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POSSIBLE BINDING PROTEINS OF RAS P21 IN HUMAN ERYTHROCYTE MEMBRANE*

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INDEXING WORDS

oncogene; *ras* p21; plasma membrane; erythrocyte

SYNOPSIS

The direct binding protein(s) of *ras* p21 was investigated in the 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 immunochemically using an anti-v-Ki-*ras* p21 monoclonal antibody. *ras* p21 was overlaid on the vesicle proteins immobilized on a nitrocellulose sheet transferred from the gel of sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *ras* p21

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**The abbreviations used are: GAP, GTPase activating protein; v-Ki-*ras*, viral Kirsten *ras*; HEPES, 4-(2-hydroxyethyl) 1-piperazineethanesulfonic acid; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl-ether)-N,N',N'-tetraacetic acid; GTPγS, guanosine 5'-(3-*O*-thio)triphosphate; BSA, bovine serum albumin.

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bound to bands 4.2 and 6. *ras* p21-binding to bands 4.2 and 6 was reduced by prior incubation of *ras* p21 with the purified band 4.2 or 6 protein. Furthermore, when *ras* p21 was mixed with inside-out vesicles and then centrifuged, *ras* p21 was coprecipitated with the vesicles. Prior digestion of the vesicles with trypsin reduced this binding significantly. These results indicate that v-Ki-*ras* p21 can bind directly to bands 4.2 and 6 of human erythrocyte membranes as fat as tested in an *in vitro* cell-free system.

INTRODUCTION

The *ras* gene family consisting of Harvey (Ha-), Kirsten (Ki-) and N-*ras* has been shown to play an important role in cell growth. All of these genes encode structurally and immunologically related proteins with molecular weight (Mr) values of about 21,000, designated as *ras* p21. *ras* p21 has been shown to induce transformation and DNA synthesis in various cell types,[11,26] differentiation of PC-12 cells,[2] maturation of *Xenopus* oocytes[5] and pinocytosis in rat embryo fibroblasts.[3] 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.[14]

ras p21 possesses GTP-binding and GTPase activities[24,25] and residues in the cytoplasmic face of the plasma membrane.[9,34] 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).[6] Recently, a cytosolic protein of Mr 116,000 - 125,000 has been identified that stimulates the GTPase activity of normal *ras* p21 but not that of oncogenic variants.[1,8,13,31] The sequence of this protein, termed GAP**, has been determined by the analysis of bovine brain and human placenta cDNAs.[32,33] Bovine brain *ras* p21 GAP is a single polypeptide with a calculated Mr of 115,742.[33] Although it has not been clarified whether *ras* p21 GAP serves as a regulatory protein or an effector protein for *ras* p21s, *ras* p21 GAP has been

proposed to serve as a possible protein interacting with the putative effector domain of *ras* p21s on the basis of the observation that a single amino acid substitution in the effector domain (residues 35-40) of *ras* p21s makes *ras* p21s insensitive to *ras* p21 GAP.[1,8,32]

It is important to identify the *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. The present studies were undertaken to investigate the *ras* p21-interacting membrane proteins. For this purpose, we used the inside-out vesicles of human erythrocyte ghosts as a model system, since (1) erythrocyte membrane proteins have been investigated and characterized most extensively;[27] (2) the inside-out vesicles are useful to study the *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 (3) the proteins functionally and structurally homologous to erythrocyte membrane proteins, such as spectrin,[16,22] ankyrin[4] and band 4.1,[10] are present in various types of cells and tissues, and erythrocyte membranes are often used as a model system to identify some novel membrane proteins.

This paper describes that the v-Ki-*ras* p21 synthesized in *E. coli* 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 the *ras* p21 these two proteins interact.

