin (Sigma) was used to detect stress fibers as described previously (1). Briefly, Swiss 3T3 cells were microinjected with each sample to be tested. At 18 to 30 h after the microinjection, the cells were incubated with 20 μM BrdU. After the BrdU labeling, the cells were fixed with PBS containing 2% paraformaldehyde and 0.2% pycric acid for 30 min at room temperature, incubated for 1 h at 30°C with an anti-BrdU antibody, and incubated for 1 h at room temperature with FITC-labeled phalloidin (1:20 dilution) and Texas red-labeled anti-mouse immunoglobulin (1:50 dilution). The specimen was then analyzed with the confocal laser scanning microscope.

Determinations. Protein concentrations were determined with bovine serum albumin as a standard protein by densitometric tracing of protein bands stained with Coomassie brilliant blue on a sodium dodecyl sulfate-polyacrylamide gel as described previously (23).

RESULTS

DNA synthesis of Swiss 3T3 cells upon stimulation with various growth factors. To investigate DNA synthesis of Swiss 3T3 cells upon stimulation with various growth factors, we analyzed BrdU incorporation into the cells in the

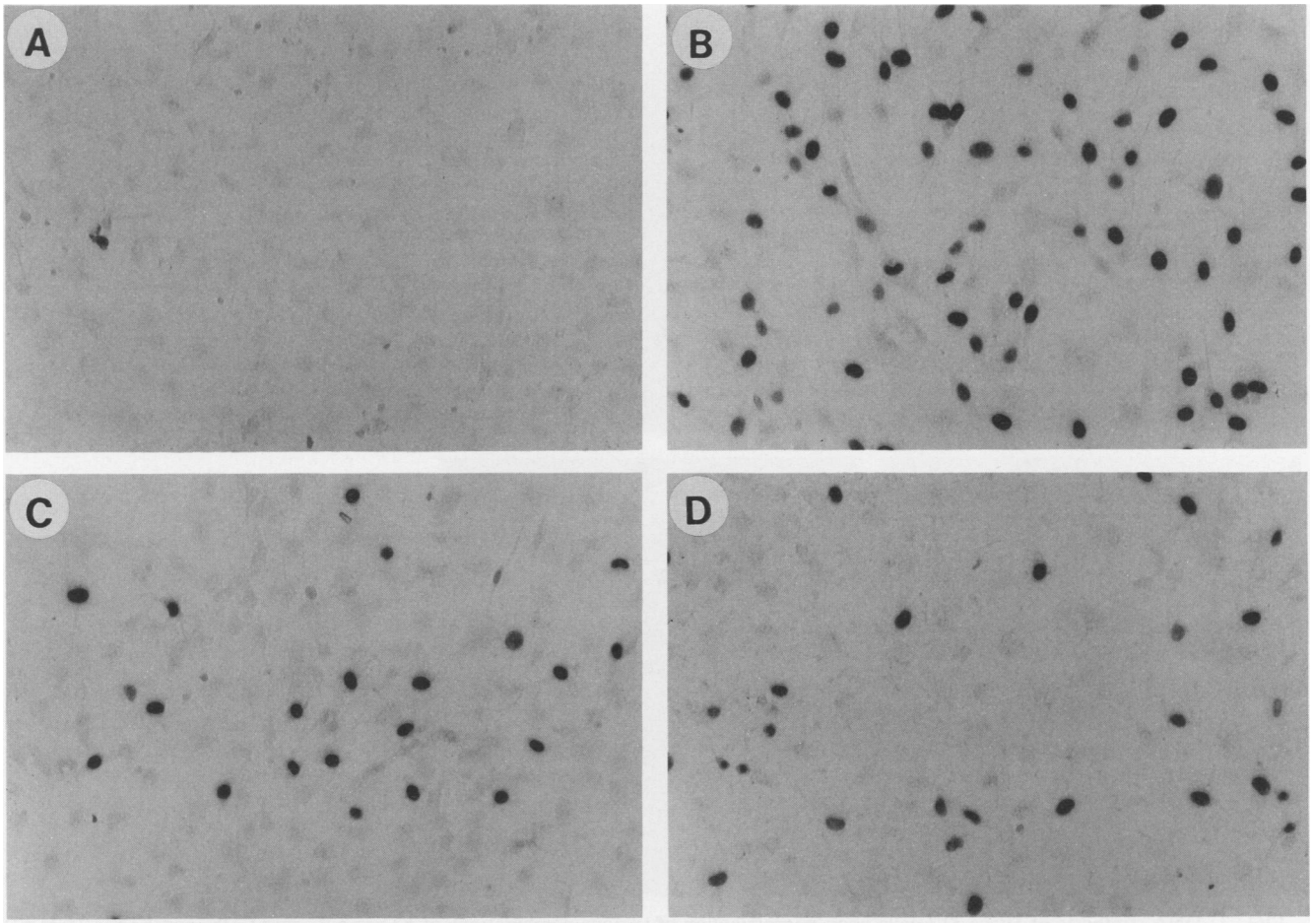

FIG. 1. DNA synthesis of Swiss 3T3 cells upon stimulation with various growth factors. Swiss 3T3 cells were incubated with no growth factor (A), 20% FCS (B), 10^{-3} M Bt_2cAMP plus 1.7×10^{-7} M insulin (C), or 7.0×10^{-11} M PDGF plus 1.7×10^{-7} M insulin (D). The results shown are representative of three independent experiments.

presence of various growth factors. We found that 20% FCS induced DNA synthesis (Fig. 1B). Dibutyryl cyclic AMP (Bt_2cAMP) and PDGF also induced DNA synthesis in the presence of insulin (Fig. 1C and D). Neither Bt_2cAMP, PDGF, nor insulin alone was active (data not shown). The cells showed no DNA synthesis in the absence of any growth factors (Fig. 1A). The FCS-induced DNA synthesis was dose dependent, and the maximal level was obtained with 20% FCS (data not shown). Both Bt_2cAMP- and PDGF-induced DNA syntheses were also dose dependent, and the maximal levels were obtained with 1.0×10^{-3} M Bt_2cAMP, and 7.0×10^{-11} M PDGF (data not shown). The