# Generation of specific antibodies against the rap1A, rap1B and rap2 small GTP-binding proteins

## Analysis of rap and ras proteins in membranes from mammalian cells

Franz-Josef KLINZ[1], Roland SEIFERT[1], Ingo SCHWANER[1], Heinrich GAUSEPOHL[2], Rainer FRANK[2] and Günter SCHULTZ[1]

[1] Institut für Pharmakologie, Freie Universität Berlin, Federal Republic of Germany
[2] European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany

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Specific antibodies against rap1A and rap1B small GTP-binding proteins were generated by immunization of rabbits with peptides derived from the C-terminus of the processed proteins. Immunoblot analysis of membranes from several mammalian cell lines and human thrombocytes with affinity-purified antibodies against rap1A or rap1B demonstrated the presence of multiple immunoreactive proteins in the 22–23 kDa range, although at strongly varying levels. Whereas both proteins were present in substantial amounts in membranes from myelocytic HL-60, K-562 and HEL cells, they were hardly detectable in membranes from lymphoma U-937 and S49.1 cyc$^-$ cells. Membranes from human thrombocytes and 3T3-Swiss Albino fibroblasts showed strong rap1B immunoreactivity, whereas rap1A protein was present in much lower amounts. In the cytosol of HL-60 cells, only small amounts of rap1A and rap1B proteins were detected, unless the cells were treated with lovastatin, an inhibitor of hydroxymethylglutaryl-coenzyme A reductase, suggesting that both proteins are isoprenylated. By comparison with recombinant proteins, the ratio of rap1A/ras proteins in membranes from HL-60 cells was estimated to be about 4:1. An antiserum directed against the C-terminus of rap2 reacted strongly with recombinant rap2, but not with membranes from tested mammalian cells. In conclusion, rap1A and rap1B proteins are distributed differentially among membranes from various mammalian cell types and are isoprenylated in HL-60 cells.

The rap proteins belong to the rapidly growing family of small GTP-binding proteins [1–3]. Up till now, amino acid sequences for four types of rap proteins have been deduced from cloned cDNA, namely rap1A [4–7], rap1B [8], rap2 [4] and rap2B [9]. Except for their C-terminal sequences, the rap proteins are highly similar to each other and share the effector domain with ras proteins [1–3].

The C-terminus of ras proteins is essential for attaching the proteins to the plasma membrane, largely by modifications to the last four amino acids, the so-called CAAX motif. These modifications include attachment of a C15 (farnesyl) isoprenoid to the cysteine of the CAAX motif, removal of the final three amino acids and methylation of the newly exposed Cα group [10]. More recently, it was shown that rap1A protein expressed in insect cells [11] and rap1B protein purified from human platelets [12] are modified by a C20 (geranylgeranyl) isoprenoid.

Little is known about the function of rap proteins in mammalian cells. Interest has focussed on the rap1A (Krev-1) protein, whose cDNA was cloned first using the feature of its homology to ras proteins [4] and more recently by its ability to suppress the transformed phenotype of fibroblasts induced by oncogenic ras protein [6]. Overexpression of rap1A *in* ras-transformed fibroblasts restores the platelet derived growth factor-mediated activation of phospholipase C and the induction of growth-related genes [13]. It has been shown that rap1A binds to ras GTPase-activating protein (GAP), although the GTPase activity of rap1A is not stimulated [14–17]. Furthermore, the GTP-bound form of rap1A is an effective competitive inhibitor of the GAP-mediated GTPase activity of ras proteins [14, 15]. This suggests that rap1A competes with ras for GAP in intact cells and blocks downstream signalling. Since GAP proteins have been identified which specifically stimulate the GTPase activity of rap1 proteins [18, 19], it is also possible that rap1A controls a signalling pathway antagonistic to ras. More recently, an antagonistic action of the rap1B protein on ras-induced germinal-vesicle breakdown *in* Xenopus oocytes was reported [20].

