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ANTIBODY AGAINST NEUROFIBROMATOSIS TYPE 1 GENE PRODUCT REACTS WITH A TRITON-INSOLUBLE GTPASE ACTIVATING PROTEIN TOWARD *ras* p21

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SUMMARY: Cellular fractionation of GTPase activating protein (GAP) activity using bovine cerebral cortex revealed that about half of GAP activity was found in membrane fraction. GAP activity of membrane was not solubilized with 0.5% (v/v) triton X-100 and was immunoprecipitated with antibody against carboxy-terminus of neurofibromatosis type 1 (NF1) gene product. In contrast, soluble GAP activity was precipitated with antibody against GAP but not with anti-NF1. These results suggest that NF1 gene product is a GTPase activating protein toward *ras* p21 with completely different intracellular distribution from that of GAP.

Neurofibromatosis type 1 (NF1) is a neurocutaneous disease affecting one in 3500 (1). NF1 gene was cloned as a gene, in which deletions, insertions, or point mutations are frequently observed among NF1 patients (2-4). Structural analysis of NF1 gene revealed that the gene codes for a polypeptide of at least 2485 amino acids, and furthermore that the polypeptide shows a sequence similarity to the catalytic domain of GTPase activating protein (GAP) (5). That part of NF1 gene was shown to have GAP activity when expressed by recombinant DNA technique (6-8). We have studied subcellular localization of GAP activity using bovine brain and found that substantial amount of GAP activity was recovered in membrane fraction. Therefore, antibodies against NF1 and GAP were made and subcellular distribution of NF1 and GAP was studied. The data in this paper suggest that NF1 is a GTPase activating protein toward *ras* p21 present in triton-insoluble fraction.

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Abbreviations: GAP, GTPase activating protein; NF1, neurofibromatosis type 1; NGFR, nerve growth factor receptor; MBS, maleimidobenzoic acid *N*-hydroxysuccinimide ester.

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MATERIALS AND METHODS

Antibodies Peptides corresponding to carboxy-termini of NF1 (2) and GAP (9), (C)GSFKRNSIKKIV and (C)NQYTKTNDVR, respectively, were coupled to bovine serum albumin using maleimidobenzoic acid *N*-hydroxysuccinimide ester (MBS). Rabbits (Japanese White, female) were immunized with the conjugates, then antisera were obtained. Antibodies were affinity purified against their respective peptides coupled to TSK gel AF-Amino Toyopearl (Tosoh) with MBS.

Subcellular fractionation of bovine brain cortex Extract from 20 g of bovine cerebral cortex was fractionated as schematically shown in Fig. 1. The ingredients of a buffer used is as follows; 10 mM Tris-HCl pH 7.5, 10 mM 2-mercaptoethanol, 1 mM EGTA, 1 μg/ml leupeptin, and 1 μg/ml antipain, and appropriate concentration of sucrose is added as shown in the figure.

Measurement of NF1 and GAP activity GAP activity was measured as described previously in the presence of 0.5% (v/v) triton X-100 and a unit of GAP activity was defined as in (10). Activities of NF1 and GAP were separately measured as follows. Extracts of bovine brain (300 to 500 μg) were reacted with 2.5 μg of anti-NF1 or anti-GAP antibody and 10 μl of 20% (v/v) protein A-Sepharose CL-4B (Pharmacia Japan) in the presence of 0.1% (v/v) triton X-100 at 4°C for 2 hr. Immunoprecipitates were washed twice with 0.6 ml of tris-buffered saline containing 0.1% triton X-100 and 5 mg/ml bovine serum albumin, then twice with 0.6 ml of the same buffer without triton X-100 and NaCl. To washed immunoprecipitates was added 80 ng of p21•[γ-^{32}P]GTP (30 Ci/mmol), and the samples were incubated at 37°C for 10 min without triton X-100 as described (10). After incubation, amount of remaining p21•[γ-^{32}P]GTP was determined as described previously (11). Extent of GTP hydrolysis (%) was calculated by dividing a decrease in radioactivity by a value obtained with a sample kept at 0°C. Standard deviations in the determination of p21•[γ-^{32}P]GTP were less than 2 % of input p21•[γ-^{32}P]GTP.

