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Binding of *ras* p21 to bands 4.2 and 6 of human erythrocyte membranes

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The direct binding protein(s) of *ras* p21 was (were) investigated in inside-out vesicles of human erythrocyte ghosts using the pure v-Kirsten (Ki)-*ras* p21 synthesized in *E. coli*. The bound *ras* p21 was detected immunochemicallly using an anti-v-Ki-*ras* p21 monoclonal antibody. *ras* p21 bound to vesicles. Prior digestion of the vesicles with trypsin reduced this binding significantly. When *ras* p21 was laid over vesicle proteins immobilized on a nitrocellulose sheet by transfer from the gel of SDS-polyacrylamide gel electrophoresis, *ras* p21 bound to bands 4.2 and 6. *ras* p21 binding to these proteins was reduced by prior incubation of *ras* p21 with the purified band 4.2 or 6 protein. These results indicate that v-Ki-*ras* p21 can bind directly to bands 4.2 and 6 of human erythrocyte membranes as far as tested in an in vitro cell-free system.

Oncogene; Plasma membrane; (*ras* p21)

1. INTRODUCTION

The *ras* oncogene product, *ras* p21, has been shown to induce transformation and DNA synthesis in various cell types [1,2], differentiation of PC-12 cells [3], maturation of *Xenopus* oocytes [4] and pinocytosis in rat embryo fibroblasts [5]. This protein has also been suggested to affect the phospholipase A_2-induced arachidonic acid liberation, the phospholipase C-mediated hydrolysis of phosphoinositides and the adenylate cyclase-mediated formation of cyclic AMP in several mammalian cell types (review [6]).

ras p21 possesses GTP-binding and GTPase activities and resides in the cytoplasmic face of the plasma membrane (review [7]). Although the mode of action of *ras* p21 is not known at all, it can be speculated, by analogy with other GTP-binding regulatory proteins for adenylate cyclase, cyclic GMP phosphodiesterase and phospholipase C, that *ras* p21 has two protein-interacting domains, the detector domain through which the function of *ras* p21 is affected by other protein(s) and the effector domain which affects the function of target protein(s) (review [8]). It is important to identify *ras* p21-interacting proteins to understand the mode of action of this oncogene product, but it has not yet been clarified whether *ras* p21 binds to some specific membrane proteins.

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Abbreviations: v-Ki-*ras*, viral Kirsten *ras*; DTT, dithiothreitol; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; GTPγS, guanosine 5′-(3-*O*-thio)triphosphate; BSA, bovine serum albumin

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The present studies were undertaken to investigate *ras* p21-interacting membrane proteins. For this purpose, we used inside-out vesicles of human erythrocyte ghosts as a model system, since [i] erythrocyte membrane proteins have been investigated and characterized most extensively (review [9]); (ii) inside-out vesicles are useful in studying *ras* p21 binding to the proteins on the cytoplasmic face of the plasma membrane and they are prepared most easily and reliably from erythrocytes; and (iii) the proteins functionally and structurally homologous to erythrocyte membrane proteins, such as spectrin [10,11], ankyrin [12] and band 4.1 [13], are present in various types of cells and tissues, and erythrocyte membranes are often used as a model system to identify novel membrane proteins.

This paper describes that v-Ki-*ras* p21 binds directly and specifically to bands 4.2 and 6 of human erythrocyte membranes in an in vitro cell-free system, although it remains to be clarified with which functional domains of *ras* p21 these two proteins interact.

