Nucleotide induced conformation determines posttranslational isoprenylation of the ras related rab6 protein in insect cells

Anke C. Schiedel, Angelika Barnekow, Thomas Mayer*

Department for Experimental Tumorbiology, Muenster University, Badestr. 9, 48149 Muenster, Germany

Received 10 August 1995; revised version received 20 October 1995

Abstract Small GTP binding proteins of the rab/YPT family are essential regulators of vectorial transport in the eukaryotic cell. Members of the rab/YPT1 family are found on the cytoplasmic surface of distinct intracellular membrane compartments. Membrane attachment is facilitated by a C-terminal geranylgeranyl moiety. In this report we investigated posttranslational modification and membrane binding of the rab6 protein, a member of the rab/YPT family located on the Golgi apparatus. A set of point mutations, which simulate the GDP or GTP bound conformation, was introduced into the rab6 cDNA. The mutated cDNAs were expressed in insect cells and the ability of the protein products to undergo geranylgeranyl modification and membrane association was assessed by Triton X-114 partition and cell fractionation. We report here that the modification of rab6 in insect cells depends on protein conformation. Only the GDP bound form, but not the GTP bound form is isoprenylated and subsequently membrane bound.

Key words: GTP; Ras protein; Intracellular transport; Rab proteins; GTPase

1. Introduction

The eukaryotic cellular protein transport between the different membrane compartments of the exo-/endocytotic pathway is mediated by vesicles (for review see [1]). These vesicles are formed from a donor compartment through the interaction of the lipid bilayer with the coat proteins and the small GTP-binding protein ARF. After budding, vesicles are targeted to the acceptor membrane. Targeting and fusion is mediated by several different factors, namely the SNAPs, NSF and the SNARE proteins, which are all complexed in the 20S particle [2,3]. The principal components of budding and fusion events are conserved in most transport processes throughout the exo/endocytotic machinery. The major regulatory event in these transport processes is GTP hydrolysis (for review see [4]). So far three classes of GTP-binding proteins involved in vesicular transport have been described. In addition to the ARF proteins and the heterotrimeric G-proteins, an increasing number of rab GTPases are known to regulate intracellular vesicle transport [5,6]. Today rab proteins represent a subfamily of the ras superfamily with more than 30 members which are localized on the cytoplasmic surface of distinct organelle membranes.

The functional status of the rab proteins is determined by the nucleotide bound to the protein. Rab proteins cycle between an active form in which GTP is bound and an inactive form in which GDP is bound. The hydrolysis of the nucleotide results in a conformational alteration of the protein. The activity of these proteins requires an array of accessory factors which regulate the GTPase cycle. At present four classes of proteins are known to control rab GTPases [7].

(1) The GTPase activating protein, GAP, which enhances the low endogenous GTPase activity.

(2) The guanosine nucleotide exchange factor, GEP, which accelerates the exchange of GDP to GTP.

(3) The rab escort protein, REP, which is a part of the rab geranylgeranyl transferase complex.

(4) The guanosine nucleotide dissociation inhibitor, GDI, a molecular chaperone of cytosolic rab proteins.

GDI is known to interact with most of the rab family members [8]. Four functions have so far been assigned to GDI: (1) it inhibits the exchange of bound GDP to GTP; (2) it accompanies cytosolic rab proteins; (3) it directs the rab proteins to their target membranes [9], where, with subsequent exchange of GDP for GTP, they become membrane bound [9]; and (4) it can extract rab proteins from the membranes [10,11].

Similar to other ras-like proteins, rab proteins also undergo posttranslational modifications, which are essential for their function [12]. The rab geranylgeranyl transferase modifies the rabs by covalently adding geranylgeranyl groups onto cysteine residues [13]. The modified cysteines are found in a CC, CXC, CXX or CXXX motif at the C-terminal part of the proteins. Nonmodified rab proteins are unable to associate with intracellular membranes and do not complex with GDI [14]. Complex formation constitutes the key event for the rab's entrance into their life cycle [8]. In contrast to isoprenylation, carboxy-methylation, a second posttranslational modification found on rab proteins is neither necessary for the GDI interaction nor for membrane attachment.