mitogenic action of insulin was also dose dependent in the presence of Bt_2cAMP or PDGF, and the maximal levels were obtained with 1.7×10^{-7} M insulin for both Bt_2cAMP and PDGF (data not shown). The maximal levels of DNA synthesis induced by Bt_2cAMP or PDGF in the presence of insulin were about 40% of that induced by 20% FCS.

DNA synthesis of Swiss 3T3 cells upon microinjection of small G proteins and *smg* GDS. Microinjection of Ki-*ras*$^{Val\text{-}12}$ p21 into Swiss 3T3 cells induced DNA synthesis in the absence of insulin, as measured by BrdU incorporation (Fig. 2D; Table 1). Comicroinjection of the GDP-bound form of Ki-*ras* p21 and *smg* GDS also induced DNA synthesis in the absence of insulin to the same extent as did injection of Ki-*ras*$^{Val\text{-}12}$ p21 (Fig. 2E; Table 1). *smg* GDS did not further stimulate Ki-*ras*$^{Val\text{-}12}$ p21-induced DNA synthesis (data not shown). When DNA synthesis was measured in the presence of insulin, labeling indices were similar to those measured in the absence of insulin (Table 1). Neither buffer A, the GDP-bound form of Ki-*ras* p21, nor *smg* GDS alone induced DNA synthesis in the absence of insulin (Fig. 2A to C; Table 1).

Microinjection of the GTPγS-bound form of *smg* p21B into Swiss 3T3 cells also induced DNA synthesis in the presence of insulin (Fig. 3D; Table 1). Comicroinjection of the GDP-bound form of *smg* p21B and *smg* GDS caused DNA synthesis to the same extent in the presence of insulin (Fig. 3E; Table 1). The GDP-bound form of *smg* p21B alone did not induce DNA synthesis even in the presence of insulin (Fig. 3B; Table 1). However, *smg* GDS alone induced DNA synthesis to a certain extent in the presence of insulin (Fig. 3C; Table 1). The mitogenic effect of the GTPγS-bound form of *smg* p21B or the GDP-bound form of *smg* p21B plus *smg* GDS was not observed in the absence of insulin (Table 1). Bt_2cAMP did not further stimulate the DNA synthesis induced by comicroinjection of the GDP-bound form of *smg* p21B and *smg* GDS (data not shown). The GTPγS-bound form of *smg* p21B did not cause DNA synthesis in the presence of PDGF or Bt_2cAMP instead of insulin (data not

