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Differential contribution of M_r 120 kDa rasGTPase-activating protein and neurofibromatosis type 1 gene product during the transition from growth phase to arrested state in human fibroblasts accompanied by a unique rasGTPase-activating activity†

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Using octyl glucoside-solubilized cell extracts from human fibroblasts during growth phase to G_0/G_1 arrest state, we found that while the number of M_r 120 kDa rasGTPase-activating protein (p120GAP) molecules per cell decreases to half its original levels, the amount of neurofibromatosis type 1 gene product (NF1, neurofibromin) remains constant during the transition. The contribution of p120GAP to the total rasGTPase-activating (rasGA) activity in growing cells was found to be larger than that observed in arrested cells (84% vs 53%). On the other hand, NF1 contributes less than 15% of the total rasGA activity in either extract. These results indicate that the qualitative changes occur in the contributors to rasGA activity during transition. They also suggest that a unique rasGA activity exists in the arrested cells, which was obtained separatedly from both p120GAP and NF1 by heparin–Sepharose column chromatography.

Ras; GTPase-activating protein; Neurofibromatosis type 1 gene product; Cell growth arrest; Human fibroblast

1. INTRODUCTION

In the last decade, studies on signal transduction systems have revealed the importance of regulatory reactions between receptors and effectors , or so-called coupling reactions, including tyrosine phosphorylation [1]. The GTPase-activating proteins of *ras* oncogene product p21 (rasGAPs) have been considered to be crucial elements in such systems for tyrosine kinase-mediated pathways. $p21^{ras}$, which binds GDP/GTP and has an intrinsic GTPase activity, is localized on the inner surface of the plasma membrane [2,3] and the amount of its GTP binding form increases upon stimulation of cells with growth factors, such as epidermal growth factor and remains at high levels in v-*src*-transformed cells [4]. At present, two different kinds of rasGAPs are known, M_r 120 kDa rasGAP (p120GAP) [5] and neurofibromatosis type 1 gene product (NF1) [6,7]. Although these two rasGAPs play important roles in the negative regulation of cell growth [8–11], several lines of evidence indicate that they play different roles in the regulation of $p21^{ras}$. For example, only p120GAP has *src* homology 2 (SH2) regions through which it can associate with tyrosine phosphorylated receptors, such as those for epidermal growth factor upon the ligand stimulation [12]. In this way, p120GAP forms oligomeric complexes with M_r 62 kDa [13] and M_r 190 kDa [14] cellular proteins resulting in a reduction in rasGA activity [15].

It is important to clarify the functional difference between p120GAP and NF1. On this point, it is noteworthy that rasGA activity is reported to increase in proportion to the cell density of BALB/c3T3 and NIH3T3 cells [16,17], a finding that might be related to contact inhibition of cell growth. These observations prompted us to investigate quantitative and qualitative changes in rasGA activity at the molecular level, focussing especially on the contribution of p120GAP and NF1 during the transition from growth phase to arrested state. In this report, we describe differential changes in p120GAP and NF1 during this process. We also suggest that a unique rasGA activity, which may be due to a new rasGAP(s) other than those attributable to p120GAP and NF1, increases in the arrested state.

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Abbreviations: GAP, GTPase-activating protein; p120GAP, GAP molecule with M_r 120 kDa; NF1, neurofibromatosis type 1 gene product; NF1-GRD, NF1 GAP-related domain; DTT, dithiothreitol; TBST, Tris-buffered saline containing Tween 20.

2. MATERIALS AND METHODS

2.1. *Cell culture*

Human fibroblasts TIG-3 [18] were obtained from the Japanese Cancer Research Resources Bank and cultured in minimum essential medium supplemented with 10% fetal calf serum (HyClone) under 5% CO_2

2.2. *Preparation of cell extracts and determination of cell number*

The cells were solubilized by sonication in extraction buffer (10 mM HEPES-NaOH, pH 7.4, 15 mM KCl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 100 μM Na_3VO_4, 1 μM Okadaic acid, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml antipain, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 10 μg/ml E-64) containing 1% (w/v) octyl glucoside to a final protein concentration of about 10 mg/ml (60–100 μl/dish). An aliquot was taken for the measurement of cell number by evaluation of P_i derived from phospholipid [19]. The homogenates were then centrifuged at $10,000 \times g$ for 15 min and glycerol was added to the resultant supernatants at a final concentration of 10%. For heparin-column chromatography, the cells were prepared by the method as described above without octyl glucoside (S-10 fraction). The protein concentrations were measured using a protein assay kit (Bio-Rad or Pierce).