Miscellaneous Activity of lactate dehydrogenase was determined according to ref. 12. Protein concentration was measured by the method of Lowry *et al.* (13) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Subcellular localization of GAP activity was studied using an extract from bovine cerebral cortex as schematically shown in Fig. 1. GAP activity was measured in the presence of 0.5% (v/v) triton X-100 considering the possibility that any GAP activity was trapped in vesicles, and the results were shown in Fig. 1A. More than half of GAP activity was recovered in fractions, P1, P2 and P3. As P2 contained major membrane GAP activity, P2 was further fractionated by sucrose density gradient (Fig. 1B). In this fractionation GAP activity was mainly recovered in band 3 (B3) fraction. To this fraction was added large excess of hypotonic buffer to separate soluble protein trapped in vesicles, then insoluble materials were collected again by centrifugation (B3P). Upon this treatment 33% of GAP activity was recovered in the supernatant (B3S) whereas 67% still remained insoluble (B3P). Activity of lactate dehydrogenase, a marker enzyme for soluble

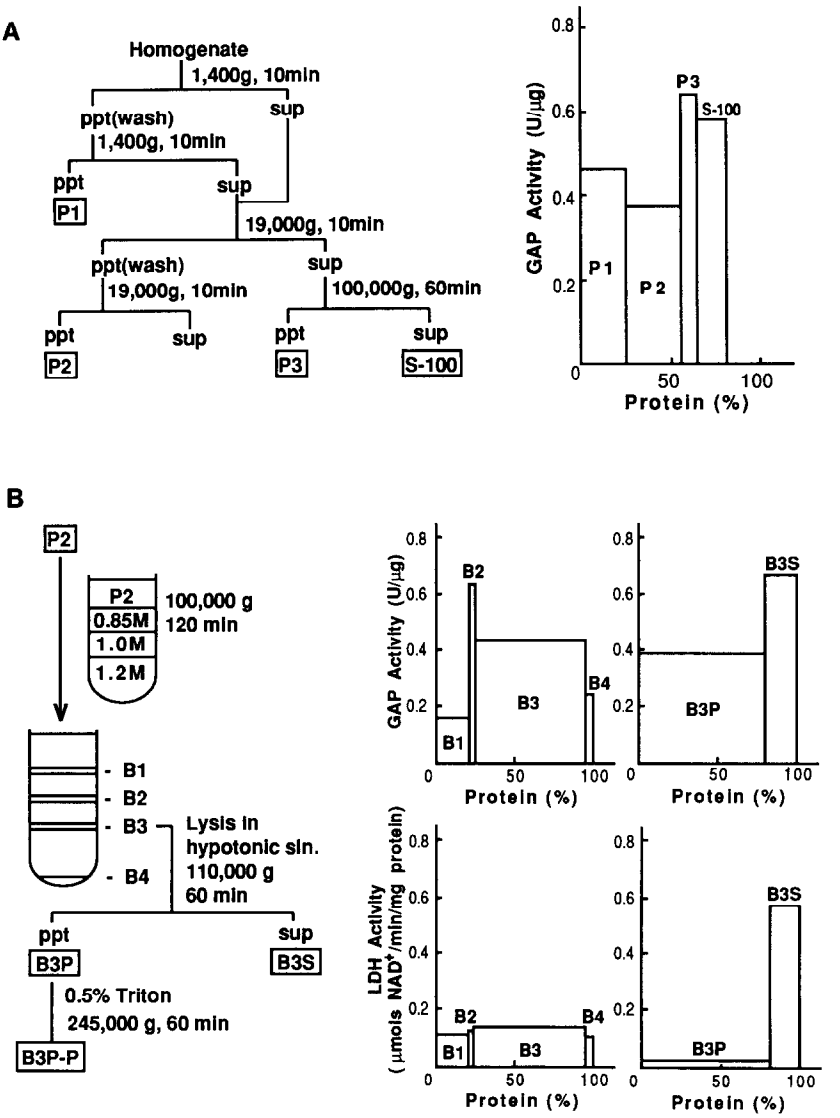

Figure 1. Subcellular fractionation of GAP activity using bovine cerebral cortex.

A. Fractionation by differential centrifugation and distribution of GAP activity.

Extract from 20 g of bovine cerebral cortex was fractionated as schematically shown left in the figure, and recovery of protein and specific activity of GAP activity are presented right. Recovery of GAP activity is therefore expressed as the area.