In neutrophils, rap1 is associated with a molecular complex at the plasma membrane which is responsible for superoxide generation [21, 22]. More recently, it was shown that the activation of NADPH oxidase from macrophages involves the small GTP-binding protein, rac1 [23]. Using an antiserum recognizing both rap1A and rap1B, Kim et al. [24] found that rap1 proteins are present in the particulate fractions, but not in the cytosol prepared from cerebrum. Beranger et al. [25] utilized an antiserum directed against both rap1A and rap1B to demonstrate the association of rap1

*Correspondence to* F.-J. Klinz, Institut für Molekulare Neurobiochemie, Ruhr-Universität Bochum, Universitätsstr. 150, W-4630 Bochum, Federal Republic of Germany
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*Abbreviation.* GAP, GTPase-activating protein.

proteins with the Golgi complex of mammalian cells. There is also evidence that rap1B associates with the platelet cytoskeleton [26].

In this paper, we report on the production of specific antibodies directed against the C-terminus of rap1A, rap1B or rap 2 and their use in immunoblot analysis of these proteins in membranes from various mammalian cell lines including HL-60 cells and human thrombocytes. Additionally, inhibition of isoprenoid biosynthesis by lovastatin was used to show that rap1A and rap1B proteins are isoprenylated and thereby attached to membranes in HL-60 cells.

## MATERIALS AND METHODS

### Materials

Materials were obtained from the following sources: nitrocellulose membranes (BA83, 0.2 μm) from Schleicher & Schüll (Dassel, FRG), [α-$^{32}$P]GTP (3000 Ci/mmol) from New England Nuclear (Dreieich, FRG), Hyperfilm-MP from Amersham Buchler (Braunschweig, FRG), Tween 20 from BioRad (Munich, FRG). Materials for gel electrophoresis were purchased from BioRad (Munich, FRG). All other chemicals were of reagent grade and obtained from Merck (Darmstadt, FRG).

Lovastatin was generously supplied by Dr A. Alberts (Merck, Sharp and Dohme, Rahway, NJ, USA).

### Preparation of antibodies specific for rap1A and rap1B proteins

Synthetic peptides derived from the C-terminal region of rap1A, rap1B and rap2 proteins were used to generate polyclonal antibodies in rabbits. Peptides were coupled via the C-terminal cysteine residue to keyhole limpet hemocyanin as described [27]. Briefly, 800 μg dissolved peptide and 500 μg activated keyhole limpet hemocyanin were stirred for 3 h at room temperature. The reaction mixture was applied to a PD-10 column (Pharmacia, Freiburg, FRG). Fractions with high protein content were collected and rechromatographed. Assuming a coupling efficiency of 100%, rabbits were immunized with 170 μg coupled peptide in complete Freund's adjuvant and boostered one month later with 100 μg coupled peptide in incomplete Freund's adjuvant. Rabbits were bled in intervals of two weeks after the booster.

Rap1A antiserum 213, rap1B antiserum 218 and rap2 antiserum 221 were subjected to affinity chromatography, using immobilized peptides coupled to Affi-Gel (BioRad, Munich, FRG).

### Expression of recombinant rap proteins in *Escherichia coli*

Recombinant rap1A [15] and rap2 proteins [28] were produced in *E. coli* using the ptaq 32 vector. Colonies were grown overnight at 37 °C in 50 ml of Luria-Bertani medium containing 50 μg/ml of both ampicillin and kanamycin. A 10 ml sample of this culture was then used to inoculate 1000 ml medium. The culture was allowed to grow at 37 °C to an $A_{600}$ of about 0.5. Isopropylthio-β-D-galactoside was added to a final concentration of 0.5 mM and the culture was incubated for 3 h at 37 °C. Bacteria were harvested by centrifugation and the cell sediment was lysed at room temperature in sample buffer according to Laemmli [29]. The lysate was cleared by centrifugation and an aliquot of the supernatant was subjected to SDS/PAGE and immunoblot analysis.