2.3. *Determination of rasGA activity*

c-Ha-*ras* protein was obtained as described previously [20]. GTP hydrolytic activity was determined in duplicate or triplicate by the cellulose membrane binding assay essentially as described previously [21].

2.4. *Measurement of the amounts of p120GAP and NF1*

The cell extracts were resolved by SDS-PAGE (6% for analysis of both p120GAP and NF1, or 7.5% for p120GAP) and transferred to polyvinylidene difluoride membranes (Bio-Rad) in the presence of 0.1% SDS In 6% SDS-PAGE, the filter was cut into two pieces at the boundary of M_r 150 kDa and both were processed for immunoblotting, the upper for the detection of NF1 and the lower for p120GAP. The filter was blocked with 10% skimmed milk in 10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.1% Tween 20 (TBST) for 2 h at room temperature, and then incubated with mono-specific anti-p120GAP peptide antibody (6 μg/ml) or anti-NF1-GRD [22] antiserum (100-fold dilution) in TBST containing 0.1% skimmed milk for 2 h. The filters were exposed to [^{125}I]Protein A (0.5 μCi/ml, Amersham) in TBST containing 0.05% skimmed milk for 1 h. The amounts of p120GAP or NF1 were assessed by measuring image density using a Fuji Image Analyzer (BAS2000).

2.5. *Preparation of antisera*

Antibody against p120GAP peptide was prepared in rabbits by injecting synthesized cysteinyl 16-mer peptide(975–990th of human p120GAP) [23] according to conventional methods. Anti-whole p120GAP antiserum was produced in mice by injecting recombinant rat p120GAP expressed in *E. coli* after purification by a combination of DEAE-ion exchange and heparin–acrylic beads column chromatographies (M.K. and S.I., unpublished data). Rabbit anti-NF1-GRD antiserum was prepared as described previously [24].

2.6. *Removal of p120GAP from cell extracts*

Cell extracts were prepared from 2-day and 9-day cultures and each extract (200 μl, about 1–2 mg) was treated with a pre-formed complex of *Staphyrococcus aureus*–goat anti-mouse IgG and either preimmune serum or anti-p120GAP antiserum. After 60 min incubation at 4°C, the supernatants and pellets were separated by centrifugation at $10,000 \times g$ for 5 min. To evaluate immunoprecipitation efficiency, the amounts of p120GAP in both the supernatants and pellets were assessed by immunoblotting with anti-p120GAP peptide antibody as described above.

2.7. *Estimation of the contribution of NF1 to the total rasGA activity by inhibition with anti-NF1-GRD antiserum*

The rasGA activities of recombinant NF1-GRD (0.48 ng), rat brain P-100 (32.4 μg), the rat brain NF1 fraction separated by Superose 6 gel filtration (24.0 μg), and p120GAP-depleted cell extracts prepared as described above (78.2 μg of 2-day cultures and 64.7 μg of 9-day cultures, respectively) were assayed in the presence of various amounts of anti-NF1-GRD antiserum supplemented with normal serum so that the total amount of serum in the reaction mixtures remained constant.

2.8. *Preparation of rat brain S-100 and P-100*

Wistar rat brains were homogenized with a Polytron homogenizer in homogenizing buffer (10 mM HEPES-NaOH (pH 7.4), 15 mM KCl, 1 mM DTT, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml of following protease inhibitors: leupeptin, antipain, E-64 and pestatin A). S-100 and P-100 were obtained by the conventional methods. P-100 fractions were prepared by resolving the pellets in homogenizing buffer containing 1% (w/v) octyl glucoside. Glycerol was added to both S-100 and P-100 fractions at a final concentration of 10%.

2.9. *Heparin–Sepharose column chromatography*

Rat brain S-100 (27 mg protein in 0.8 ml) or S-10 fractions of cells prepared as described above (6.6 mg protein in 1 ml) were applied to HiTrap Heparin (Pharmacia, 1 ml) equilibrated with buffer A (10 mM HEPES-NaOH (pH 7.4), 1 mM EGTA, 1 mM $MgCl_2$, 1 mM DTT, 10% glycerol, and protease inhibitors used in the preparation of brain extracts). Elution was achieved with a 40-ml (for rat brain) or 20-ml (for cells) linear NaCl gradient (0–1.0 M) in buffer A. Fractions (0.5 ml for rat brain or 0.25 ml for cells) were collected the rasGA activity (5 μl aliquots) was determined as described above. NaCl concentration was determined by titration of Cl^- with $AgNO_3$.