B. Fractionation of P2 by sucrose density gradient and hypotonic treatment.

P2 fraction (48 ml, 9.4 mg of protein per ml) was layered on 6 tubes of discontinuous sucrose density gradient in Hitachi RPS27-2 rotor tube, and the samples were centrifuged at 100,000 x g for 2 h. B1, B2, B3 observed at interfaces and B4 at the bottom were saved and collected by centrifugation. B3 fraction (4.8 ml, 34 mg protein/ml) was diluted with 78 ml of 5 mM Tris-HCl (pH 7.5) and homogenized, then the sample was centrifuged at 110,000 x g for 60 min. The activities of GAP and lactate dehydrogenase (LDH) in solubilized (B3S) and precipitate (B3P) fractions were measured and expressed as in A.

proteins, was recovered in the supernatant (B3S) and was negligible in B3P fraction, which suggested that GAP activity in B3S may be due to soluble GAP trapped in vesicles (Fig. 1B). B3P was further purified by sucrose density gradient, and then added 0.5% (v/v) triton X-100. The sample was centrifuged at 245,000 x g for 60 min, then GAP activities of supernatant and precipitates (B3P-P) were measured. By this procedure, 75% of GAP activity was recovered in the precipitates with concomitant increase in specific activity of about 2-folds, which indicated that the GAP activity was not solubilized by 0.5% triton X-100.

To see whether this triton-insoluble GAP activity was attributable to GAP, immunoblot analysis using anti-GAP antibody was carried out and the result is shown in Fig. 2A. Extracts with the equal GAP activity were subjected to the analysis. A band corresponding to GAP was clearly observed in soluble fraction, whereas very faint in B3P fraction, suggesting that a molecule with GAP activity other than GAP may exist in the B3P fraction. *ras* gene product p21 was solubilized upon triton X-100 treatment as shown in Fig. 2B (lane 6) which indicates effectiveness of the treatment.

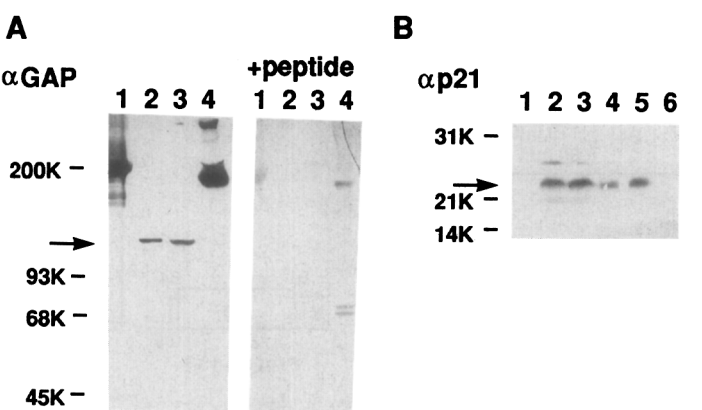

Figure 2. Immunoblot analysis of GAP and p21.
A. GAP immunoblot.
Extracts with the same GAP activity (30 units, a unit of GAP activity was defined as described (10)) were subjected to immunoblot analysis with anti-GAP antibody (left) or with anti-GAP in the presence of GAP peptide (50 μg/ml) (right). Molecular weight of marker proteins in kilodaltons is shown left. Lane 1, 3 μg each of marker proteins (Amersham, Rainbow Marker); lane 2, purified porcine GAP (5 ng); lane 3, bovine GAP partially purified by DEAE Sephacel (10 μg); lane 4, B3P fraction (56 μg). Arrow indicates GAP bands. Cross reaction of the antibody to myosin was observed (lanes 1 and 4).
B. p21 immunoblot.
Extracts with the same GAP activity (30 units) were analyzed as in A with anti-p21 antibody NCC-RAS-004 (14). Lane 1, partially purified bovine GAP (10 μg); lane 2, P2 (70 μg); lane 3, B2 (66 μg); lane 4, B3P (56 μg); lane 5, B3P fraction purified by sucrose density gradient (59 μg); lane 6, triton-insoluble materials (B3P-P)(34 μg). Arrow indicates p21 bands.

