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Characterization of a Factor That Stimulates Hydrolysis of GTP Bound to *ras* Gene Product p21 (GTPase-Activating Protein) and Correlation of Its Activity to Cell Density

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The postmicrosomal fraction of the extract from NIH 3T3 and BALB/c 3T3 cells stimulated the hydrolysis of GTP bound to H-*ras* gene product p21 by severalfold. The stimulation was observed with normal p21 but not with p21 with valine as the 12th residue. This specificity is similar to that of GTPase-activating protein (GAP) for N-*ras* p21 described by M. Trahey and F. McCormick (Science 238:542–545, 1987). Consistent with this specificity, analysis of p21-bound nucleotides in living cells revealed that almost all normal p21 bound GDP, whereas oncogenic mutant p21s bound both GTP and GDP. Similar activity was also found in various mouse tissues, with brain tissue showing the highest specific activity. When cell extracts were prepared from cultured cells, there was a linear relationship between GAP activity and cell density. These results suggest the factor is involved in the regulation of cell proliferation.

ras genes are a multigene family which encodes 21,000-dalton proteins termed collectively as p21 (reviewed in references 2 and 28). *ras* genes were first identified as transforming genes of Harvey and Kirsten murine sarcoma viruses. Subsequently, homologous proto-oncogenes have been found in most eucaryotic cells with high homology from yeasts to humans, suggesting that *ras* genes have an important role in the development of the organisms. DNA-mediated gene transfer techniques revealed that the *ras* genes are activated by point mutations (6, 30, 34). Activated *ras* genes have been detected in numerous human and experimentally induced animal tumors. Involvement of *ras* gene function in cell proliferation and differentiation was directly shown by experiments employing microinjection of p21 or its antibody into cultured cells or *Xenopus* oocytes (3, 4, 9, 14, 24).

Biochemical properties of p21 that may be relevant to its normal functions and transforming activities have been described (12, 16, 22, 23, 29). The p21 of all *ras* genes binds GTP and GDP with high affinity and displays a low GTPase activity which is further reduced by point mutations. Similarities of p21 to GTP-binding proteins of the adenylate cyclase system in biochemical properties and cellular localization (13) have led to the assumption that p21 may also control the cellular signal transduction through GTP-GDP conversion. Trahey and McCormick (31) and Satoh et al. (25) recently demonstrated that only the GTP form of p21 is functionally active when microinjected into living cells.

The cellular factors that directly interact with p21 have never been identified, and the mode of action of p21 remains to be clarified. Trahey and McCormick (31) reported that a cytoplasmic factor in *Xenopus* oocytes stimulates the GTPase of normal N-*ras* p21 but not that of activated p21s. This factor (GTPase-activating protein [GAP] [31]) may accelerate the rate of GTPase of p21 in vivo and may have an important role in cellular signal transduction through controlling the ratio of p21-GTP and p21-GDP. Therefore, we examined whether there is a similar factor which may affect GTPase of p21 of c-H-*ras*. Here we report the presence of GAP activity which stimulated hydrolysis of GTP bound to c-H-*ras* p21 in cultured cells and various mouse tissues and further demonstrated that GAP activity is closely correlated with cell density.

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MATERIALS AND METHODS

Cells and culture conditions. BALB/c 3T3 cells (clone A31-1-1; T. Kakunaga [18]) were obtained from Japanese Cancer Research Resources Bank and were grown in Eagle minimum essential medium containing 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.). NIH 3T3, RS-485 (8), EJ (32), and Ha-821 (27) cells (generous gifts of T. Y. Shih, National Cancer Institute-Frederick, Frederick, Md.) were cultured in Dulbecco modified Eagle medium containing 10% calf serum (GIBCO). Cell growth was monitored by counting the cell number with a hemocytometer.

Preparation of p21. *Escherichia coli* YN2261 cells carrying the *ras* gene expression plasmid consisted of pJL6 (20) and the c-H-*ras* gene or EJ *ras* gene were the generous gifts of Y. Kaziro at Tokyo University, Tokyo (26). p21s were overexpressed and purified as described previously (16).