3. RESULTS

3.1. *Decrease in the amount of p120GAP but not NF1 during the transition from growth phase to arrested state*

In order to obtain quiescent cells, human fibroblasts (2.5×10^6 cells per 100-mm dish) were cultured without medium change. Under the culture conditions, the cells stopped growing around day 6 (data not shown). These cells were homogenized in octyl glucoside-containing buffer and the amounts of p120GAP and NF1 were measured by immunoblotting methods. A comparison of the distributions between the soluble and insoluble fractions shows that all of the p120GAP (at least 94%), as well as most of the NF1 (at least 83%), could be well solubilized (Fig. 1a), implying that the vast majority of both p120GAP and NF1 in the cells is taken into account for each experiment.

It is noteworthy that while the total rasGA activity decreases slightly during the transition from growth phase to arrested state (Fig. 1b), the amounts of p120GAP and NF1 change differently ; the amount of p120GAP decreases gradually to about 50% of that in the growing state, while NF1 protein levels remain constant (Fig. 1a, lanes 1 and 2 vs. 3 and 4, and Fig. 1b). Although another protein with M_r 78 kDa, which localizes preferentially in the detergent-insoluble fraction (Fig. 1a, lanes 5–8), crossreacts specifically with the anti-p120GAP peptide antibody, it hardly crossreacts with antiserum against recombinant p120GAP.