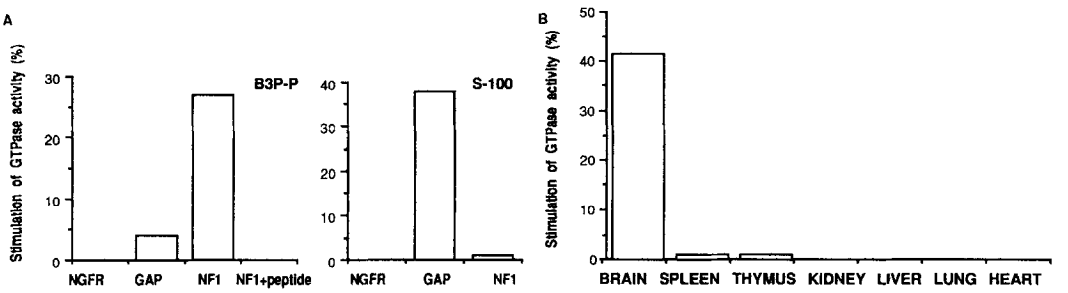

Figure 3. Immunoprecipitation assay of NF1 and GAP activities (A) and tissue specificity of NF1 activity (B).
A. B3P-P (300 μg) or S-100 (500 μg) fraction was reacted with 2.5 μg of anti-NF1 (NF1), anti-GAP (GAP), or anti-NGF receptor (NGFR) antibodies as described in MATERIALS AND METHODS, and GAP activity in immunoprecipitates was measured. NF1 peptide (30 μg) was added to see the specificity of immunoprecipitation (NF1 + peptide). Mean values of triplicate experiments are presented.
B. NF1 activity of triton-insoluble fractions (400 μg) prepared from various tissues of rat was measured as in A.

Last year a part of NF1 gene having similarity to GAP was shown to possess GAP activity when the GAP-related domain was expressed (6-8). Therefore anti-NF1 antibody was prepared and reactivity of the antibody with the triton-insoluble GAP activity was tested (Fig. 3A). The anti-NF1 immunoprecipitated GAP activity from triton-insoluble fraction (B3P-P). Triton-insoluble GAP activity was not immunoprecipitated when the reaction was carried out in the presence of excess NF1 peptide or with unrelated antibody, anti-nerve growth factor receptor (NGFR). In contrast soluble GAP from S-100 fraction was immunoprecipitated with anti-GAP but not with anti-NF1, which excluded the possibility that anti-NF1 cross-reacted with GAP molecule. These results suggest that NF1 is present in insoluble fraction but not in soluble fraction, and that NF1 molecule expressed *in vivo* also possses GAP activity as does GAP related domain expressed by recombinant DNA technique (6-8). Immunoblot analysis with the anti-NF1 showed a single band of about 250 kilodalton in triton-insoluble fraction (data shot shown). However the molecular identity of this band as NF1 gene product is remained to be clarified. Small amount of GAP activity was immunoprecipitated by anti-GAP antibody from insoluble fraction. Whether this activity is due to a trace amount of GAP detected by immunoblot is currently under investigation.

We raised NF1 antibody against human sequence since this is the only sequence so far determined. However the antibody immunoprecipitated GAP activity from triton-insoluble fraction prepared from human cell lines as well as rat brain. These results suggest that carboxy-terminal sequence may be conserved beyond species. When NF1 activity of rat whole brain was measured by immunoprecipitation assay as above, more

than 90% of NF1 activity of the homogenate was recovered in triton-insoluble fraction, which confirmed the results described above. The reason why insoluble protein could be immunoprecipitated is not clear at present. One explanation is that binding of antibody might change the solubility of the antigen.

Tissue specificity of NF1 activity was also studied with triton-insoluble fraction prepared from various tissues of rat (Fig. 3B). Among seven tissues tested, only brain showed high NF1 activity, in contrast to the results of Wallace *et al.* (2) that NF1 is expressed ubiquitously. They mostly analyzed cell lines or cancerous tissues, which might be the reason for the discrepancy. Because GAP activity is also high in brain (10), two different molecules with GAP activity may be present in brain. It is quite interesting to examine histochemical localization of NF1 and GAP in brain and to identify signaling pathways that may differentially couple with.

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