MATERIALS AND METHODS

Materials

v-Ki-*ras* p21 was synthesized in *E. coli* containing pHN121, a derivative of the Kirsten murine sarcoma virus clone HiHi3, a kindly gift from Dr. H. Nakano (Tokyo Research Laboratories, Kyowa Hakko Kogyo Co., Tokyo, Japan), as described.[30] This preparation (5 mg of protein) was purified by a DEAE-Sephacel column (0.7 x 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 of 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 through 30. These fractions were pooled and diluted with 10 volumes of Buffer A and applied on the same column (0.7 x 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, 0.6 mg of protein) was nearly homogeneous, judged from the stained protein with Coomassie Brilliant Blue on SDS-PAGE and was stocked at -20C in the presence of 50% glycerol until used. An anti-v-Ki-*ras* p21 monoclonal antibody was kindly supplied by Dr. H. Shiku (Department of Oncology, Nagasaki University School of Medicine, Nagasaki, Japan). This antibody was made by injections of the purified antigen into mice. The isolation and screening of hybridomas were done by the standard procedures.[17] Among the several antibodies thus obtained, the antibody designated as RASK-4 was used in this study.[35]

The sealed inside-out vesicles of human erythrocyte ghosts were prepared by the method of Steck and Kant.[28] The 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, the 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 37C, and the reaction was terminated by the addition of 1 mg/ml of trypsin inhibitor. Band 4.2 protein was purified from fresh human erythrocyte ghosts by the method of Korsgren and Cohen[18] 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 pH8.5 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 obtained from Boehringer Mannheim. Band 6 protein of human erythrocyte membranes was indentified 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 obtained from Vector Laboratories. Other materials and chemicals were obtained from commercial sources.

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% or 11% polyacrylamide

slab gel by the method of Laemmli.[21] The proteins on the gel were transferred to a nitrocellulose sheet by the method of Burnette.[7] Tranfer was carried out in a transfer buffer (25 mM Tris/glycine at pH 8.3 and 20% methanol) for 10 h at 4C 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.

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.[15] 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 of 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 40C. 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 of T-TBS (TBS containing 0.05% Tween 20) for 15 min at 40C and incubated for 1 h at 40C with 75 ml of 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 of T-TBS for 10 min at 40C and incubated with 75 ml of T-BSA-TBS for 1 h at 40C. The strips were then incubated with avidin-biotin peroxidase complex (1:50 dilution) in BSA-TBS for 10 h at 4C. They were washed 3 times with 150 ml of T-TBS for 10 min at 40C and once with 150 ml of TBS for 10 min at 40C. *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.

Overlay of 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 of T-BSA-TBS. After the incubation for 4.5 h at 40C, the strips were overlaid with the GTPγS-bound form of *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 temperature. The GTPγS-bound form of *ras* p21 was prepared by incubating *ras* p21 in the solution for 1 h at 30C. The strips were washed 6 times with 150 ml of T-TBS containing 1 mM $MgCl_2$ for 15 min at 40C, and incubated with 75 ml of T-BSA-TBS containing 1 mM $MgCl_2$ for 1 h at 40C. *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 $MgCl_2$.

Assay for ras p21-binding to the inside-out vesicles

In this experiment, the GTPγS- and GDP-bound forms of *ras* p21s were used. The GTPγS- and GDP-bound forms of *ras* p21s were prepared by incubating *ras* p21 (3.3 μg of protein) with 1.7 mM GTPγS and GDP, respectively, in 30 μl of 80 mM HEPES at pH 7.5 containing 2 mM $MgCl_2$, 1.7 mM DTT, 0.17% BSA and 0.17 mM EDTA for 1 h at 30C. All subsequent procedures were performed at 4C. *ras* p21 was centrifuged at 16,000 x 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 at 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 the incubation, the mixture was centrifuged at 16,000 x g for 1.5 min. The pellet was washed with 200 μl of cold Buffer B and centrifuged at 16,000 x g for 1.5 min. The pellet was dissolved in 35 μl of 62 mM Tris/HCl at 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 above.

Determinations

Protein was determined by the method of Lowry et al.[23] The relative intensity of Coomassie Brilliant Blue staining bands and immunostaining bands was estimated by scanning at 560 nm using a Shimadzu dual wavelength chromatogram scanner, Model CS-930.

RESULTS

To investigate the direct binding protein(s) of the GTPγS-bound form of *ras* p21 in the inside-out vesicles of human erythrocyte ghosts, *ras* p21 was overlaid on the proteins immobilized on a nitrocellulose sheet transferred from the gel of SDS-PAGE of the vesicles, and *ras* p21 bound to the proteins was detected by its specific monoclonal antibody. The vesicle proteins were well resolved on this SDS-PAGE as judged by protein staining with Coomassie Brilliant Blue (Fig. 1A). *ras* p21 specifically bound to two proteins with Mrs of 72-kDa and 35-kDa, which corresponded to protein bands designated as bands 4.2 and 6, respectively (Fig. 1B). The GDP-bound form of *ras* p21 similarly bound to these two proteins (data not shown). In the absence of *ras* p21, the antibody did not react with any proteins of the vesicles (Fig. 1C).