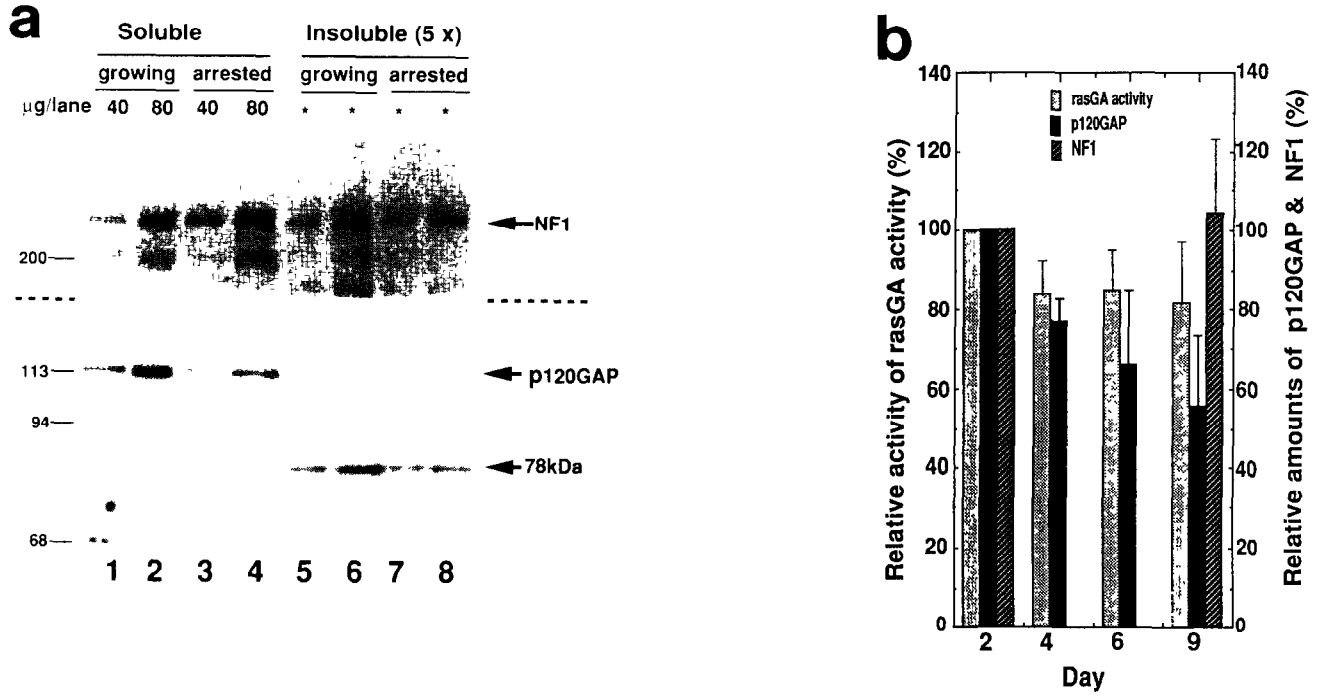

Fig. 1. Differential changes in the amounts of p120GAP and NF1 during the transition from growing phase to arrested state in human fibroblasts. Cells were cultured for 2, 4, 6, or 9 days and solubilized. 40 or 80 μg of protein in the soluble fraction (lanes 1–4) and constant volumes of insoluble fraction from the pellets solubilized with SDS (lanes 5–8) were resolved by 6% SDS-PAGE. The amounts of insoluble fraction applied were equivalent to five-times the amounts of the corresponding soluble fraction compared at the level of the volume of the original cell homogenates (indicated as 5×). Only the data from 2-day and 9-day cell cultures are shown in panel a. (b) The amounts of p120GAP (■), NF1 (▨) and rasGA activity (□) per cell were assessed as described in section 2 and are expressed relative to those in the 2-day cultures (100%). Results are means ± S.E.M. of at least four independent experiments (n = 4–11).

3.2. *Removal of p120GAP from cell extracts by immunoprecipitation and the contribution of p120GAP to the total rasGA activity*

Next we evaluated the contribution of p120GAP to the total rasGA activity by measuring the residual rasGA activity in the supernatants after immunoprecipitating p120GAP molecules from the cell extracts with anti-p120GAP antibody. This antibody efficiently immunoprecipitates more than 75% of the p120GAP molecules in each cell extract (Fig. 2a). As shown in Fig. 2b, whereas more than two-thirds of the total rasGA activity was removed from growing cell extracts, less than half of the activity was removed from extracts of arrested cells. These activities correspond to 84% (± 13%, n = 3) and 53% (± 9%, n = 3) of the total rasGA activity, respectively, when corrected for immunoprecipitation efficiency.

3.3. *Contribution of NF1 to the total rasGA activity in cell extracts*

In addition to the difficulty in evaluating exactly the immunoprecipitation efficiency by immunoblotting due to the large size of the NF1 molecule, the antiserum against NF1-GRD did not efficiently immunoprecipitate NF1 in cell extracts. Therefore, we estimated the contribution of NF1 to the total rasGA activity by measuring the decrease in the rasGA activity of p120GAP-depleted cell extracts in the presence of anti-NF1-GRD antiserum. This antiserum completely inhibits the rasGA activity of recombinant NF1-GRD and NF1 partially purified from rat brain S-100 by Superose 6 (Fig. 3). In the case of rat brain P-100 fractions, a rich source of NF1[30], the rasGA activity is inhibited by up to 40%; thus the contribution of NF1 to the total rasGA activity can be estimated as 40%. Under these conditions, a significant inhibition in the rasGA activity of cell extracts from either growing phase- or arrested state cells could not be detected (data not shown). Therefore, we used cell extracts from which the p120GAP molecules had been mostly removed by immunoprecipitation, despite some p120GAP contamination. As shown in Fig. 3, about a quarter of the rasGA activity was inhibited in the presence of anti-NF1-GRD antiserum in both p120GAP-depleted growing and arrested cell extracts. These inhibition values correspond to 5–15% of each total rasGA activity, although these values are almost within the range of experimental error. Taken together with the results on the contribution of p120GAP, the rasGA activity of growing cell extracts consists entirely of p120GAP and NF1. In contrast, arrested cells may contain a unique rasGA activity (designated as X) attributable to a species other than