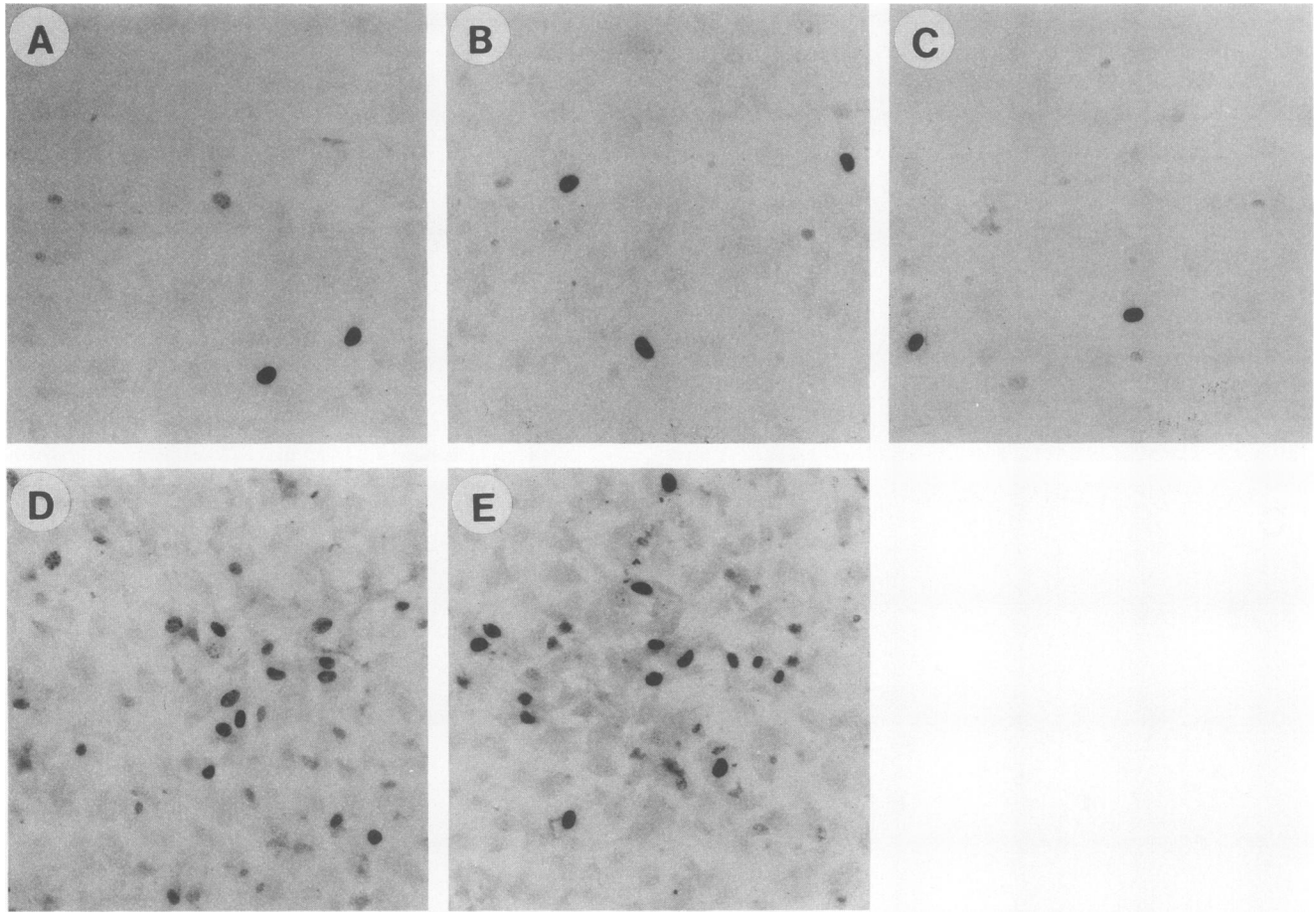

FIG. 2. DNA synthesis of Swiss 3T3 cells upon microinjection of various combinations of Ki-*ras* p21, *smg* GDS, and Ki-*ras*-$^{Val-12}$ p21. Swiss 3T3 cells were microinjected with buffer A (A), the GDP-bound form of Ki-*ras* p21 (4 mg of protein per ml) (B), *smg* GDS (8 mg of protein per ml) (C), Ki-*ras*$^{Val-12}$ p21 (4 mg of protein per ml) (D), or the GDP-bound form of Ki-*ras* p21 (4 mg of protein per ml) plus *smg* GDS (8 mg of protein per ml) (E) and incubated in the absence of insulin. The results shown are representative of three independent experiments.

shown). The sample preparation of the GTPγS-bound form of *smg* p21B contained 60 nM free GTPγS. Buffer A containing 60 nM GTPγS alone showed no effect on DNA synthesis in the presence of insulin (Fig. 3A; Table 1).