The rab geranylgeranyl transferase (GG transferase), originally termed geranylgeranyl transferase II, is a two component enzyme [15]. Nonmodified rab proteins are presented by the component A, also called rab escort protein (REP), to the catalytic component B [16]. Component A can be recycled by passing the modified rab proteins onto GDI [16]. Several recent studies have investigated the structural requirements for geranylgeranyl addition to rab proteins. In addition to the cysteines at the C-terminus, internal parts of the rab proteins are required for geranylgeranyl modification. For rab6 it was shown that the structures of both the effector domain and the hypervariable domain determine a modification by the rab GG

*Corresponding author. Fax: (49) (251) 83-8390.

Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; SNAP, soluble NSF attachment protein; SNARE, SNAP receptor; GAP, GTPase activating protein; GDI, guanosine nucleotide dissociation inhibitor; GDS, guanosine nucleotide dissociation stimulating protein; GTPγS, guanosine 5′-*O*-thiophosphate; REP rab escort protein.

SSDI 0014-5793(95)01258-3

transferase [17]. The same structures are also involved in targeting the rab6 protein to the Golgi apparatus [17]. Studies on the related rab5 protein revealed that addition of geranylgeranyl residues depends on the nucleotide bound and thus on the protein conformation [18]. The aim of our study was to elucidate how nucleotides liganded to rab6 determine modification and subsequent target membrane association. We used the rab6 protein as a model to study the influence of the protein's conformation on the process of isoprenylation. Different point mutations were introduced into the rab6 cDNA resulting in proteins which showed conformations corresponding to GTP or GDP bound forms of rab6. To study modification and membrane binding, mutant cDNAs were introduced into recombinant baculoviruses and the proteins were expressed in insect Sf9 cells. The resulting polypeptides were analyzed for their ability to undergo posttranslational modification and subsequent membrane binding.

2. Material and methods

2.1. Chemicals

Low melting point agarose was purchased from FMC BioProducts (Rockland) and nitrocellulose was from Schleicher and Schüll (Dassel, Germany). Secondary goat anti-mouse IgG coupled to peroxidase and [α-^{32}P]GTP (3000 Ci/mmol) were from Amersham (Braunschweig, Germany) and [^{3}H]mevalonolactone (20–30 Ci/mmol) from DupontNEN (Bad Homburg, Germany). Reticulocyte lysate was obtained from Serva (Heidelberg, Germany).

2.2. Cells and antibodies

Spodoptera frugiperda (Sf9) cells were grown in Grace insect medium supplemented with yeastolate, lactalbumin and 10% heat inactivated fetal calf serum (Life Technologies, Eggenstein, Germany). Cells were grown at 27°C and subcultured twice a week. *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV) was purchased from Invitrogen (San Diego). The rab6 specific monoclonal antibody was raised against the peptide: NH_2-Cys-Tyr-Gly-Met-Glu-Ser-Thr-Gln-Asp-Arg-Ser-Arg-Glu-Asp-Met-Ile-Asp-Ile-Lys-Leu-Glu-Lys-Pro-Gln-Glu-Gln-Pro-Val-Ser-Glu-Gly-Gly-COOH corresponding to amino acids Gly^{176} to Gly^{206} of the human rab6 protein [11]. The *myc*-specific monoclonal antibody 9E10 was described earlier and the hybridoma cells were obtained from ATCC [19].

2.3. DNA constructs

The source for the rab6 cDNA was a human cDNA clone [20]. A *Nde*I restriction site was introduced at the start ATG to facilitate the insertion of an oligonucleotide coding for the *myc*-epitope tag. The sequence NH_2-Met-Glu-Gln-Lys-Leu-Ile-Ser-Glu-Glu-Asp-Leu-His-COOH corresponds to an epitope for the monoclonal antibody 9E10, originally raised against the c-*myc* protein [19], and thus allows immunodetection of the rab6 protein. The structure of the resulting fusion protein is depicted in Fig. 1B. Point mutations were introduced by subcloning the rab6 cDNAs into pTZ19 and by site-directed mutagenesis using the thioanalogue method as described by Taylor [21]. Wildtype and mutated cDNA were inserted into the pBlueBacHis A transfer vector (Invitrogen, San Diego) using the *Bam*HI site.