### Culture of HL-60 cells

HL-60 cells were grown in suspension culture in RPMI-1640 medium supplemented with 10% (by vol.) horse serum, 1% (by vol.) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified atmosphere with 7% $CO_2$ at 37 °C.

HL-60 cells, at a density of about $5 \times 10^5$ cells/ml, were treated for 72 h with 10 μM lovastatin or its vehicle. Lovastatin at this concentration was not cytotoxic to HL-60 cells, as revealed by trypan blue exclusion and did not effect cell growth, as revealed by the analysis of cell growth curves (data not shown). Additionally, lovastatin did not induce morphological maturation of HL-60 cells or functional differentiation, as revealed by the analysis of Pappenheim-stained smears of cells (J. Oertel, unpublished results) and measurement of phorbol-myristate-acetate-induced superoxide formation (data not shown).

### Preparation of membranes and cytosol from HL-60 cells

HL-60 and other cultured cells were disrupted by nitrogen cavitation and membranes ($40000 \times g$ sediment) were prepared as described [30]. Cytosol ($180000 \times g$ supernatant) was isolated by spinning the $40000 \times g$ supernatant for 1 h at $180000 \times g$ and 4 °C. Samples were frozen in aliquots in liquid nitrogen and stored at −80 °C. Protein concentrations were determined by the method of Peterson [31], using bovine serum albumin as a standard.

### SDS/polyacrylamide gel electrophoresis

Before application to the gel, proteins were precipitated by acetone, dissolved in sample buffer according to Laemmli [29] and heated for 5 min at 95 °C. 50 μg protein/lane, together with molecular-mass-marker proteins (Sigma, Deisenhofen, FRG) were separated on SDS/polyacrylamide gels prepared by the method of Laemmli [29]. The slab gels (1 mm thick) consisted of a 5% stacking gel (10 mm long) and a 12.5% separating gel (50 mm long).

### Immunoblot analysis

Subsequently to SDS/PAGE, proteins were electrotransferred onto nitrocellulose membranes for 2 h at 100 V [32]. Proteins on blots were stained with Poinceau S to ascertain that comparable amounts of protein were loaded in each lane. The nitrocellulose sheets were then reacted with the appropriate dilution of rabbit antiserum followed by anti-(rabbit-IgG)–alkaline-phosphatase conjugate, or mouse monoclonal antibody followed by anti-(mouse-IgG)–alkaline-phosphatase conjugate, as specified by the manufacturer's instructions (Promega, Madison, WI, USA). Two mouse monoclonal antibodies were used for this study, Tumark-ras 11 (NEN, Dreieich, FRG) [33] and 142-24EO5 (Microbiological Associates, Bethesda, MD, USA) [34]. The intensity of the bands on immunoblots was evaluated using a video densitometer (Biotec-Fischer, Reiskirchen, FRG).

## RESULTS

We selected peptides covering the C-terminal sequences of rap1A (amino acid residues 168–181), rap1B (amino acid residues 168–181) and rap2 (amino acid residues 164–177)

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c-Ha-ras (human)   VREIRQHKLRKLNPPDESGPGCMSCKCVLS
rap1A    (human)   NEIFYDLVRQINRKTPVEKKKPKKKSCLLL
rap1B    (human)   NEIFYDLVRQINRKTPVPGKARKKSSCQLL
rap2     (human)   DELFAEIVRQMNYAAQPDKDDPCCSACNIQ
rap2B    (human)   DELFAEIVRQMNYAAQSNGDEGCCSACVIL
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**Fig. 1. Comparison of C-terminal amino acid sequences of c-Ha-ras and rap proteins.** C-terminal amino acid sequences of primary proteins encoded by human cDNA clones for c-Ha-ras [1], rap1A [4], rap1B [8], rap2 [4], and rap2B [9] are compared. Ras and rap primary proteins undergo complex C-terminal processing events, e.g. the last three amino acids are proteolytically removed, so that cysteine becomes the C-terminal residue. Sequences of synthetic peptides used for generation of rap antibodies are underlined.