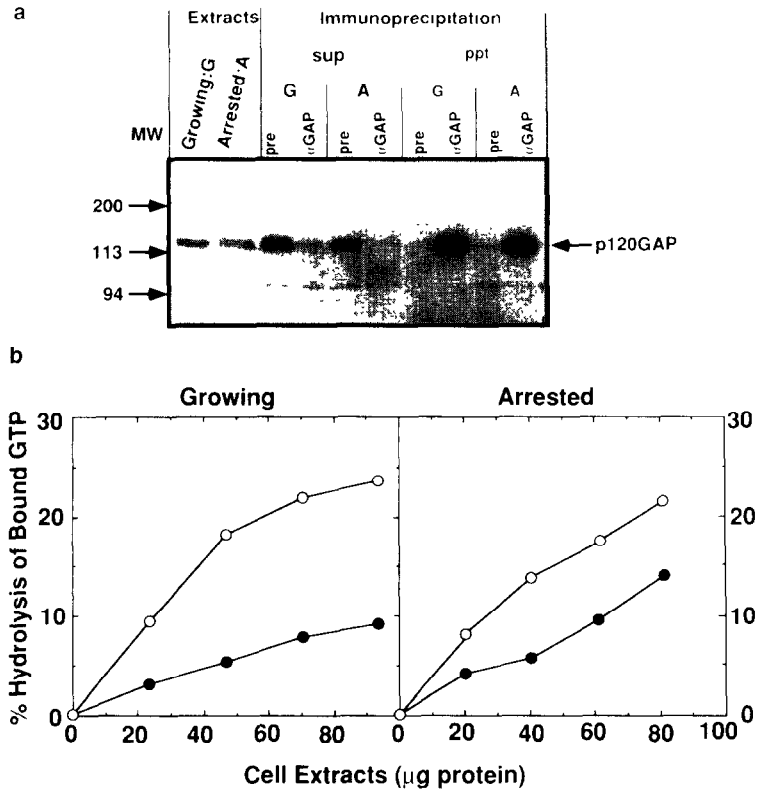

Fig. 2. Removal of p120GAP from cell extracts by immunoprecipitation and the contribution of p120GAP to the total rasGA activity Cell extracts were prepared from 2-day and 9-day cultures and p120GAP molecules were removed from each cell extract. The amounts of p120GAP in both the supernatants and immunoprecipitates were assessed as shown in panel a The rasGA activity of each supernatant after treatment with preimmune serum (○) or anti-p120GAP antiserum (●) was measured using various amounts of cell extracts as shown in panel b.

p120GAP and NF1, because the sum contribution of NF1 and p120GAP to the total rasGA activity does not exceed at most 80%.

3.4. *Separation of X(s), which also exists in rat brain S-100 and S-10 fractions of TIG-3 cells in the arrested state, by column chromatography*

We found that rat brain S-100 also contains a rasGA activity other than those attributable to p120GAP and NF1 which accounts for half the total rasGA activity ([24], M.K. and S.I. unpublished data). Therefore we applied S-100 to a heparin–Sepharose column in order to separate this unique rasGA activity. As shown in Fig. 4a–c, a significant rasGA activity (nearly 32% of the total rasGA activity) elutes between 0.5–0.73 M NaCl with no detectable amounts of either p120GAP or NF1, which elute at 0.4 M NaCl. Essentially the same profile of rasGA activity was observed when S-10 fractions of cells in the arrested state were applied to the same column (Fig. 4d–f). These data strongly suggests the existence of a new rasGAP molecule(s) other than p120GAP and NF1.

4. DISCUSSION

In this paper we have described changes in the com-

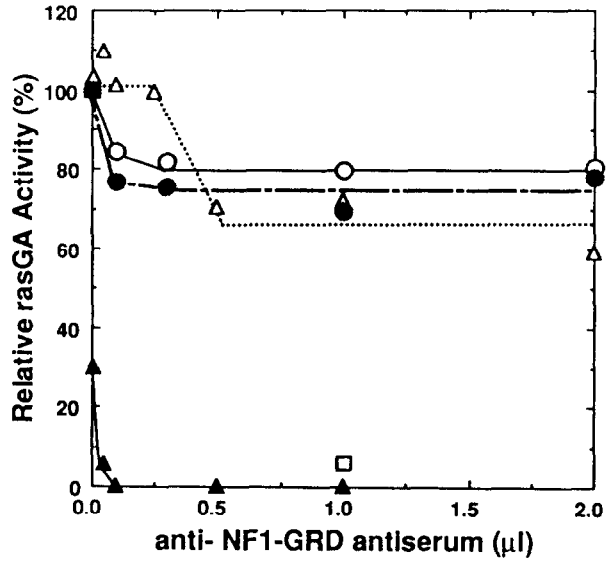

Fig 3. Estimation of the contribution of NF1 to the total rasGA activity by inhibition with anti-NF1-GRD antiserum. The rasGA activities from different sources were measured in the presence of various amounts of anti-NF1-GRD antiserum as described in section 2. The activities are expressed relative to the activity found in the presence of preimmune serum (100%) instead of anti-NF1-GRD antiserum. Recombinant NF1-GRD (▲), rat brain NF1 fraction without p120GAP (□), rat brain P-100 (△) and p120GAP-depleted cell extracts prepared from 2-day cultures (○) or 9-day cultures (●).