2. MATERIALS AND METHODS

2.1. *Materials*

The sealed inside-out vesicles of human erythrocyte ghosts were prepared by the method of Steck and Kant [14]. Vesicles were used after they were washed once in 50 mM Hepes at pH 8.0 and resuspended to 10 mg/ml of protein in the same buffer. Where indicated, vesicles (10 mg/ml of protein) were digested with 0.2 mg/ml of trypsin in 100 μl of 50 mM Hepes at pH 8.0 for 1 h at 37°C, and the reaction was terminated by the addition of 1 mg/ml of trypsin inhibitor. v-Ki-*ras* p21 was synthesized in *E. coli* containing pHN121, a derivative of the Kirsten murine sarcoma virus clone HiHi3, as described in [15]. This preparation (5 mg of protein) was purified by a DEAE-Sephacel column (0.7 × 2.5 cm) equilibrated with buffer A (50 mM Hepes at pH 7.5, 1 mM EDTA and 1 mM DTT). After the column was washed with 3 ml buffer A, *ras* p21 was eluted with a 30-ml linear concentration gradient of NaCl (0–0.8 M) in buffer A. Fractions of 0.5 ml each were collected. *ras* p21 appeared in fractions 16–30. These fractions were pooled and diluted with 10 vols of buffer A and resubjected to the same column (0.7 × 1.2 cm) under the same conditions. *ras* p21 was eluted with 1 ml of buffer A containing 1 M NaCl. This preparation of *ras* p21 (1 ml of 0.6 mg of protein) was nearly homogeneous as judged by protein staining with Coomassie brilliant blue on SDS-PAGE and was stocked at −20°C in the presence of 50% glycerol until use. The anti-v-Ki-*ras* p21 monoclonal antibody was made by injections of the purified antigen into mice. The isolation and screening of hybridomas were done by the standard procedures [16]. Among the several antibodies thus obtained, the antibody designated as CL 25–94 was used here. The detailed properties of this antibody will be described elsewhere (Shiku, H., in preparation). The band 4.2 protein was purified from fresh human erythrocyte ghosts by the method of Korsgren and Cohen [17] except that 0.3 mM sodium phosphate at pH 8.0 containing 0.2 mM EDTA was used instead of 0.1 mM EGTA at pH 8.5 which was used previously for the solubilization of spectrin and actin. The purified band 4.2 protein was over 80% pure as judged by the same method described above. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was from Boehringer, Mannheim. The band 6 protein of human erythrocyte membranes was identified to be this enzyme. The preparation of this enzyme was over 95% pure as judged by the same method described above. Biotinylated anti-mouse IgG, avidin and biotinylated peroxidase were from Vector Laboratories. Other materials and chemicals were obtained from commercial sources.

2.2. *Assay for the* ras *p21 binding to the inside-out vesicles*

In this experiment, GTPγS-bound *ras* p21 was used. GTPγS-bound *ras* p21 was prepared by incubating *ras* p21 (3.3 μg of protein) in 30 μl of 80 mM Hepes at pH 7.5 containing 1.7 mM GTPγS, 2 mM $MgCl_2$, 1.7 mM DTT, 0.17% BSA and 0.17 mM EDTA for 1 h at 30°C. All subsequent procedures were performed at 4°C. *ras* p21 was centrifuged at 16000 × *g* for 1 h to remove the aggregated form. The indicated amounts of *ras* p21 were incubated for 1.5 h with the inside-out vesicles (15 μg of protein) in 50 μl of buffer B (50 mM Hepes, pH 7.5, 1.2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.12 M KCl) containing 1 mM GTPγS and 0.1% BSA. After incubation, the mixture was centrifuged at 16000 × *g* for

1.5 min. The pellet was washed with 200 μl of cold buffer B and centrifuged at 16000 × *g* for 1.5 min. The pellet was dissolved in 35 μl of 62 mM Tris-HCl, pH 6.8, containing 3% SDS, 2% 2-mercaptoethanol, 5% glycerol and 0.05% bromophenol blue, and a 15-μl aliquot was subjected to SDS-PAGE at room temperature. *ras* p21 (0–300 ng of protein) was separately subjected to the same SDS-PAGE as a reference protein. After the electrophoresis, proteins on the gel were transferred to a nitrocellulose sheet, and *ras* p21 was detected immunochemically as described below.

2.3. *SDS-PAGE and electrophoretic transfer of the proteins from the slab gel to a nitrocellulose sheet*

SDS-PAGE was carried out on a 5–18%, 11% or 8% polyacrylamide slab gel by the method of Laemmli [18]. The proteins on the gel were transferred to a nitrocellulose sheet by the method of Burnette [19]. Transfer was carried out in a transfer buffer (25 mM Tris-glycine, pH 8.3, and 20% methanol) for 10 h at 4°C, at a constant voltage of 40 V (0.4 A). Under these conditions, the efficiency of electrophoretic transfer was greater than 70% for all proteins. The sheet was cut into strips corresponding to the individual lanes, and *ras* p21 was detected as described below.