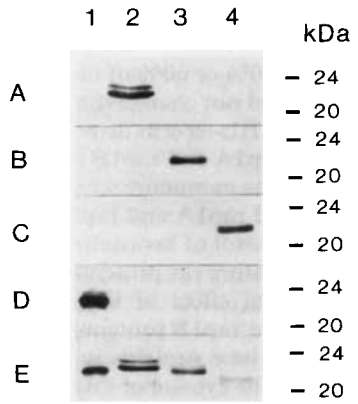


**Fig. 2. Characterization of affinity-purified antibodies raised against rap proteins by immunoblot analysis.** 100 ng purified recombinant c-Ha-ras protein (lane 1), a lysate from *E. coli* producing rap1A (lane 2), an extract from yeast *S. cerevisiae* expressing rap1B (lane 3) and a lysate from *E. coli* producing rap2 (lane 4) were subjected to SDS/PAGE and transferred to nitrocellulose. Blots were reacted with affinity-purified rap1A-antiserum 213 (A), rap1B-antiserum 218 (B) and rap2-AS 221 (C), ras11 mAb (D) and 142-24EO5 mAb (E). Values on the right indicate the position of molecular-mass-marker proteins.

(Fig. 1) to generate antibodies in rabbits. The specificity of affinity-purified antibodies against rap1A (antiserum 213), rap1B (antiserum 218) and rap2 (antiserum 221) was tested in immunoblot experiments with recombinant rap proteins. Affinity-purified antibodies against rap1A, rap1B and rap2 exclusively reacted with the corresponding recombinant rap proteins and showed no cross-reactivities (Fig. 2). Rap1A-antiserum 213 recognized two bands of approximately 22 kDa and 23 kDa in lysates from *E. coli* cells producing rap1A protein (see Fig. 2). Rap1B-antiserum 218 exclusively reacted with a band of approximately 22 kDa in extracts from yeast *Saccharomyces cerevisiae* producing rap1B protein (Fig. 2). Rap2-antiserum 221 showed strong reactivity towards a single

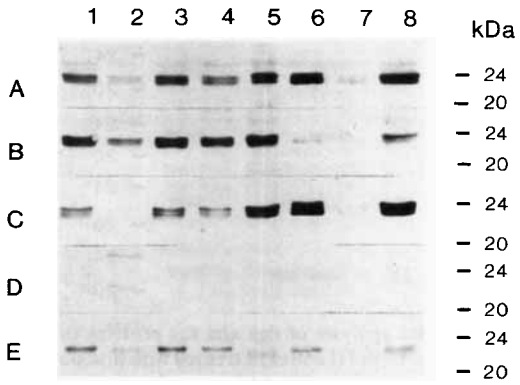


**Fig. 3. Immunoblot analysis of rap and ras proteins in membranes from mammalian cell lines and human thrombocytes.** 50 μg membrane proteins from HL-60 cells (lane 1), U-937 cells (lane 2), P388D$_1$ cells (lane 3), K-562 cells (lane 4), HEL cells (lane 5), human thrombocytes (lane 6), S49.1 cyc$^-$ cells (lane 7) and 3T3-Swiss Albino fibroblasts (lane 8) were subjected to SDS/PAGE and transferred to nitrocellulose. Blots were reacted with 142-24EO5 mAb (A), affinity-purified rap1A-antiserum 213 (B), rap1B-antiserum 218 (C), rap2-antiserum 221 (D) and ras11 mAb (E). Values on the right indicate the position of molecular-mass-marker proteins.

band of approximately 21 kDa in lysates from *E. coli* cells producing rap2 protein (Fig. 2). Binding of affinity-purified rap antibodies to recombinant rap proteins and to membranes from HL-60 cells was blocked by prior incubation of antibodies with the peptides used for immunization (data not shown).

Purified recombinant c-Ha-ras protein [35], which strongly reacted with the panreactive monoclonal antibody ras11, was not recognized by the affinity-purified antibodies against rap1A, rap1B and rap2 proteins (Fig. 2). ras11 monoclonal antibody showed only minor cross-reactivity with recombinant rap proteins (see Fig. 2).