2.4. Generation of recombinant baculoviruses

Recombinant baculoviruses were generated as described by Guarino and Summers [22]. Briefly, 2×10^6 cells were cotransfected with 1 μg Ac*M*NPV virus DNA and 2 μg plasmid DNA using Ca-phosphate transfection. Six days after transfection supernatant was harvested. From the supernatant recombinant viruses were plaque purified using 5-brom-4-chlor-3-indolyl-β-D-galactopyranoside (Xgal) as a substrate to detect the β-galactosidase activity of the recombinant proteins.

2.5. Expression of proteins

1.8×10^7 Sf9 cells were infected with wildtype or recombinant baculoviruses at a multiplicity of infection (moi) of 1. 72 h after infection cells were harvested. Cells were homogenized with 20 strokes in a tight fitting Dounce homogenizer in extraction buffer (20 mM Tris-HCl, 150 mM KCl containing 1 mM PMSF and 1 mM NaF).

2.6. Cell fractionation

Cell lysate from rab6 wt, T27N or Q72R infected Sf9 cells was supplemented to 1 M KCl. Membrane and cytosolic forms of the proteins were separated by a $100{,}000 \times g$ centrifugation step (SW60 rotor) for 30 min at 4°C. Corresponding aliquots were analyzed on 15% SDS-PAGE.

2.7. Triton X-114 separation

Rab6 protein from cell lysates of infected cells were analyzed for their geranylgeranyl modification by Triton X-114 partition as described by Bordier [23].

2.8. SDS-Page and Western blotting

Samples were analyzed on 15% SDS-PAGE and blotted onto nitrocellulose. Blots were incubated with 1:100 dilutions of supernatant from 9E10 or 5B10 cultures and horseradish peroxidase conjugated secondary antibody. Antibodies were detected using chemiluminescence (Amersham, Braunschweig) and documented on X-ray film (Fuji Photo Film Co., Japan).

2.9. GTP binding assays [24]

Purified rab6 wt, T27N and Q72R proteins (2 μg each) were separated by SDS-PAGE and blotted onto nitrocellulose. The membranes were preincubated in GTP-binding buffer (50 mM NH_2PO_4 pH 7.5, 10 μM $MgCl_2$, 2 mM DTT, 0.2% Tween 20, 4 μM ATP) for 30 min at room temperature. GTP binding was done with [α-^{32}P]GTP (3000 Ci/mmol) at 1 μCi/ml in binding buffer for 1 h at room temperature. Membranes were washed three times for 5 min in binding buffer and exposed to X-ray film for 10 min.

2.10. GTPase assays [25]

6 μg of purified rab6 wt or mutant proteins were loaded on ice with 10 μCi [α-^{32}P]GTP (3000 Ci/mmol) in 50 mM Tris-HCl pH 7.5, 0.2 mM EDTA, 1 mM DTT, 10 μM GTP. The reaction was started by adding 10 μM $MgCl_2$ and raising the temperature to 37°C. Aliquots were removed at various time points and the reaction was stopped by adding 0.2% SDS, 5 mM EDTA, 50 mM GDP and 50 mM GTP. The samples were separated by PEI thin-layer chromatography. The amount of converted GDP was quantified by scintillation counting.

2.11. Labeling of Sf9 cells with [^{3}H]mevalonolactone

48 h after infection (moi = 5), Sf9 cells were incubated with 50 μM compactin for 1 h. Cells were labeled by addition of 50 μCi of [^{3}H]mevalonolactone (20–30 Ci/mmol) for 16 h. Cells were harvested and lysed as described above.