Preparation of cell extracts. Packed cultured cells were suspended in 3 volumes of a solution containing 10 mM Tris hydrochloride (pH 7.5), 10 mM NaCl, 10 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg of antipain per ml, and 1 μg of leupeptin per ml and were homogenized manually with a Teflon homogenizer. The homogenate was centrifuged at 5,000 × *g* for 5 min and then 100,000 × *g* for 90 min. The postmicrosomal fraction (S-100 fraction) was saved, 10% (vol/vol) glycerol was added, and the preparation was stored at −70°C until use.

Various mouse tissues taken from ICR/JCL mice (female, 6 months old) and porcine brain were homogenized in the same buffer by waring blender for 1 min three times at 5-min intervals. The homogenate was centrifuged as described above, and the supernatant was saved.

In the experiments shown in Fig. 5 and 6, cells were disrupted in a hypotonic buffer (10 mM Tris hydrochloride [pH 7.5], 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 μg of antipain per ml, 1 μg of leupeptin per ml) by vortex

mixing for 1 min, and the supernatant after centrifugation at 10,000 × *g* (S-10 fraction) was used.

Protein concentration was determined as described by Bradford (5) with bovine serum albumin as a standard.

Assay for GAP activity. p21 (250 ng) was incubated at 37°C for 2 min with 1.5 μM [α-^{32}P]GTP (750 Ci/mmol; ICN Radiochemicals, Irvine, Calif.) in the presence of 20 mM EDTA to facilitate the guanine nucleotide exchange (15). p21–[α-^{32}P]GTP binary complex (1.4 ng) was incubated with various amounts of cell extracts in 20 μl of a solution containing 50 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM GTP, 5% glycerol, 0.5 mM β-glycerophosphate, 5 μg of antipain per ml, and 5 μg of leupeptin per ml for 10 min at 37°C.

p21 was immunoprecipitated by monoclonal antibodies (Y13-259 or YA6-172, 0.46 μg) (10) with 2 μg of anti-rat immunoglobulin G (Cooper Biomedical, Inc., West Chester, Pa.) and 1 mg of Formalin-fixed *Staphylococcus aureus* cells (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The radioactive nucleotides were liberated from p21 by incubating the immunoprecipitates in 10 μl of 2% sodium dodecyl sulfate–20 mM EDTA–10 μM GDP–10 μM GTP at 80°C for 2 min and were analyzed by polyethyleneimine thin-layer chromatography in 0.5 M potassium phosphate buffer (pH 3.5). The plate was subjected to autoradiography.

Labeling of cells with $^{32}P_i$. Logarithmically growing cells in 35-mm dishes (0.8×10^5 to 1.4×10^5 cells per cm^2) were labeled with $^{32}P_i$ in 1 ml of phosphate-free Eagle minimum essential medium supplemented with 0.5 mCi of $^{32}P_i$ (Amersham Corp., Arlington Heights, Ill.) and 10% dialyzed fetal calf serum for 16 h. Cells were washed three times with cold Tris-buffered saline and collected with a rubber policeman. Packed cells were lysed with 50 μl of a buffer containing 20 mM Tris hydrochloride (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 2 μg of antipain per ml, 2 μg of leupeptin per ml, and 1% (vol/vol) Triton X-100. Immunoprecipitation of p21 and analysis of p21-bound nucleotides were carried out as described above, except that protein A–Sepharose CL-4B (Pharmacia Fine Chemicals, Piscataway, N.J.) was used instead of *S. aureus* cells and that 2 nmol of GDP was added to the complex of Y13-259, anti-rat immunoglobulin G, and protein A-Sepharose before the addition of the labeled extracts.

RESULTS

Stimulating factor for hydrolysis of GTP bound to c-H-*ras* p21. p21–[α-^{32}P]GTP binary complex was added to the solution containing various amounts of the S-100 fraction from NIH 3T3 cells and an excess of nonradioactive GTP. After incubation at 37°C for 10 min, p21-bound radioactive nucleotides were recovered by immunoprecipitation and were analyzed by polyethyleneimine thin-layer chromatography. The S-100 fraction from NIH 3T3 cells markedly increased the hydrolysis of GTP bound to p21 (Fig. 1A). To obtain quantitative data, the GTP and GDP spots were excised, and the radioactivity was measured with a liquid scintillation counter (Fig. 1B). In the presence of the S-100 fraction, GTPase activity was increased by about fourfold after 10 min of incubation. The sum of the radioactivities of [α-^{32}P]GTP and [α-^{32}P]GDP did not change as a result of additing the extract, which indicated that neither the degradation of p21 nor the nucleotide exchange reaction between p21-bound and exogenous GTP occurred during the incubation. About 20% of the total GAP activity was recovered in the particulate fraction (P-100) (data not shown). Whether these two activities are the same remains to be clarified.