We also tested monoclonal antibody 142-24EO5, which was raised against a synthetic peptide consisting of amino acid residues 96–118 of ras proteins [34] and which cross-reacts with rap1 proteins [21]. This antibody displayed high reactivity against recombinant c-Ha-ras, rap1A and rap1B, whereas recombinant rap2 was only weakly recognized (Fig. 2). This result is in line with a comparison of amino acid sequences between ras and rap proteins, showing that the amino acid sequence corresponding to c-Ha-ras residues 96–118 is less conserved in rap2 compared to rap1A and rap1B [1].

To investigate the distribution of small GTP-binding proteins in different mammalian cell lines, membranes were first probed with 142-24EO5 mAb, which is strongly reactive against ras and rap1 proteins (Fig. 2). This antibody detected proteins in the 22–23-kDa range in all cells tested, although at different levels (Fig. 3). Whereas in membranes from human thrombocytes and 3T3-Swiss Albino fibroblasts immunoreactivity was high, intermediate levels were seen in membranes from the leukemic cell lines HL-60, P388D$_1$, K-562 and HEL, and little immunoreactivity was detected in membranes from lymphoma cell lines U-937 and S49.1 cyc$^-$. HL-60, U-937, K-562, and HEL cell lines are of human origin, whereas P388D$_1$, S49.1 cyc$^-$ and 3T3-Swiss Albino cell lines are of mouse origin. To characterize the subtypes of rap proteins present in the membranes, we next reacted affinity-purified antibodies against subtypes of rap proteins. Rap1A-antiserum 213 showed high levels of immunoreactive 22–23 kDa pro-

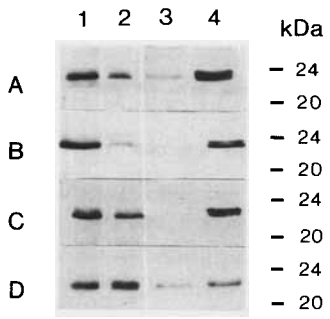


**Fig. 4. Immunoblot analysis of rap and ras proteins in membrane and cytosolic fractions from HL-60 cells treated with lovastatin.** HL-60 cells were treated for 72 h with 10 μM lovastatin or its vehicle (control). 50 μg of membrane proteins from control (lane 1) and lovastatin-treated HL-60 cells (lane 2) and 50 μg cytosol proteins from control (lane 3) and lovastatin-treated HL-60 cells (lane 4) were subjected to SDS/PAGE and transferred to nitrocellulose. Blots were reacted with 142-24EO5 mAb (A), affinity-purified rap1A-antiserum 213 (B), rap1B-antiserum 218 (C), ras11 mAb (D). Values on the right indicate the position of molecular-mass-marker proteins.

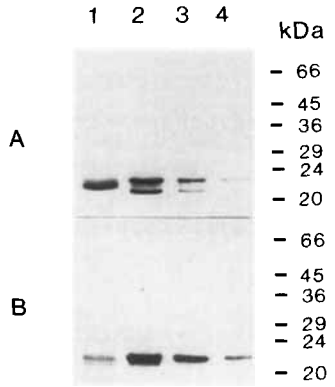


**Fig. 5. Estimation of rap1A and ras protein levels in membranes from HL-60 cells by immunoblot analysis.** (A) 50 μg membrane proteins from HL-60 cells (lane 1) and 30 ng (lane 2), 10 ng (lane 3), 3 ng (lane 4) of recombinant rap1A protein were subjected to SDS/PAGE, transferred to nitrocellulose and reacted with affinity-purified rap1A-AS 213. (B) 50 μg of membrane proteins from HL-60 cells (lane 1) and 30 ng (lane 2), 10 ng (lane 3) and 3 ng (lane 4) of recombinant c-Ha-ras protein were subjected to SDS/PAGE, transferred to nitrocellulose and reacted with ras11 mAb. Values on the right indicate the position of molecular-mass-marker proteins.