Prior digestion of the vesicles with trypsin caused disapearance of several protein bands including band 4.2, judged from the stained protein with Coomassie Brilliant Blue on SDS-PAGE (Fig.1D), 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 the new *ras* p21-bound bands (Fig. 1E). The essentially same results were obtained when the human erythrocyte ghosts were used instead of their vesicles (data not shown). The vesicles were mostly depleted of spectrin and actin whereas the ghosts contained them in a large amount.

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).

In the next set of experiments, the GTPγS-bound form of v-Ki-*ras* p21 was mixed with the inside-out vesicles of human erythrocyte ghosts, and then the mixture was centrifuged, *ras* p21 was coprecipitated with the vesicles (Fig. 2A). Under the same conditions, *ras* p21 was not precipitated in the absence of the vesicles (Fig. 2B). Prior digestion of the vesicles with trypsin reduced the *ras* p21-binding to the vesicles to the level of 70-80% of the intact vesicles (Fig. 2C), indicating that *ras* p21 interacted at least partly with the vesicle protein(s). The

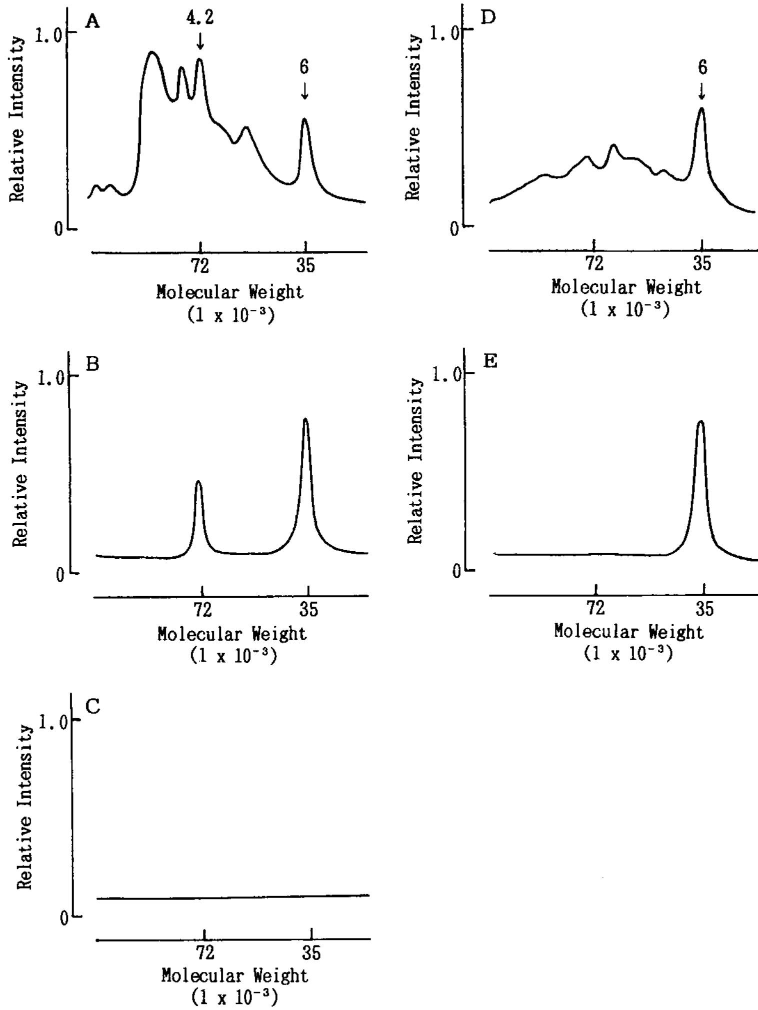

Fig. 1

Immunostaining of *ras* p21 overlaid on the intact and trypsin-digested vesicle proteins immobilized on a nitrocellulose sheet. The intact and trypsin-digested (30 μg of protein) were subjected to SDS-PAGE (polyacrylamide: 11%). 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 overlaid on this sheet and *ras* p21 bound to the proteins was stained immunochemically. The relative intensity of each band stained was quantitated by densitometric scanning at 560 nm. A, protein staining of the inatct vesicle proteins; B, immunostaining of *ras* p21 overlaid on the intact vesicle proteins; C, immunostaining of the intact vesicle proteins without overlay with *ras* p21; D, protein staining of the trypsin-digested vesicle proteins; E, immunostaining of *ras* p21 overlaid on the trypsin-digested vesicle proteins.