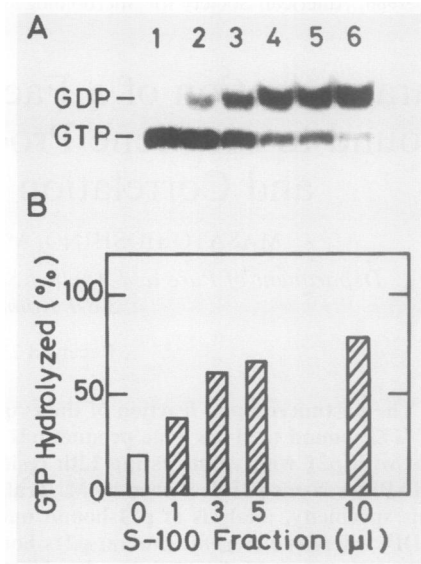

FIG. 1. Stimulation of GTPase activity of p21 by the S-100 fraction of a lysate from NIH 3T3 cells. (A) p21–[α-^{32}P]GTP (1.4 ng) was incubated with various amounts of the S-100 fraction of a lysate from NIH 3T3 cells for 10 min at 37°C. p21 was recovered by immunoprecipitation, and the p21-bound nucleotides were analyzed as described in Materials and Methods. Lanes: 1, no incubation at 37°C without the cell extract; 2 through 6, incubation at 37°C for 10 min with 0, 1, 3, 5, and 10 μl of the S-100 fraction (5.5 mg of protein per ml), respectively. (B) The GTP and GDP spots were cut out, and the radioactivity was measured with a liquid scintillation counter. The percentage of GTP hydrolyzed was determined.

Figure 2 shows the time course of net GTP hydrolysis by p21s encoded by normal and EJ *ras* genes. The rate of GTP hydrolysis by normal p21 was again markedly increased by the addition of the S-100 fraction. More than 70% of GTP was hydrolyzed during 10 min of incubation at 37°C. p21 with valine as the 12th residue instead of glycine did exhibit a very low but definite GTPase activity. However, this low GTPase activity was not stimulated by the S-100 fraction. These results indicate that the stimulation is specific to normal p21. This specificity is the same as that described by Trahey and McCormick for N-*ras* p21 (31). The effect of monoclonal antibodies against p21 on the interaction between p21 and GAP was studied to examine whether GAP

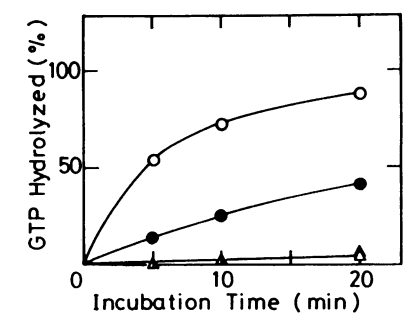

FIG. 2. Effect of the S-100 fraction from NIH 3T3 cells on the rate of GTP hydrolysis by normal and activated p21. The time courses of GTP hydrolysis by normal (○, ●) and activated (△, ▲) p21s were followed as described in the legend to Fig. 1 in the presence (○, △) or absence (●, ▲) of 55 μg of the S-100 protein. GTP hydrolyzed at time zero (normal p21 [10%], activated p21 [4%]) was subtracted from each value.