The stimulatory effect of Ki-*ras*$^{Val-12}$ p21 or the GTPγS-bound form of *smg* p21B on DNA synthesis was not linear concentration dependent. Ki-*ras*$^{Val-12}$ p21 at any doses between 0.75 and 4 mg/ml induced DNA synthesis irrespective of the presence or absence of insulin to the same extent, but Ki-*ras*$^{Val-12}$ p21 at less than 0.5 mg/ml was totally inactive (data not shown). The GTPγS-bound form of *smg* p21B at any doses between 2 and 6 mg/ml induced DNA synthesis in the presence of insulin to the same extent, but the GTPγS-bound form of *smg* p21B at less than 1.5 mg/ml was totally inactive (data not shown). Comicroinjection of Ki-*ras*$^{Val-12}$ p21 and the GTPγS-bound form of *smg* p21B at 4 mg/ml each induced DNA synthesis in the presence of insulin to extents similar to those observed upon microinjection of either Ki-*ras*$^{Val-12}$ p21 or the GTPγS-bound form of *smg* p21B alone (Fig. 3F; Table 1). Comicroinjection of Ki-*ras*$^{Val-12}$ p21 and the GTPγS-bound form of *smg* p21B at 0.5 and 1.5 mg/ml, respectively, did not induce DNA synthesis in the presence of insulin (data not shown).

Membrane ruffling of Swiss 3T3 cells upon microinjection of small G proteins and *smg* GDS. To investigate membrane

TABLE 1. Labeling indices of Swiss 3T3 cells upon microinjection with small G proteins and *smg* GDS

Substance microinjected	Labeling index (%)[a]	
	In the absence of insulin	In the presence of 1.7×10^{-7} M insulin
Buffer A	3.3 ± 1.5	3.7 ± 1.5
Buffer A containing 60 nM GTPγS	2.0 ± 1.1	4.2 ± 1.6
GDP-bound form of Ki-*ras* p21	2.0 ± 1.1	3.3 ± 1.5
smg GDS	2.0 ± 1.1	17 ± 3.1
Ki-*ras*$^{Val-12}$ p21	29 ± 3.7	30 ± 3.7
GDP-bound form of Ki-*ras* p21 + *smg* GDS	29 ± 3.7	28 ± 3.7
GDP-bound form of *smg* p21B	3.3 ± 1.5	3.3 ± 1.5
GTPγS-bound form of *smg* p21B	2.0 ± 1.1	29 ± 3.7
GDP-bound form of *smg* p21B + *smg* GDS	2.7 ± 1.3	31 ± 3.8
GTPγS-bound form of *smg* p21B + Ki-*ras*$^{Val-12}$ p21	27 ± 3.6	29 ± 3.7

[a] About 50 cells were usually microinjected with small G proteins and *smg* GDS. The concentrations of small G proteins and *smg* GDS used in each experiment were about 4 and 8 mg/ml, respectively. Three separate experiments were performed at different times, using cells derived from the same culture. Three separate experiments were not statistically different ($P < 0.05$). Results are presented as $P \pm \sqrt{P(1-P)/N}$ (P, mean probability; N, total number of the injected cells [about 150]).