2.12. Geranylgeranylation of bacterial rab6 proteins by reticulocyte lysate [26]

To determine the K_m value increasing amounts of bacterially expressed rab6 proteins were incubated with 20 μl of untreated reticulocyte lysate in a 25 μl incubation mix containing 0.5 mM $MgCl_2$, 1 mM DTT, 50 μM $ZnCl_2$, 1 mM NP-40, 40 μM mevalonolactone and 20 μCi [^{3}H] mevalonolactone (20–30 Ci/mmol) for 1 h at 37°C. To investigate modification of rab6 Q72R 0,3 μg of wt or mutant protein were incubated in a 50 μl assay with 5 μCi of [^{3}H]mevalonolactone (20–30 Ci/mmol). Reactions were stopped by addition of 0.5 ml of 4% SDS and 0.5 ml of 30% trichloracetic acid. After 45 min on ice, samples were filtered on glass fiber filters (Whatman GF/B). After washings the retained radioactivity was determined by scintillation counting.

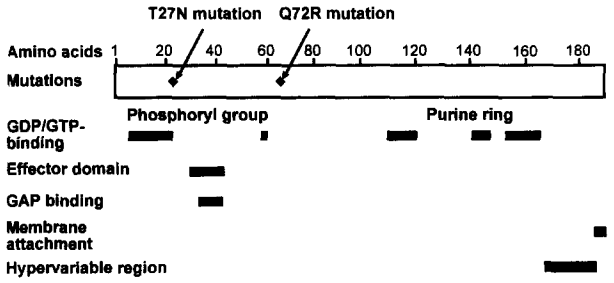
3. Results

To study the influence of guanosine nucleotide attachment to rab6 on the geranylgeranyl modification, two different mutants were generated (Fig. 1A). Rab6 T27N has an exchange of threonine-27 to asparagine. This mutation results in a protein that binds GDP with a much higher affinity than GTP, thus permanently blocking the GTPase activity. The change of Gln^{72} to Arg in rab6 Q72R abolishes the intrinsic GTPase activity of

A

name of the mutant	changes in the amino-sequence	effect of the mutation
rab6 T27N	Thr 27 → Asn 27	binds GDP → inactive
rab6 Q72R	Gln 72 → Arg 72	no GTPase activity → permanently active

B



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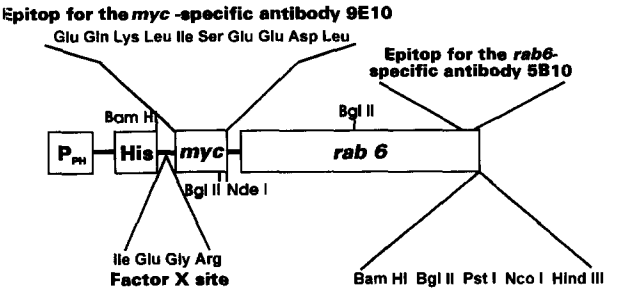

Fig. 1. Structure of the rab6 cDNAs. (A) The table displays the rab6 mutants and the resulting phenotypes and summarizes the amino acid changes in the mutant proteins rab6 T27N and Q72R and the resulting nucleotide binding phenotypes. (B) The structure displays of the point mutations within rab6 T27N and Q72R and the functional domains in the rab6 polypeptide. The model was modified after Barbacid [12]. (C) Structure of the rab6 cDNAs in the recombinant baculovirus. The His-tag (His) and the *myc*-epitope (myc) are located at the 5'-end of the rab6 coding region. Transcription is initiated at the polyhydrin promoter (P_{PH}). Translation is started at an ATG preceding the His-tag. The recognition sequence for the factor X protease was inserted between the His-tag and the *myc*-epitope.

the resulting polypeptide; therefore, the protein remains in a permanently active form. The topology of the mutants is summarized in Fig. 1B. To detect the protein after expression in Sf9 cells, a *myc* antigenic epitope tag was added to the NH_2 domain. The resulting *Bam*HI fragment, encoding the *myc*-tagged human rab6 protein, was introduced into the pBlueBacHisA transfer vector and fused to a polyhistidine region provided by the vector. The structure of the rab6 DNAs is depictured in Fig. 1C.