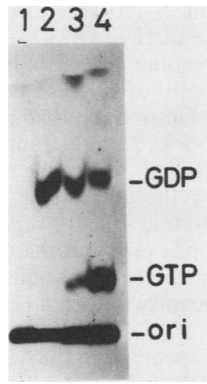

FIG. 3. Analysis of guanine nucleotides associated with p21 in vivo. Cells grown in 35-mm dishes were cultured in phosphate-free minimum essential medium supplemented with 500 μCi of $^{32}P_i$ per ml and 10% dialyzed fetal calf serum for 16 h. After lysis of the cells, p21 was immunoprecipitated with Y13-259 antibody, and the p21-bound nucleotides were analyzed as described in Materials and Methods. Lanes: 1 and 2, RS-485 cells; 3, EJ cells; 4, Ha-821 cells. For the negative control, recombinant p21 (5 μg) was preincubated with Y13-259 before the addition of labeled extracts (lane 1). The spots above the GDP spots seen in lanes 3 and 4 are not reproducible.

directly interacted with p21. YA6-172 and Y13-238 (10) did not inhibit the GTPase activity of p21 but completely abolished the stimulation of GTPase activity by the cell extract (data not shown). In contrast, Y13-259 (10) decreased the basal GTPase activity, but the residual activity was still definitely stimulated by the cell extract. Neither the various phosphatase inhibitors nor phosphatase from calf intestine affected the stimulation of p21 GTPase by the extract, suggesting that it is unlikely that a phosphatase activity is involved in the stimulation of hydrolysis of p21-bound GTP.

Analysis of p21-bound nucleotides in vivo. Cells were labeled with $^{32}P_i$ and p21-bound nucleotides were recovered by immunoprecipitation. Radioactive nucleotides were analyzed as in Fig. 1. RS-485 (8), EJ (32), and Ha-821 (27) cells are NIH 3T3 cells transformed by normal c-H-*ras* genes linked to a viral promoter, human EJ DNA, and Ha-MuSV, respectively. Normal p21 formed complexes exclusively with GDP (Fig. 3, lane 2). In contrast, p21 with valine as the 12th residue bound both GDP and GTP (lane 3), and viral p21 bound GTP as a major nucleotide (lane 4). This observation was consistent with the results described above that the stimulation of GTPase was specific to normal p21. When total nucleotides extracted from the labeled cells were analyzed by Polyanion-SI FPLC (Pharmacia) with a linear gradient from 10 to 850 mM potassium phosphate buffer (pH 7.0), the differences in the specific activity of GTP (counts per minute per unit of A_{254}) of three cell lines were less than 5%. Although the amount of GDP was too little to draw a solid conclusion on its specific activity, the ATP/ADP ratio and the specific activities of these nucleotides did not differ significantly among the cell lines, suggesting that the specific activity of GDP of these cell lines may also be similar (data not shown).

Tissue specificity of GAP activity. Cell extracts were prepared from various mouse tissues including brain, lung, spleen, kidney, heart, and liver, and GAP activity was measured (Fig. 4). Among six tissues tested, brain showed the highest specific activity.

Because the GTPase reaction measured in the experiments described above is a single turnover reaction, the hydrolysis of GTPase could not exceed 100%. This is why the stimulation of GTPase was not linearly proportional to the amount of S-100 fraction added in the present assay conditions. Furthermore, GAP activity from cultured cells was affected by culture conditions as described below. Therefore, we chose S-100 fraction from porcine brain as a standard material, and the standard curve was obtained by an experiment similar to that shown in Fig. 1. With this standard curve, GAP activity of a given cell extract was expressed in terms of the amount of S-100 protein from porcine brain that gave an equivalent stimulation in the GTP hydrolysis. One arbitrary unit of GAP activity was defined as the activity that was observed with 1 μg of S-100 protein from porcine brain. This arbitrary unit is used in the experiments shown in Fig. 5 and 6.

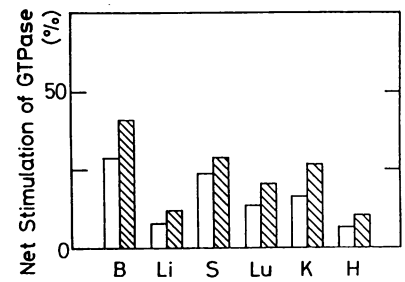

FIG. 4. Tissue distribution of GAP activity. The S-100 fraction was prepared from various mouse tissues including brain (B), liver (Li), spleen (S), lung (Lu), kidney (K), and heart (H), and GAP activity was measured with 30 μg (□) and 60 μg (▨) of the S-100 fraction. The net stimulation was calculated by subtracting the basal GTP hydrolysis observed by the sample without the S-100 fraction (35.2%).