teins in membranes from HEL and P388D$_1$ leukemic cells, intermediate levels in membranes from HL-60 and K-562 leukemia cells and low levels in membranes from U-937 and S49.1 cyc$^-$ lymphoma cells as well as in membranes from human thrombocytes and 3T3-Swiss Albino fibroblasts (Fig. 3). In contrast, with rap1B-antiserum 218 high levels of immunoreactive 22–23 kDa proteins were demonstrated in membranes from human thrombocytes, 3T3-Swiss Albino fibroblasts and HEL cells (Fig. 3). Intermediate levels of rap1B were found in membranes from HL-60, P388D$_1$ and K-562 leukemia cells, whereas, as in the case of rap1A, rap1B immunoreactivity was barely detectable in membranes from U-937 and S49.1 cyc$^-$ lymphoma cells (Fig. 3). Interestingly, affinity-purified rap2-antiserum 221, which has been shown to display strong immunoreactivity against recombinant rap2 protein (Fig. 2), detected only minor immunoreactive bands in the 20–24 kDa range in membranes of some cells tested (Fig. 3). We used ras11 mAb to analyze the levels of ras proteins in membranes from mammalian cell lines and human thrombocytes. As shown in Fig. 3, ras immunoreactivity in the 21–22 kDa range was clearly detectable in membranes from HL-60, P388D$_1$, K-562, 3T3-Swiss Albino cells and human thrombocytes, whereas ras proteins were present at lower levels or were barely detectable in membranes from HEL, U-937 and S49.1 cyc$^-$ cells.

It has been demonstrated for ras proteins that isoprenylation of the C-terminus is a prerequisite for proper membrane insertion. To test whether rap1A and rap1B proteins are isoprenylated in membranes from HL-60 cells, we treated HL-60 cells with lovastatin, an inhibitor of hydroxymethyl-glutaryl-coenzyme A reductase, and thereby, of isoprenylation. Membranes and cytosol from control and lovastatin-treated HL-60 cells were subjected to immunoblot analysis with several antibodies. Application of 142-24EO5 mAb, which is highly reactive against ras and rap1 proteins (Fig. 2), showed that levels of immunoreactive proteins were decreased in membranes from lovastatin-treated HL-60 cells, whereas immunoreactivity in the cytosol was strongly increased (Fig. 4). Specific antibodies against rap1A, rap1B and ras proteins (Fig. 4) showed that treatment of HL-60 cells with lovastatin affected the membrane concentration of small GTP-binding proteins in a different way. After lovastatin treatment, the membrane content of rap1A and rap1B protein was reduced to about 20% or 60% of the control respectively, whereas the ras level did not change significantly. Analysis of cytosol from untreated HL-60 cells demonstrated the presence of low levels of both rap1A and rap1B immunoreactivity and intermediate levels of ras immunoreactivity. Whereas concentrations of unprocessed rap1A and rap1B proteins increased very strongly in the cytosol of lovastatin-treated HL-60 cells, concentrations of immature ras proteins increased only moderately. The differential effect of lovastatin on membrane levels of ras, rap1A and rap1B proteins is most likely due to different half-lives of these proteins in HL-60 cells. Rap1A and rap1B proteins in the cytosol of lovastatin-treated HL-60 cells consist of two immunoreactive bands, the upper one, with slightly decreased electrophoretic mobility, suggesting an unprocessed form of these proteins.

Differentiation of HL-60 cells towards neutrophils with dibuturyl cAMP (0.2 mM) for 48 h did not lead to significant changes in the levels of rap1A and rap1B proteins in membranes or cytosol (data not shown).

We used our specific antibody against rap1A to measure the amount of rap1A protein in membranes from HL-60 cells (Fig. 5). Comparison with recombinant rap1A protein [15] is hampered by the fact that the purified protein consists of two well-separated immunoreactive bands. By evaluating the intensity of rap1A (ras) immunoreactivity in membranes and purified recombinant rap1A (c-Ha-ras), we calculated that rap1A constitutes about 0.04% of membrane proteins, whereas ras proteins constitute about 0.01% of membrane proteins.