2.4. *Immunochemical detection of* ras *p21 on a nitrocellulose sheet*

ras p21 on a nitrocellulose sheet was detected immunochemically using the anti-*ras* p21 monoclonal antibody by the method of Hsu et al. [20]. After the electrophoretic transfer of the proteins from the gel to a nitrocellulose sheet, its strips (50 cm^2 of total area) were incubated in 75 ml TBS (50 mM Tris-HCl at pH 7.5 and 0.2 M NaCl) containing 0.05% Tween 20 and 5% BSA for 1 h at 40°C. The strips were then incubated with the antibody in BSA-TBS (TBS containing 5% BSA) for 1 h at room temperature. They were washed 4 times with 150 ml T-TBS (TBS containing 0.05% Tween 20) for 15 min at 40°C and incubated for 1 h at 40°C with 75 ml T-BSA-TBS (TBS containing 0.05% Tween 20 and 5% BSA). The strips were then incubated with biotinylated anti-mouse IgG (1:50 dilution) in BSA-TBS for 1 h at room temperature. They were washed 3 times with 150 ml T-TBS for 10 min at 40°C and incubated with 75 ml T-BSA-TBS for 1 h at 40°C. The strips were then incubated with avidin-biotin peroxidase complex (1:50 dilution) in BSA-TBS for 10 h at 4°C. They were washed 3 times with 150 ml T-TBS for 10 min at 40°C and once with 150 ml of TBS for 10 min at 40°C. *ras* p21 was detected by peroxidase reaction with 0.5 mg/ml of 4-chloro-1-naphthol in TBS containing 17% methanol and 0.01% H_2O_2 for 15–30 min at room temperature.

2.5. *Overlay of the* ras *p21 on a nitrocellulose sheet*

Following the electrophoretic transfer of the proteins from the gel to a nitrocellulose sheet, its strips (50 cm^2 of total area) were placed in 75 ml T-BSA-TBS. After the incubation for 4.5 h at 40°C, the strips were overlaid with the GTPγS-bound *ras* p21 (6 μg of protein/ml) in BSA-TBS containing 1 mM GTPγS, 1.2 mM $MgCl_2$, 1 mM DTT and 0.1 mM EDTA, and incubated for 1 h at room

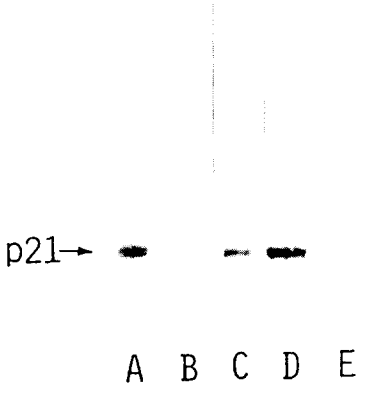

Fig.1. The *ras* p21 binding to vesicles. *ras* p21 (2 μg protein) was mixed with the intact or trypsin-digested vesicles (15 μg protein). The mixture was centrifuged and an aliquot of the pellet (6.4 μg of vesicle protein) was subjected to SDS-PAGE (polyacrylamide: 5–18%). After electrophoresis, the proteins were transferred to a nitrocellulose sheet and *ras* p21 immobilized on this sheet was stained immunochemically. Lanes: A, *ras* p21 plus intact vesicles; B, *ras* p21 without vesicles; C, *ras* p21 plus the trypsin-digested vesicles. In another experiment, *ras* p21 (200 ng protein) and intact vesicles (6.4 μg protein) were directly subjected to the same SDS-PAGE. After electrophoresis, *ras* p21 was detected as described above. Lanes: D, *ras* p21; E, intact vesicles.

temperature. The GTPγS-bound *ras* p21 was prepared by incubating the *ras* p21 in the same solution for 1 h at 30°C. The strips were washed 6 times with 150 ml T-TBS containing 1 mM $MgCl_2$ for 15 min at 40°C, and incubated with 75 ml T-BSA-TBS containing 1 mM $MgCl_2$ for 1 h at 40°C. *ras* p21 bound to the proteins was detected immunochemically as described above except that all the washing and blocking buffers used in this experiment contained additionally 1 mM $MgCl_2$.

2.6. *Determinations*

Protein was determined by the method of Lowry et al. [21]. The relative intensity of immunostaining bands was estimated by scanning at 560 nm using a Shimadzu dual-wavelength chromatogram scanner, model CS-930.