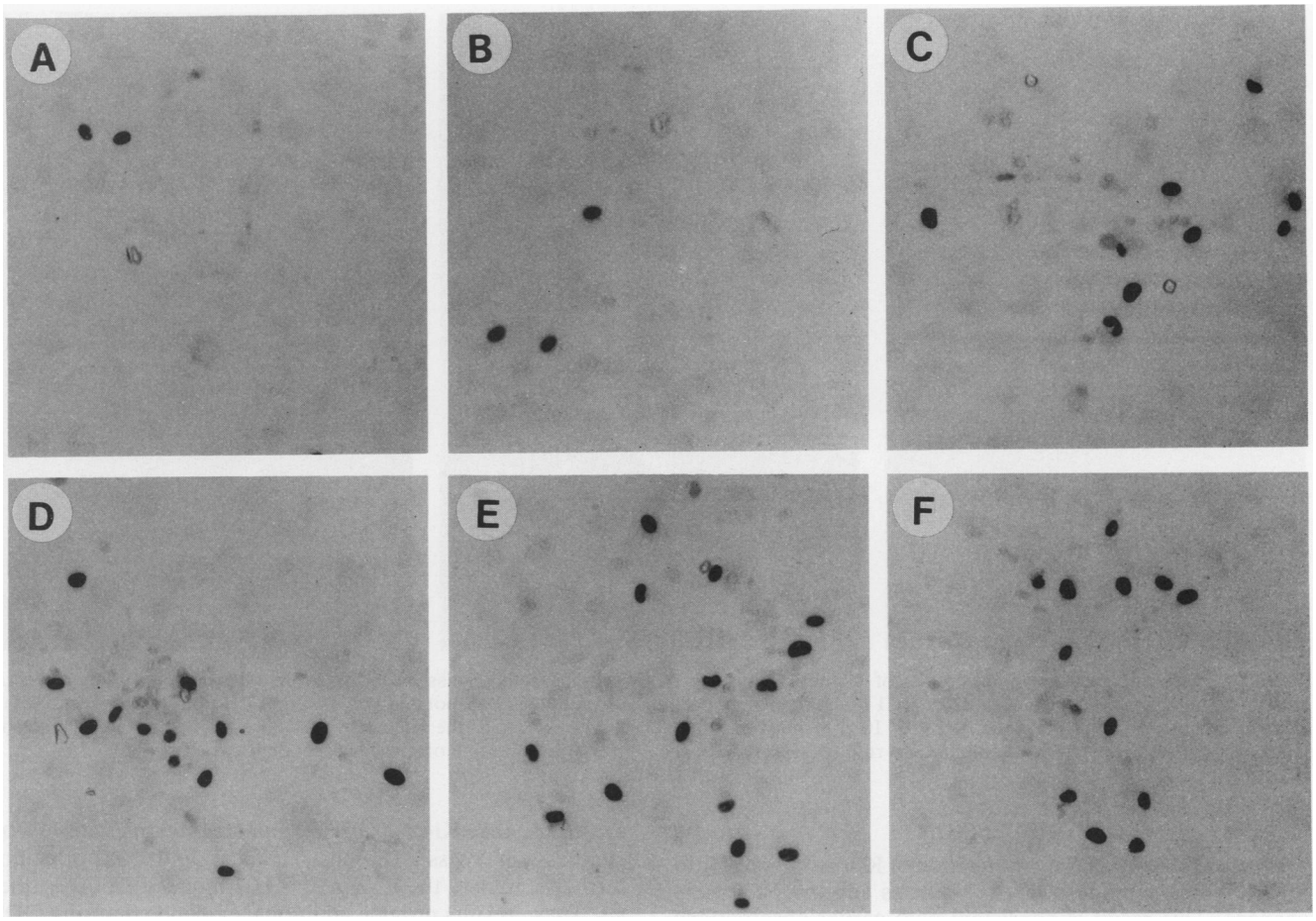

FIG. 3. DNA synthesis of Swiss 3T3 cells upon microinjection of various combinations of *smg* p21B, *smg* GDS, and Ki-*ras*$^{Val-12}$ p21. Swiss 3T3 cells were microinjected with buffer A containing 60 nM GTPγS (A), the GDP-bound form of *smg* p21B (4 mg of protein per ml) (B), *smg* GDS (8 mg of protein per ml) (C), the GTPγS-bound form of *smg* p21B (4 mg of protein per ml) (D), the GDP-bound form of *smg* p21B (4 mg of protein per ml) plus *smg* GDS (8 mg of protein per ml) (E), or Ki-*ras*$^{Val-12}$ p21 plus the GTPγS-bound form of *smg* p21B (4 mg of each protein per ml) (F) and incubated in the presence of 1.7×10^{-7} M insulin. The results shown are representative of three independent experiments.

ruffling of Swiss 3T3 cells, we used scanning electron microscopy and video tape recording of phase-contrast microscopy. By scanning electron microscopy, membrane ruffling was observed when the cells were microinjected with either Ki-*ras*$^{Val-12}$ p21 or the GTPγS-bound form of *smg* p21B and incubated in the presence of insulin (Fig. 4B and C). Membrane ruffling was not observed for the cells microinjected with buffer A containing 60 nM GTPγS (Fig. 4A). Even in the absence of insulin, membrane ruffling was observed upon microinjection of Ki-*ras*$^{Val-12}$ p21 but not of the GTPγS-bound form of *smg* p21B (data not shown). When the cells were microinjected with the GDP-bound form of Ki-*ras* p21 (4 mg of protein per ml), the GDP-bound form of *smg* p21B (4 mg of protein per ml), or *smg* GDS (8 mg of protein per ml), membrane ruffling was not detected in the presence of 1.7×10^{-7} M insulin (data not shown).

By video tape recording of phase-contrast microscopy, membrane ruffling was observed in the presence of 1.7×10^{-7} M insulin when Swiss 3T3 cells were microinjected with Ki-*ras*$^{Val-12}$ p21 (4 mg of protein per ml) or the GTPγS-bound form of *smg* p21B (4 mg of protein per ml) (data not shown). Even in the absence of insulin, membrane ruffling was observed upon microinjection of Ki-*ras*$^{Val-12}$ p21 but not of the GTPγS-bound form of *smg* p21B (data not shown). When the cells were microinjected with buffer A containing 60 nM GTPγS, the GDP-bound form of Ki-*ras* p21 (4 mg of protein per ml), the GDP-bound form of *smg* p21B (4 mg of protein per ml), or *smg* GDS (8 mg of protein per ml), membrane ruffling was not detected in the presence of 1.7×10^{-7} M insulin (data not shown).