Correlation between GAP activity and cell density. It was of interest to examine a correlation between GAP activity and cell growth. BALB/c 3T3 cells were seeded at a density of 0.25×10^5 cells per cm^2, and the cell growth was followed. The medium was changed daily to minimize the possible effects of the difference in nutritional conditions or soluble growth inhibitor. Figure 5 shows typical results, and similar results were obtained in three independent experiments. Logarithmic growth with a mean doubling time of 24.3 h was observed until the cells reached confluence (2×10^5 to 3×10^5 cells per cm^2, Fig. 5A). Cell extracts were prepared from these cells, and GAP activity was measured. The specific activity of GAP (units [described above] per microgram of protein) was proportional to the cell density, and the activity was maximum when the cells ceased to grow (Fig. 5B). When the concentration of fetal calf serum was reduced to 0.5% at 24 h after seeding, cells stopped growing, but GAP activity remained at a low level. Thus, the GAP activity of cell extracts from serum starved cells and restimulated cells fell on the same straight line (Fig. 5B), suggesting that the specific activity of GAP may not be affected significantly by serum concentration. When the confluent culture was treated for 24 h with spent medium, which was prepared by exposing the fresh medium to another confluent culture for 3 days, the specific activity did not change significantly (data not shown). A similar relation between the cell density and GAP activity was also observed with rat 3Y1 cells (a generous gift of Y. Kaziro).

To ensure the relation between GAP activity and cell density, confluent cells were seeded at various densities and harvested after 24 h, and then GAP activity was determined

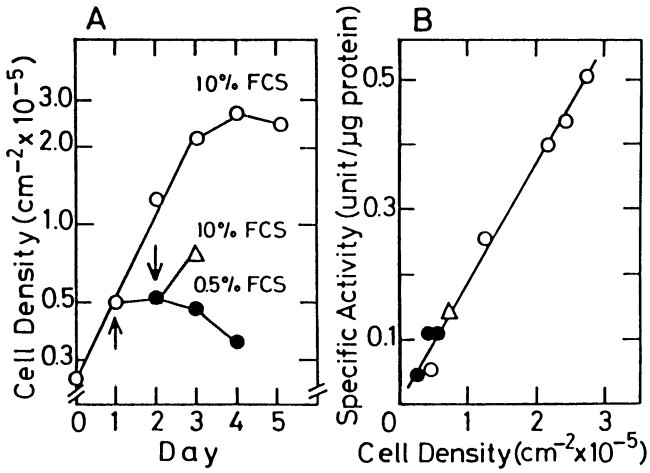

FIG. 5. Relationship between GAP activity and cell growth. (A) Growth curve. BALB/c 3T3 cells were seeded on day 0 at a density of 0.25 × 10^5 cells per cm^2 and were cultured in Eagle minimum essential medium supplemented with 10% fetal calf serum (○). The other cultures were starved by decreasing the serum concentration to 0.5% at 24 h after seeding (●) or stimulated by the addition of serum to 10% at 24 h after serum starvation (△). Cell growth was monitored by counting the cell number by hemocytometer. (B) GAP activity was determined as in Fig. 1, and units were determined by using the standard curve with S-100 fraction from porcine brain (see text). The specific activity (units per microgram of protein of the cell extract) was calculated and plotted versus the cell density.

(Fig. 6). Under these conditions, the specific activity of GAP activity was again proportional to cell density. Furthermore, in another experiment, the specific activity decreased rapidly within 2 h when confluent cells were dispersed by trypsinization (data not shown). These results strongly suggest that GAP activity correlates with cell density.

DISCUSSION

There are several lines of evidence that the *ras* gene product p21 is involved in the regulation of cell proliferation and differentiation (3, 4, 9, 14, 24), and that only the GTP-bound p21 is functionally active (25, 31). Therefore, it is conceivable that GAP activity may be involved in the signal transduction pathway through regulating the ratio between p21-GTP and p21-GDP complexes.