Sf9 cells were infected with the recombinant viruses and assayed for expression of rab6 proteins. Fig. 2 shows the results of Western blot analyses using the *myc* epitope specific 9E10 antibody (Fig. 2B) or the rab6 specific mab 5B10 (Fig. 2A). Neither the mock-infected nor the Ac*M*NPV wt infected cells revealed a signal. Cross-reaction of endogenous *myc* or rab6 protein is therefore excluded. In rab6 wt, rab6 T27N and rab6 Q72R infected cells, recombinant rab6 proteins with a molecular weight of about 30 kDa could clearly be detected by either the *myc* tag specific mab 9E10 (Fig. 2B) or the rab6 specific mab 5B10 (Fig. 2A). For rab6 wt and rab6 T27N proteins, the bands can be resolved into a double band using shorter exposure times (Fig. 2). The relative amounts of both bands vary between different experiments and seem to correlate with the amount of recombinant proteins in the infected cells. At higher levels of expression the lower band becomes more prominent. In rab6 Q72R virus infected cells only a single band could be observed. The appearance of bands with different molecular weights can result either from addition of one or two geranylgeranyl groups or from proteolytic cleavage of the rab6 protein. Addition of geranylgeranyl groups to different rab proteins alter the electrophoretic mobility. While the geranylgeranylated form of rab1b displays a slower mobility than the nonmodified form, for rab3a the opposite behavier was described [27]. It is unlikely that the two bands represent the result of proteolytic activities inside the *myc*-rab6 fusions protein since our two antibodies recognize both the NH_2-terminal part (9E10) and the very COOH-terminal part (5B10) of the molecule.

To confirm the integrity and proposed phenotypes of the rab6 wt and mutant proteins, Sf9 cells were infected with the corresponding recombinant viruses. Proteins were isolated from the 100,000 × *g* supernatants of the cell lysates by chromatography on Ni-affinity matrix. Fig. 3A shows that the rab6 wt, T27N and Q72R proteins can be purified as a single band of about 30 kDa from the lysates of the infected cells. The

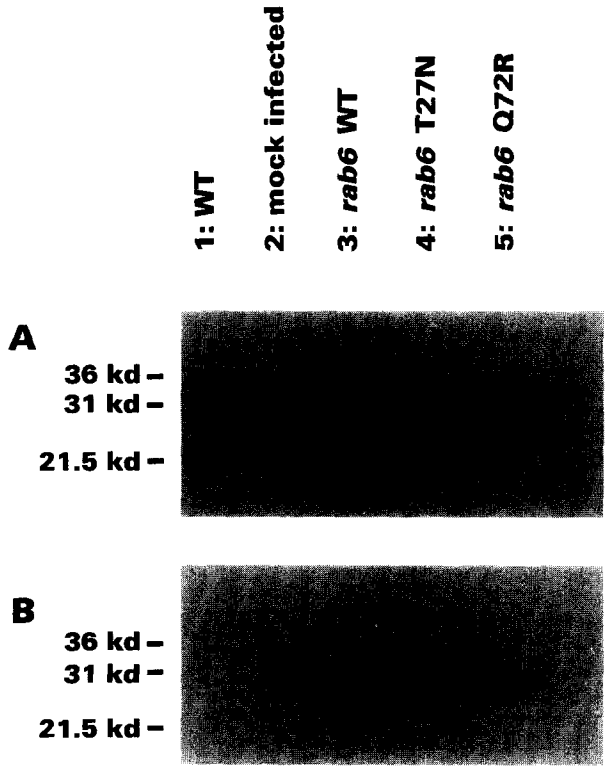

Fig. 2. Detection of rab6 proteins in baculovirus-infected cells Sf9 cells were mock infected (mock) or infected with wildtype Ac*M*NPV (wt) or recombinant viruses containing rab6 wt, rab6 T27N or rab6 Q72R cDNAs at a moi of 1. 72 h after infection cells were homogenized. Cell lysates were separated on 15% SDS-gels and blotted onto nitrocellulose. Blots were incubated with the rab6 specific mab 5B10 (A) or the *myc*-specific mab 9E10 (B) and peroxidase coupled secondary antibodies. Proteins were visualized using chemiluminescence as described in section 2.