3. RESULTS

When the GTPγS-bound v-Ki-*ras* p21 was mixed with inside-out vesicles of human erythrocyte ghosts, and then centrifuged, *ras* p21 coprecipitated with the vesicles (fig.1, lane A). Under the same condition, *ras* p21 did not precipitate in the absence of the vesicles (fig.1, lane B). Prior digestion of vesicles with trypsin reduced the *ras* p21 binding to vesicles to a level 70–80% of that in intact vesicles (fig.1, lane C), indicating that *ras* p21 interacted at least partly with the vesicle protein(s). The antibody used in this experiment reacted specifically with *ras* p21 and did not react with proteins of the vesicles (fig.1, lanes D,E). Fig.2 shows the dose-dependent effect of the *ras* p21 binding to intact and trypsin-digested vesicles. *ras* p21 bound to both vesicles in a dose-dependent manner, but the levels of binding trypsin-digested vesicles were 70–80% of those to intact vesicles. Particularly, the binding of *ras* p21 in smaller amounts to vesicles was more sensitive to tryptic digestion.

In the next set of experiments, the direct binding

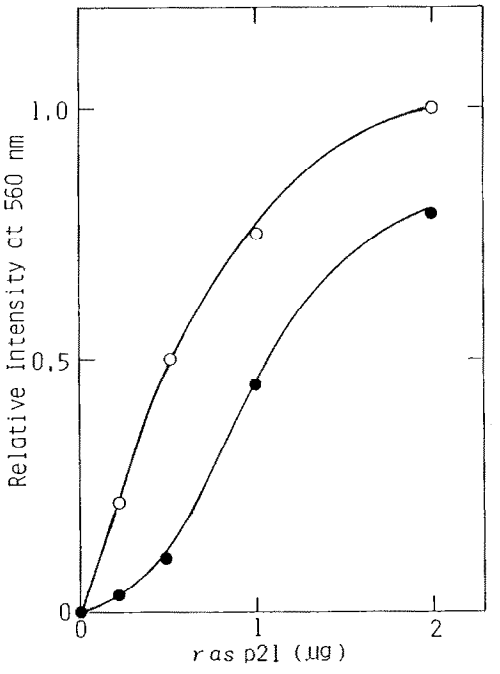

Fig.2. Dose-dependent effect of the *ras* p21 binding to intact and trypsin-digested vesicles. The *ras* p21 binding to intact or trypsin-digested vesicles was performed as described in the legend to fig.1 except that various amounts of *ras* p21 were added as indicated. (○—○) Intact vesicles; (●—●) the trypsin-digested vesicles.

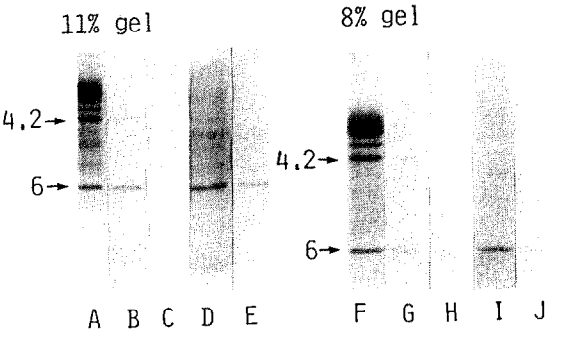

Fig.3. Immunostaining of *ras* p21 laid over intact and trypsin-digested vesicle proteins immobilized on a nitrocellulose sheet. The intact and trypsin-digested vesicles (30 μg protein) were subjected to SDS-PAGE (polyacrylamide: 11 or 8%). One of the lanes from the intact or trypsin-digested vesicle proteins was stained with Coomassie brilliant blue. The proteins in other lanes were transferred to a nitrocellulose sheet. *ras* p21 was then laid over this sheet and protein-bound *ras* p21 was stained immunochemically. Lanes: A–E, 11% polyacrylamide gel; F–J, 8% polyacrylamide gel; A and F, protein staining of the intact vesicle proteins; B and G, immunostaining of *ras* p21 laid over the intact vesicle proteins; C and H, immunostaining of the intact vesicle proteins without *ras* p21 overlay; D and I, protein staining of the trypsin-digested vesicle proteins; E and J, immunostaining of the *ras* p21 laid over the trypsin-digested vesicle proteins.