Changes of stress fibers and morphology of Swiss 3T3 cells upon microinjection of small G proteins. To observe both DNA synthesis and stress fibers of Swiss 3T3 cells, we performed double staining of the cells with an anti-BrdU antibody and FITC-labeled phalloidin, respectively, and analyzed the results by confocal laser scanning microscopy. Microinjection of Ki-*ras*$^{Val-12}$ p21 induced DNA synthesis and remarkably decreased stress fibers, whereas microinjection of the GTPγS-bound form of *smg* p21B also induced DNA synthesis but did not remarkably decrease stress fibers (Fig. 5B and C). Microinjection of buffer A containing 60 nM GTPγS neither induced DNA synthesis nor decreased stress fibers (Fig. 5A). The cells microinjected with Ki-*ras*$^{Val-12}$ p21 became round and piled up, but the cells microinjected with the GTPγS-bound form of *smg* p21B or with buffer A containing 60 nM GTPγS did not show these morphological changes (Fig. 5).

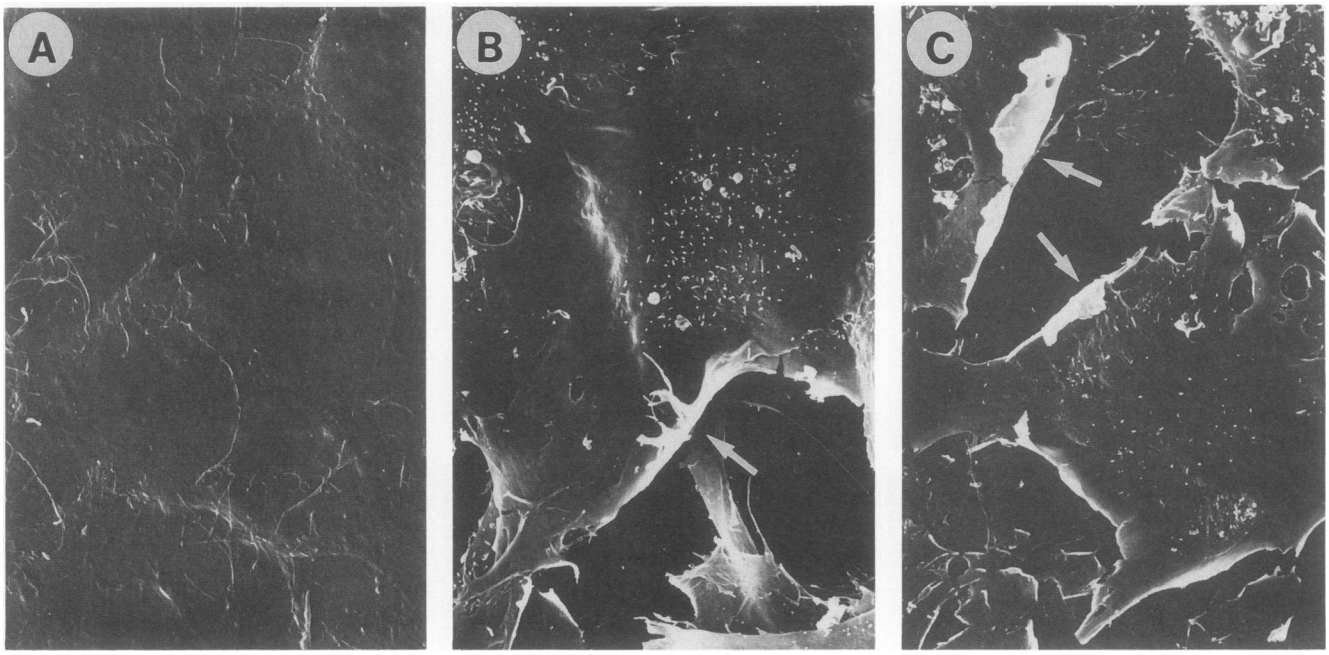

FIG. 4. Scanning electron micrographs of membrane ruffling of Swiss 3T3 cells. Swiss 3T3 cells were microinjected with buffer A containing 60 nM GTPγS (A), Ki-*ras*$^{Val-12}$ p21 (4 mg of protein per ml) (B), or the GTPγS-bound form of *smg* p21B (4 mg of protein per ml) (C) and incubated in the presence of 1.7×10^{-7} M insulin. After the cells were fixed, the specimen was observed by scanning electron microscopy. The arrows indicate membrane ruffling. The results shown are representative of three independent experiments.