purified proteins were separated by SDS-PAGE, transferred to nitrocellulose and the ability to bind [^{32}P]GTP was assessed by an overlay assay. As seen in Fig. 3B rab6 wt and rab6 Q72R bind GTP on the blot. In contrast, no GTP-binding activity could be observed for the rab6 T27N protein. To detect the endogenous GTPase activity, isolated rab6 wt and rab6 Q72R proteins were preloaded with [α-^{32}P]GTP. After incubation for different times at 37°C the conversion of GTP to GDP was determined by thin-layer chromatography. As seen in Fig. 3C, rab6 Q72R displayed a drastically reduced GTPase activity. The results presented in Fig. 3A and B confirmed that the three different rab6 proteins are expressed in the Sf9 cells in similar amounts and in an native form as demonstrated by the GTP-binding and GTPase assays.

To analyze the modification of the different rab6 proteins we used Triton X-114 extraction. It has previously been described that isoprenylated ras-like proteins are separated into the detergent-containing phase after lipid modification [17]. The results of the separation experiments are shown in Fig. 4A. Sf9 cells were infected with the recombinant viruses indicated above and harvested 72 h after infection. Equal aliquots were extracted with Triton X-114. The aqueous supernatant (S) and the detergent pellet (P) fractions were analyzed by SDS page and Western blot analysis using the 5B10 antibody. Fig. 4A shows that almost the entire amount of the expressed rab6 wt protein migrates into the detergent phase (Fig. 4A, lanes 1 and 2). The low molecular weight bands in Fig. 4A most likely represent proteolytic degradation production. Since the Triton X-114 separation procedure includes several incubations at elavated temperatures, degradation is much more prominent than in the other experiments. Thus insect cells are clearly able to modify the rab6 wt protein posttranslationally. Modification of rab proteins in insect cells has been shown before [28]. Rab6 wt and rab6 T27N become modified to a similar extent as shown in Fig. 4A, lanes 1–4. The Triton X-114 extraction demonstrates that most of the rab6 wt and rab6 T27N molecules are isoprenylated in infected insect cells [17]. The extent of modification depends on the period between infection and cell harvest. At postinfection times later than 72 h a lower rate of modification is observed (data not shown). Rab6 Q72R shows a different behavior. After detergent extraction the total amount of the rab6 Q72R protein remains in the supernatant. Thus rab6 Q72R is not posttranslationally modified by an isoprene in Sf9 cells. We in addition analyzed cell lysates at different postinfection times, but were unable to detect a modification of rab6 Q72R at any time. Furthermore, we labeled Sf9 cells infected with the recombinant rab6 baculoviruses with [^{3}H]mevalonolactone, a precursor of the geranylgeranyl moiety. Cell lysates were separated on a 15% SDS gel and subjected to fluorography. Fig. 4B shows