## DISCUSSION

We have generated specific antibodies against the small GTP-binding proteins rap1A, rap1B and rap2. Although the production of antibodies against rap1 proteins has been described by several groups [24, 25, 36], no subtype-specific

antibodies against rap1A or rap1B have been described so far. Rap1A and rap1B proteins share 95% amino acid similarity, but the C-terminal sequences are less conserved. This in line with the observation that C-terminal amino acid sequences of ras proteins show only little similarity between Ha-ras, Ki-ras and N-ras. To generate specific antibodies against rap1A and rap1B, we synthesized peptides comprising the 14 amino acids from the extreme C-terminus of processed rap1A and rap1B proteins. These antibodies specifically recognized the corresponding recombinant proteins and, furthermore, reacted with proteins of 22–23 kDa molecular mass in membranes from various mammalian cell lines and human thrombocytes.

To generate rap2-specific antibodies, we synthesized a peptide comprising amino acids 164–177 of the rap2 protein. Although the rap2 antibody was strongly reactive against recombinant rap2 protein, we found no clear immunoreactive bands in the 20–24 kDa region using membranes from several mammalian cell lines and human thrombocytes. We cannot exclude the possibility that rap2 levels in membranes from cells tested were beyond the sensitivity of our immunoblot analysis. Alternatively, yet unknown posttranslational modifications may inhibit recognition of rap2 in membranes from mammalian cells, whereas the antiserum is highly reactive against recombinant rap2.

An interesting picture emerges from our analysis of rap1A and rap1B immunoreactivity in membranes prepared from various leukemia and lymphoma cell lines. Whereas rap1A and rap1B proteins were present in substantial amounts in membranes from human leukemia cell lines HL-60, K-562, HEL and mouse leukemia cell line $P388D_1$, both proteins were barely detectable in membranes from human histiocytic lymphoma cell line U-937 and mouse lymphoma cell line S49.1 $cyc^-$. In most of these cell lines, rap1A immunoreactivity is higher compared to rap1B immunoreactivity. The situation is different for membranes prepared from human thrombocytes and mouse 3T3-Swiss Albino fibroblasts, where rap1B is present at much higher levels compared to rap1A.

By subcellular fractionation and reaction with an antiserum recognizing both rap1A and rap1B proteins, rap1 immunoreactivity was localized exclusively in the particulate fraction of Rat-1 fibroblasts and HL-60 cells [25]. In contrast to these data, we find low but detectable rap1A and rap1B immunoreactivity in the cytosol of HL-60 cells, as was also demonstrated for the cytosol of human platelets [36].

Culine et al. [37] studied the expression of rap genes in various human tumors and cell lines by Northern-blot analysis. rap1A and rap1B mRNA are apparently expressed at similar levels in the various cells examined in this study. This fits our data showing that rap1A and rap1B immunoreactivity in membranes of several cell lines is comparable. Additionally, expression of rap2 mRNA is low in all cells examined [37]. Our failure to detect rap2 immunoreactivity in membranes from tested mammalian cell lines and human thrombocytes may be explained by the presence of low levels of rap2 mRNA, and thereby rap2 protein, in these cells.

With both rap1A and rap1B antibodies we find multiple immunoreactive bands in the 22–23 kDa range in membranes from all cells tested including HL-60 cells. This heterogeneity appears not to rely on proteolysis during membrane preparation, since the addition of various commonly used protease inhibitors did not reduce the number of immunoreactive bands in membranes prepared from HL-60 cells (data not shown). Winegar et al. [36] recently reported that a rap1-common antiserum detects immunoreactive bands of 22 kDa and 24 kDa in membranes from human platelets. In this study, an antiserum recognizing the unprocessed form of rap1B protein reacts exclusively with a 24-kDa band [36]. Our data using antibodies specific for rap1A or rap1B proteins, but not selective for the unprocessed or processed forms of these proteins, demonstrate that rap1A is only present in low amounts in platelet membranes. The upper 23 kDa band stained by our rap1B antibody may consist of unprocessed rap1B. It should be noted, however, that the 23 kDa bands recognized by rap1A or rap1B antibodies are not restricted to differentiated cells like human thrombocytes, but can also be seen in membranes from various mammalian cell lines. In addition to C-terminal processing, heterogeneity of rap1 proteins could also rely on other posttranslational modifications such as phosphorylation. Indeed, it has been shown, that the rap1 proteins are phosphorylated by cAMP-dependent protein kinase and that the electrophoretic mobility of the protein is altered by phosphorylation [38, 39].