DISCUSSION

We have shown that microinjection of Ki-*ras*$^{Val-12}$ p21 into Swiss 3T3 cells induces DNA synthesis and morphological changes. This result is consistent with earlier observations (27). We have moreover shown that comicroinjection of the GDP-bound form of Ki-*ras* p21 and *smg* GDS induces DNA synthesis, that Ki-*ras*$^{Val-12}$ p21 maximally stimulates DNA synthesis in Swiss 3T3 cells, and that *smg* GDS does not further stimulate the Ki-*ras*$^{Val-12}$ p21-induced DNA synthesis. These results provide additional evidence that *smg* GDS converts Ki-*ras* p21 from the GDP-bound inactive form to the GTP-bound active form, which then exerts its actions in

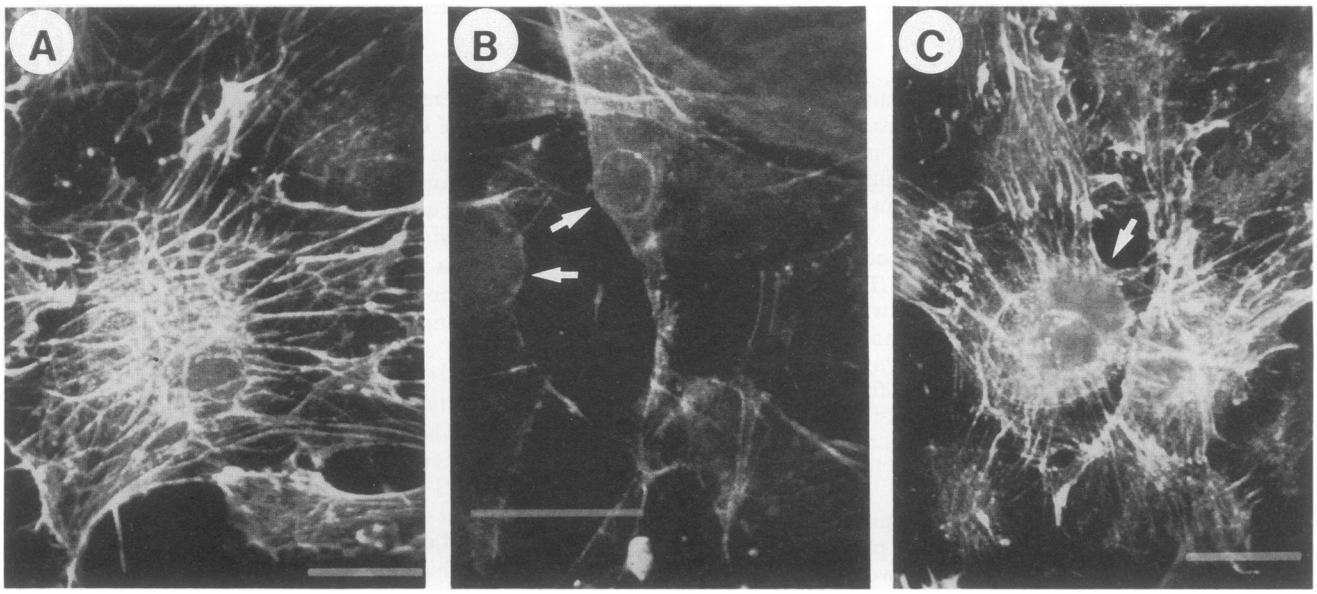

FIG. 5. Confocal laser scanning micrographs of stress fibers and morphology of Swiss 3T3 cells. Swiss 3T3 cells were microinjected with buffer A containing 60 nM GTPγS (A), Ki-*ras*$^{Val-12}$ p21 (4 mg of protein per ml) (B), or the GTPγS-bound form of *smg* p21B (4 mg of protein per ml) (C) and incubated in the presence of 1.7×10^{-7} M insulin. After the cells were fixed and stained, the specimen was analyzed by confocal laser scanning microscopy. Arrows indicate the BrdU-labeled nuclei of the cells. Bars, 25 μm. The results shown are representative of three independent experiments.

intact cells. These facts are consistent with our former observation that cotransfection of Ki-*ras* p21 cDNA and *smg* GDS cDNA into NIH 3T3 cells induces cell proliferation and transformation to the same extent as does transfection of Ki-*ras*$^{Val-12}$ p21 cDNA alone and that transfection of *smg* GDS cDNA does not further stimulate these activities of Ki-*ras*$^{Val-12}$ p21 (11).