protein(s) of the GTPγS-bound *ras* p21 in vesicles was investigated. For this purpose, *ras* p21 was laid over the proteins immobilized on a nitrocellulose sheet by transfer from the SDS-PAGE gel of the vesicles, and protein-bound *ras* p21 was detected as described above. The vesicle proteins were well resolved on this SDS-PAGE as judged by protein staining with Coomassie brilliant blue (fig.3, lanes A,F). *ras* p21 specifically bound to two proteins with molecular masses of 72 kDa and 35 kDa, which corresponded to protein bands designated as bands 4.2 and 6, respectively (fig.3, lanes B,G). The GDP-bound form of *ras* p21 similarly bound to these two proteins (not shown). The GDP-bound form of *ras* p21 was prepared by the same method as the GTPγS-bound form. In the absence of *ras* p21, the antibody did not react with proteins of the vesicles (fig.3, lanes C,H). Prior digestion of vesicles with trypsin caused disappearance of several protein bands including band 4.2 as judged by protein staining with Coomassie brilliant blue on SDS-PAGE (fig.3, lanes D,I), but did not affect the protein staining of band 6. This treatment abolished the *ras* p21 binding to band 4.2, but did not affect the *ras* p21 binding to band 6 nor produced new *ras* p21-bound bands (fig.3, lanes E,J). Essentially the same results were obtained when the human erythrocyte ghosts were used instead of their vesicles (not shown). The vesicles were mostly depleted of spectrin and actin whereas the ghosts contained them in large amounts.

The addition of either the purified band 4.2 or 6 protein to *ras* p21 during the overlay period inhibited the *ras* p21 binding to bands 4.2 and 6 immobilized on a nitrocellulose sheet in a dose-dependent manner (table 1).

Table 1

Inhibition by the purified band 4.2 and 6 proteins of the *ras* p21 binding to these bands immobilized on a nitrocellulose sheet

Addition	μg	Relative intensity at 560 nm (%)	
		Band 4.2	Band 6
Control		100	100
Band 4.2 protein	6	75	69
	12	41	37
	24	20	17
Band 6 protein	3	65	62
	6	38	35
	12	20	18

The intact vesicles (30 μg of protein) were subjected to SDS-PAGE (polyacrylamide: 8%). After electrophoresis, proteins were transferred to a nitrocellulose sheet. *ras* p21 (0.6 μg protein) was preincubated with indicated amounts of band 4.2 protein or band 6 protein in 0.1 ml BSA-TBS containing 1 mM GTPγS, 1.2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.1 mM EGTA for 10 min at 30°C, laid over a nitrocellulose sheet, and stained immunochemically as described in the legend to fig.3. Band 4.2 and 6 proteins were used after dialysis for 12 h at 4°C against a large volume of 5 mM sodium phosphate, pH 8.0, containing 0.5 mM EGTA and 0.5 mM DTT. The values are means (n = 3) expressed as percentage of control

4. DISCUSSION

The present results indicate that *ras* p21 binds directly to bands 4.2 and 6 of the inside-out vesicles of human erythrocyte ghosts as far as tested in an in vitro cell-free system. This binding is not simply non-specific, because *ras* p21 binds specifically to bands 4.2 and 6 among many vesicle proteins and this binding is inhibited by the purified intact band 4.2 and 6 proteins.

It has been speculated that *ras* p21 has two protein-interacting domains, the detector and effector domains [8]. It is unknown with which functional domains of *ras* p21 bands 4.2 and 6 interact. However, since both the GTPγS- and GDP-bound forms of *ras* p21 similarly bind to bands 4.2 and 6 and the GTP- and GDP-bound forms are considered to be active and inactive forms, respectively [7], it is likely that bands 4.2 and 6 interact with domain(s) other than the effector domain. One such possibility is the detector domain. It is also conceivable that *ras* p21 has an additional protein-interacting domain to the detector and effector domains, which determines the specific localization of this oncogene product on the plasma membrane and that bands 4.2 and 6 interact with such a domain.

Since it has not been shown whether *ras* p21 functions in mature erythrocytes, the physiological significance of the binding of this protein to bands 4.2 and 6 in this particular cell type is not known at present. Band 6 is a glyceraldehyde-3-phosphate

dehydrogenase, one of the glycolytic enzymes, and is present in most mammalian tissues. It is not known whether band 4.2 is also present in other tissues, but it is possible that band 4.2 or a band 4.2-like protein is present in other cell types since the proteins structurally and immunologically related to spectrin [10,11], ankyrin [12] and band 4.1 [13] have been identified in various cell types. Therefore, it is conceivable that *ras* p21 may interact with bands 4.2 and 6 in the cells containing these proteins. Tissue distribution of band 4.2 or a band 4.2-like protein and the *ras* p21 binding to bands 4.2 and 6 in cells other than erythrocytes are now being investigated.

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