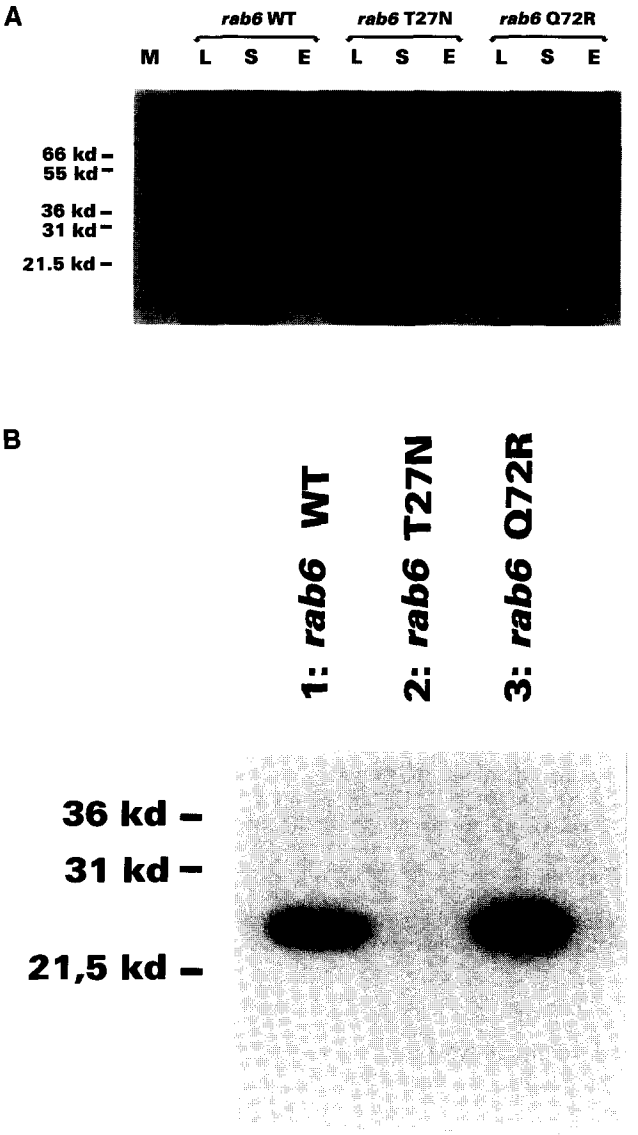

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Fig. 3. Characterization of recombinant rab6 proteins. (A) Sf9 cells were infected with recombinant rab6 wt, T27N and Q72R baculoviruses (moi = 1). 72 h after infection cells were harvested. Cell extracts (2.5 mg/ml) were cleared by centrifugation at 100,000 × g for 1 h. Supernatants were loaded on a Ni-NTA affinity column. Rab proteins were eluted with 50 mM imidazole pH 7.0. Corresponding fractions of the total lysates (L), supernatants (S) and eluted rab6 proteins (E) were separated on a 15% SDS gel and stained with Coomassie blue. (B) To determine the GTP binding properties 2 μg of rab6 wt, rab6 T27N and Q72R were purified from Sf9 cells infected with the recombinant viruses and separated on 15% SDS gels and transferred to nitrocellulose. The membranes were incubated with [α-^{32}P]GTP (3000 Ci/mmol) and exposed to X-ray film for 10 min. (C) The GTPase activity of rab6 wt and rab6 Q72R protein was assessed by preloading of 6 μg of purified protein with 10 μCi of [α-^{32}P]GTP (3000 Ci/mmol). The loaded proteins were incubated at 37°C. Aliquots were taken at the time points indicated. GTP and GDP were separated by thin-layer chromatography. The radioactivity in the converted GDP was determined by scintillation counting.

that the rab6 wt and the rab6 T27N proteins were labeled with the tritiated geranylgeranyl residue while no labeled rab6 protein could be detected in Sf9 cells infected with the Q72R virus. Parallel labeling experiments using [^{35}S]methionine revealed that all three proteins were expressed in equal amounts in the infected cells (data not shown).

To investigate whether the lack of modification of rab6 Q72R is limited to the Sf9 insect cells we investigated the ability of a mammalian in vitro system to modify the rab6 proteins.