We have also shown that microinjection of the GTPγS-bound form of *smg* p21B into Swiss 3T3 cells induces DNA synthesis and membrane ruffling. We have moreover shown that comicroinjection of the GDP-bound form of *smg* p21B and *smg* GDS induces DNA synthesis. It is generally believed that the GDP-bound and GTP-bound forms of small G proteins are inactive and active, respectively, but direct evidence for *smg* p21B has not been obtained (41). We have shown that DNA synthesis and membrane ruffling are observed upon microinjection of not the GDP-bound form but the GTPγS-bound form of *smg* p21B. These results indicate that the GTP-bound and GDP-bound forms of *smg* p21B are active and inactive, respectively, and that *smg* GDS converts *smg* p21B from the GDP-bound inactive form to the GTP-bound active form, which then initiates DNA synthesis in intact cells.

In many types of cells, including NIH 3T3 cells and Swiss 3T3 cells, PDGF induces DNA synthesis. PDGF stimulates phospholipase Cγ, which then activates the diacylglycerol-protein kinase C system and inositol trisphosphate-Ca^{2+} system (for reviews, see references 37 and 41). The activity of *ras* p21 is also regulated by PDGF, and *ras* p21 mediates at least a part of the mitogenic actions of PDGF (7, 18, 32, 40). *smg* p21 is phosphorylated by protein kinase A (9, 12, 14, 20, 21, 26), and this phosphorylation makes *smg* p21 sensitive to the action of *smg* GDS and thereby initiates the conversion from the GDP-bound inactive form to the GTP-bound active form (12, 15, 19). It has been reported that Bt_2cAMP and cyclic AMP-elevating agents, such as prostaglandins and cholera toxin, stimulate DNA synthesis in the presence of insulin in Swiss 3T3 cells (for reviews, see references 5 and 38). Therefore, it can be speculated that *smg* p21 mediates at least a part of the mitogenic action of cyclic AMP in Swiss 3T3 cells, but further studies are necessary to establish the physiological function of *smg* p21 in the mitogenic action of cyclic AMP.

We have shown that the active form of *ras* p21 markedly decreases stress fibers and makes the cells round and pile up, whereas the active form of *smg* p21 does not show these effects. The reason for these different effects of *ras* p21 and *smg* p21 on morphology is not known, but it is possible that they share only a part of their multiple effector proteins in Swiss 3T3 cells. Moreover, we have shown that the active form of *ras* p21 induces DNA synthesis or membrane ruffling even in the absence of insulin, whereas the active form of *smg* p21 induces DNA synthesis or membrane ruffling only in the presence of insulin. The reason for these phenomena is not known at present, but two explanations are possible: (i) the active form of *ras* p21 can mediate the actions of both PDGF and insulin, whereas the active form of *smg* p21 cannot mediate the action of insulin, and (ii) the active form of *ras* p21 can produce the insulinlike growth factor that substitutes for insulin, whereas the active form of *smg* p21 is inactive in this capacity. Although the precise modes of action of *ras* p21 and *smg* p21 in Swiss 3T3 cells are unknown, our results indicate that both of these small G proteins are mitogenic in this cell line and that their actions are slightly different.

There are two lines of evidence that *smg* p21 is antimitogenic in NIH 3T3 cells: (i) K*rev*-1 p21 suppresses the transforming activity of v-Ki-*ras* p21 in NIH 3T3 cells (24), and (ii) overexpression of *smg* p21 in NIH 3T3 cells inhibits the *ras* p21-, PDGF-, and 12-*O*-tetradecanoylphorbol-acetate-induced activation of the c-*fos* promoter/enhancer element (39). These results together with those presented above indicate that *smg* p21 acts differently on different cell types, being antimitogenic in NIH 3T3 cells and mitogenic in Swiss 3T3 cells. The mechanisms for these opposite actions of *smg* p21 in the two cell lines are not known, but the effector proteins or their modulatory proteins may be different in the two cell lines. Further studies are essential for our understanding of the physiological function and mode of action of *smg* p21 in various types of cells.

ACKNOWLEDGMENTS

We thank A. Mizoguchi (Department of Anatomy, Kobe University School of Medicine) and S. Uga (Department of Medical Zoology, Kobe University School of Medicine) for preparing the confocal laser scanning micrographs and the scanning electron micrographs, respectively. We are grateful to J. Yamaguchi for skillful secretarial assistance.

This investigation was supported by Grants-in-Aid for Scientific Research and for Cancer Research from the Ministry of Education, Science, and Culture, Japan (1991), by Grants-in-Aid for Abnormalities in Hormone Receptor Mechanisms and for Aging and Health from the Ministry of Health and Welfare, Japan (1991), and by grants from the Human Frontier Science Program (1991), the Yamanouchi Foundation for Research on Metabolic Disease (1991), and the Research Program on Cell Calcium Signal in the Cardiovascular System (1991).

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