Like ras proteins [10], rap proteins are expected to undergo a complex processing of C-terminal sequences. To examine whether rap1A and rap1B were isoprenylated in membranes from HL-60 cells, HL-60 cells were treated with lovastatin, an inhibitor of hydroxymethylglutaryl-coenzyme A reductase and thereby of isoprenoid biosynthesis. Whereas rap1A and rap1B immunoreactivity was reduced in membranes from cells treated with lovastatin, we observed a strong accumulation of immunoreactivity in the cytosol. Ras immunoreactivity in membranes did not change significantly after treatment of HL-60 cells with lovastatin. The fact that ras immunoreactivity was slightly increased in the cytosol of lovastatin-treated HL-60 cells is in line with results showing that ras proteins are isoprenylated in mammalian cells. The different action of lovastatin on ras and rap1 proteins suggests that the half-life for ras proteins in membranes from HL-60 cells is much longer than that for rap1 proteins. As a consequence of different half-lives for isoprenylated proteins, drugs like lovastatin, which unspecifically block the isoprenylation of these proteins, may selectively decrease membrane levels of defined small GTP-binding proteins in short-term experiments. Treatment of HL-60 cells with 10 μM lovastatin for 72 h did not affect cell growth (data not shown), a result that is in line with unchanged levels of ras proteins in membranes from lovastatin-treated HL-60 cells.

Overexpression of rap1A (Krev-1) cDNA in ras-transformed fibroblasts leads to suppression of the transformed phenotype, but membrane levels of ras and rap1A proteins were not measured in this study [6]. In comparison with purified recombinant rap1A and c-Ha-ras protein, we estimated the ratio of rap1A/ras in membranes from HL-60 cells and found the rap1A protein to be present in about fourfold greater amounts than ras proteins. It has been shown that HL-60 cells possess an activated N-ras oncogene together with altered versions of the myc oncogene [40]. If the activated N-ras oncogene were linked to oncogenic transformation in these cells, one would expect that overexpression of the rap1A protein inhibits the transformed phenotype, as has been described for fibroblasts [6]. Corresponding to our data, a moderate excess (about fourfold) of the potential tumor suppressor protein rap1A above ras is apparently not sufficient to keep HL-60 cells in an untransformed state.

In conclusion, we have generated specific antibodies against the rap1A, rap1B and rap2 small GTP-binding proteins. By immunoblot analysis, we have shown that concentrations of rap1A and rap1B are highly variable in membranes from different mammalian cell types and that both proteins are isoprenylated.

We thank Dr A. Wittinghofer (Heidelberg, FRG) for purified recombinant c-Ha-ras and rap1A proteins and *E. coli* cells producing rap1A, Dr V. Pizon (Paris, France) for an extract from yeast expressing rap1B and *E. coli* cells producing rap2, and Dr A. Söling and B. Schein for membranes from human thrombocytes. We are grateful to Dr K.-D. Hinsch for help in analyzing the hydrophobicity of rap proteins and Dr M. Guschmann for help during coupling of peptides. The technical assistance of R. Bonnet, M. Bigalke, I. Reinsch and I. Tychowiecka is gratefully acknowledged. We thank Dr H.-H. Kiltz (Bochum, FRG) for help with video densitometer analysis and K. Grabert (Bochum, FRG) for photographic work.

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