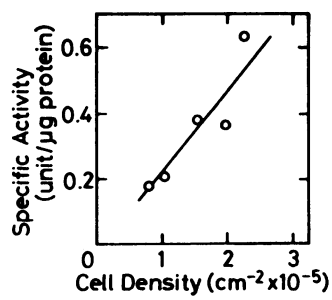

FIG. 6. Effect of cell density on GAP activity. BALB/c 3T3 cells were seeded at various densities (0.5 × 10^5 to 1.5 × 10^5 cells per cm^2) in 35-mm culture dishes and incubated for 24 h. Cell numbers were counted, and GAP activity was determined as described in the legend to Fig. 1. The specific activity was calculated as described in the legend to Fig. 5 and plotted against the cell density.

A factor present in S-100 fraction of NIH 3T3 cells stimulated the GTPase of H-*ras* p21 by severalfold (Fig. 1). When an excessive amount of partially purified GAP was added, the initial velocity of GTPase became much greater (data not shown). The factor did not stimulate the GTPase of oncogenic mutant p21 (Fig. 2), indicating that the stimulation of GTPase is not due to a nonspecific effect, such as stabilization of p21 by the cell extract. This specificity to normal p21 is similar to that of GAP reported by Trahey and McCormick on N-*ras* p21 (31), but the identity of these two factors is not clear at present. A similar effect of GAP on H-*ras* p21 was also recently reported (1, 7) after submission of our manuscript.

Consistent with the specificity of GAP, normal p21 almost exclusively bound GDP in living cells, whereas the oncogenic mutant p21s bound both GTP and GDP (Fig. 3). Because the nucleotide exchange reaction with normal p21 is faster than that with EJ p21 (26), this difference in the ratio between GDP and GTP may be attributable to the difference in GTPase activity in vivo. Viral p21 bound GTP as a major nucleotide. This may be due to the facts that viral p21 displays the lowest GTPase activity (16) and that the rate of the nucleotide exchange reaction of viral p21 is much faster than that of c-*ras* p21 (15, 17, 19).

The amount of p21-GTP in RS cells was so low that it has escaped from the present immunochemical detection. RS cells are transformed by overexpression of the normal *ras* gene, and the intracellular level of p21 is much higher than that in normal cells (8). Thus, the ratio of p21-GDP to p21-GTP in RS cells would be extremely high if the amount of p21-GTP in RS cells did not differ significantly from that of untransformed cells. Gibbs et al., using a yeast system, also reported that normal *RAS* 1 and *RAS* 2 product almost exclusively bound GDP in vivo (11).

GAP activity was detected in all tissues examined with different specific activity (Fig. 4), suggesting that GAP may play a significant role in cellular function. Among six tissues tested, brain showed the highest activity, whereas lung, kidney, and spleen were moderate, and liver and heart were the lowest. It is worthwhile to point out that this tissue specificity in specific activity seems to correlate with the extent of *ras* gene expression in various mice tissues except heart (21).

To explore the role of GAP in the regulation of p21 function in vivo, we examined the relationship between GAP activity and cell growth. In BALB/c 3T3 cells, the specific activity of GAP was proportional to the cell density (Fig. 5 and 6). GAP activity is lower in actively growing cells than in density-arrested cells, suggesting that the amount of p21-GTP complex that is functionally active in confluent cells may be less than that in growing cells. Thus, it is an interesting hypothesis that GAP activity might be involved in the cell growth, particularly in the density-dependent growth inhibition of normal cells. In contrast, serum starvation did not affect GAP activity significantly. The difference in the specific activity is not due to the difference in the time of culture, because cells seeded at different densities showed specific activities proportional to the cell densities at the same time after seeding (Fig. 6).

Adari et al. (1) and Calés et al. (7) recently reported that the region recognized by GAP is the site through which p21 interacts with yet unidentified cellular target molecule (33) based on the results employing site-directed mutagenesis. In this regard it is noteworthy that Y13-238, which abolishes the stimulation by GAP (1; this paper), does not suppress p21 function in vivo when microinjected into NIH 3T3 cells

(24). To further characterize the properties of GAP and to demonstrate directly the involvement of the factor in the density-dependent cell growth, it is necessary to purify the factor and to prepare antibodies against it. It would be interesting to examine an effect of microinjection of the factor itself or its antibodies into living cells. It would also be interesting to study how GAP activity is regulated by the cell density.

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