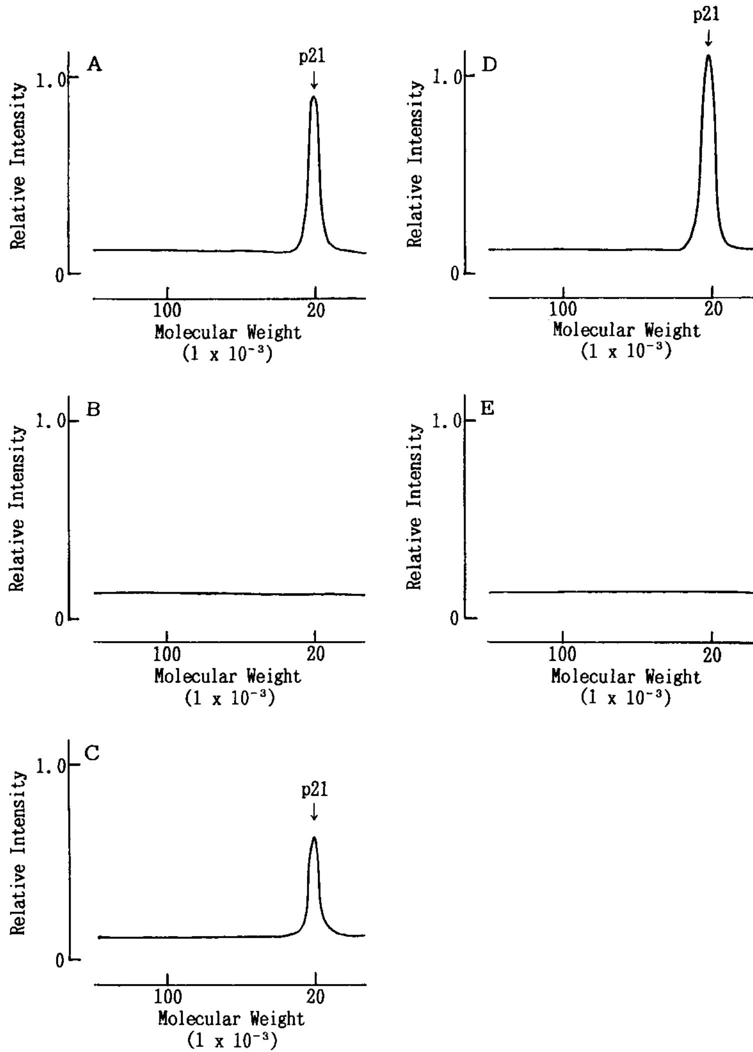

Fig. 2
The *ras* p21-binding to the vesicles. *ras* p21 (2 mg of protein) was mixed with the intact or trypsin-digested vesicles (15 μg of protein). The mixture was centrifuged and an aliquot of the pellet (6.4 μg of vesicle protein) was subjected to SDS-PAGE (polyacrylamide: 5-18%). After the electrophoresis,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

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		relative intensity at 560 nm	
		band 4.2	band 6
	(μg)	(%)	
control		100	100
	6	75	69
band 4.2 protein	12	41	37
	24	20	17
	3	65	62
band 6 protein	6	38	35
	12	20	18

The intact vesicles (30 μg of protein) were subjected to SDS-PAGE (polyacrylamide: 11%). After the electrophoresis, the proteins were transferred to a nitrocellulose sheet. *ras* p21 (0.6 μg of protein) was preincubated for 10 min at 30C with indicated amounts of band 4.2 protein or band 6 protein in 0.1 ml of BSA-TBS containing 1 mM GTPγS, 1.2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.1 mM EGTA, overlaid to the nitrocellulose sheet, and stained immunochemically as described in the legend to Fig. 1. Band 4.2 and 6 proteins were used after dialysis for 12 h at 4C against a large volume of 5 mM sodium phosphate at pH 8.0 containing 0.5 mM EGTA and 0.5 mM DTT. The values are means (n=3) expressed as percentage of control.

antibody used in this experiment reacted specifically with *ras* p21 and did not react with any proteins of the vesicles (Fig. 2, D and E). Fig. 3 shows the dose-dependent effect of the *ras* p21-binding to the intact and trypsin-digested vesicles. *ras* p21 bound to both vesicles in a dose-dependent manner, but the binding levels to the trypsin-digested vesicles were 70-80% of those to the intact vesicles. Particularly, the binding of *ras* p21 in smaller amounts to the vesicles was more sensitive to tryptic digestion.