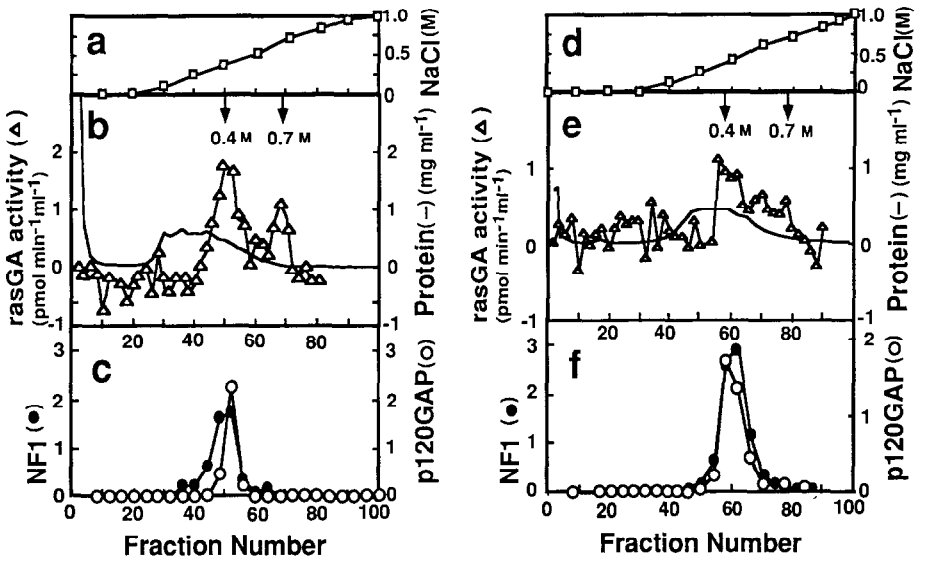

Fig. 4. Detection of a unique rasGA activity other than those of p120GAP or NF1 in the fractions from heparin–Sepharose column chromatography. Rat brain S-100 or S-10 fractions from arrest state cells were separated and both the rasGA activity and the amounts of p120GAP and NF1 were determined as described in section 2. The left part of the figure (a–c) shows the data for rat brain and the right (d–f) for TIG-3 cells: Cl^- concentrations in panels a and d; rasGA activity and protein concentrations in panels b and e; and the amounts of p120GAP and of NF1 in panels c and f.

position of the rasGA activity during the transition from growth phase to arrested state under conditions where the total activity does not change very much; the amount of p120GAP decreases, while NF1 remains constant. Including the present data, several lines of evidence indicate that p120GAP and NF1 regulate $p21^{ras}$ differently despite their common rasGA activity. In an in vitro study, the biochemical characteristics of p120GAP and NF1, including their enzymatic sensitivities to lipids [25], salts [26], and detergents [27], have been shown to differ, although the truncated NF1-GRD, rather than full-length NF1, was used for comparison. We found that the rasGA activity of NF1-GRD is more sensitive to octyl glucoside than that of p120GAP, and that the final concentration of octyl glucoside in the reaction mixtures should be below 0.1%. This situation makes it difficult to estimate the small contribution of NF1 to the total rasGA activity even after removal of the major contributor, p120GAP. In vivo, the levels of $p21^{ras}$-GTP in tumor cell lines derived from malignant Schwanomas have been reported to be high, probably due to a decrease in NF1 levels, while p120GAP levels are normal [10]. While p120GAP also has potent activity as an effector of $p21^{ras}$ [28–30], such potent effector activity has not been recognized in the function of IRA, a homolog of NF1in *Saccharomyces cerevisiae* [31].

We also suggest the existence of a new rasGAP(s) in both rat brain S-100 and the extracts of TIG-3 cells in the arrested state by demonstrating rasGA activity in another heparin–Sepharose column chromatography fraction (eluted at 0.7 M NaCl) separate from the fraction containing both p120GAP and NF1 (eluted at 0.4 M NaCl). In addition to these data, the possibility that X(s) is an isomer of NF1 (NF1 type II) [32] or a nucleotide exchange factor [33] appears to be excluded (M.K. and S.I., data not shown).

It has been shown that the machinery of growth factor receptor in quiescent cells is negatively regulated in a similar way by increases in phosphotyrosine phosphatase activity [34]. Therefore it is reasonable to speculate that NF1 and an unidentified rasGAP(s) contribute to the total rasGA activity to shut off tyrosine kinase–$p21^{ras}$-mediated pathway and to keep the cells in the G_1/G_0 state.

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