These results together with those shown in Fig. 1 suggest that 20-30% of the total binding to the vesicles is due to *ras* p21 binding to band 4.2. The residual 70-80% of the total binding may reflect the *ras* p21 binding to band 6 and non-specific binding. It was difficult from these data to estimate accurately the Kd values and the stoichimetry of the *ras* p21 binding to bands 4.2 and 6, since there are many procedures for immunochemical detection of *ras* p21 bound to bands 4.2 and 6. However, it was roughly

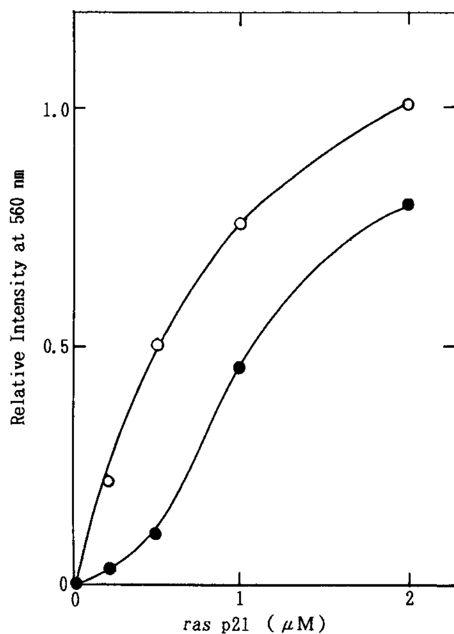

Fig. 3
Dose-dependent effect of the *ras* p21-binding to the intact and trypsin-digested vesicles. The *ras* p21-binding to the intact or trypsin-digested vesicles was performed as described in the legend to Fig. 2 except that various amounts of *ras* p21 were added as indicated. (○), the intact vesicles; (●), the trypsin-digested vesicles.

estimated that *ras* p21 binds to band 4.2 with a Kd value in the range of 10^{-7} M. On the assumption that the residual 70-80% of the total binding of *ras* p21 to the intact vesicles reflects only the *ras* p21 binding to band 6, a Kd value of the *ras* p21 binding to band 6 was in the range of 10^{-6} M, but the real Kd value might be less than this value. The stoichiometry was 0.2-0.5 mole of *ras* p21 per 1 mole of band 4.2 or 6 protein.

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 specifically binds 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.[6] 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* p21s similarly bind to bands 4.2 and 6 and the GTP- and GDP-bound forms are considered to be active and inactive forms, respectively,[24,25] it is likely that bands 4.2 and 6 interact with other domain(s) than the effector domain. One of possible domains is the detector domain. It is also conceivable that *ras* p21 has other protein-interacting domain than 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 has been shown that band 4.2 binds to the cytoplasmic domain of band 3, the erythrocyte anion transport protein.[18,19] Band 4.2 also can bind to erythrocyte ankyrin *in vitro* and may associate with band 4.1 and spectrin.[19] Proteins closely related to spectrin,[16,22] ankyrin[4] and band 4.1[10] are also present in various nonerythroid cells. Recently, it was immunochemically shown that band 4.2 is also associated with the plasma membrane of various nonerythroid cells and tissues such as platelets, brain and kidney.[12] Therefore, it is conceivable that *ras* p21 may interact with bands 4.2 and 6 in the cells containing these proteins. The cDNA of band 4.2 was also isolated from a human reticulocyte cDNA library, and its primary structure was determined.[20,29] The amino acid sequence of band 4.2 has homology with two closely related Ca^{2+}-dependent cross-linking proteins, guinea pig liver transglutaminase and the α subunit of human coagulation factor XIII. It is tempting to speculate that the *ras* p21-binding to band 4.2 may affect the function of this protein and thereby causes morphological changes of the cells. The *ras* p21-binding to bands 4.2 and 6 in other cells than erythrocytes and its binding to transglutaminase are now being investigated.

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