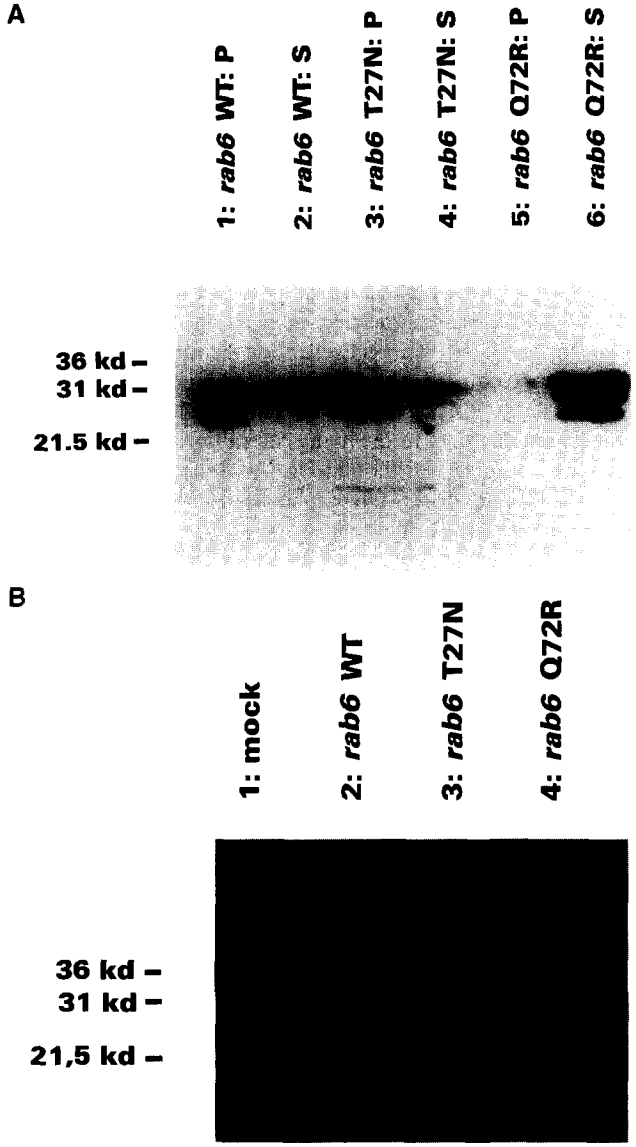

Fig. 4. Isoprenylation of rab6 proteins in infected Sf9 cells Sf9 cells were infected with rab6 wt, rab6 T27N or rabQ72R recombinant Ac*M*NPV. (A) 72 h after infection, cell lysates were extracted with Triton X-114 as described in section 2 and separated into the detergent-containing pellet (P) and the aqueous supernatant (S). Under these conditions modified rab6 proteins separate into the detergent phase while unmodified apoproteins remain in the supernatant. Aliquots of the pellet and supernatant phases were separated on 15% SDS-gels and transferred to nitrocellulose. Rab6 proteins were detected using the rab specific antibody 5B10. Blots were developed as described in Fig. 2. (B) 48 h after infection 50 μM compactin was added to the medium. Cells were labeled for 16 h with 50 μCi/ml of [^{3}H]mevalonolactone (20–30 Ci/mmol). Cells were harvested and total cell lysates were separated on 15% SDS gels. After treatment with DMSO/PPO gels were exposed for 24 h.

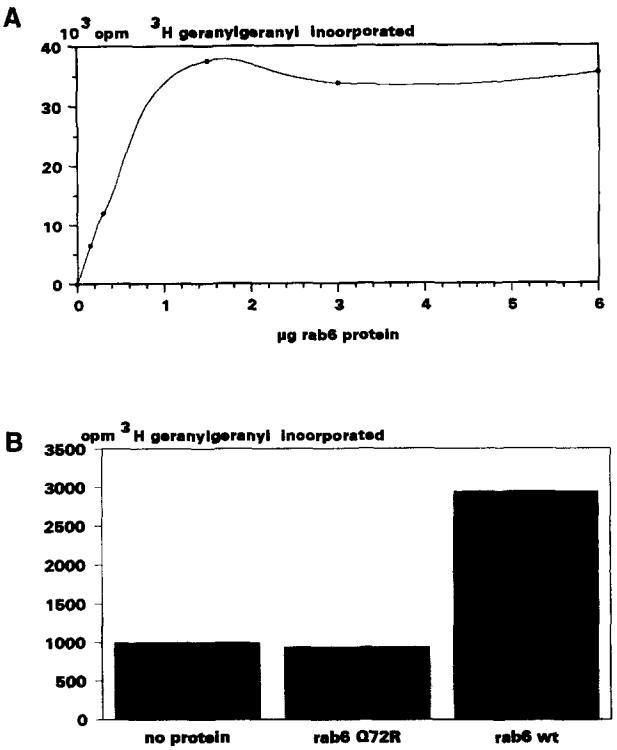

Fig. 5. In vitro isoprenylation of rab6 proteins. Rab6 wt and rab6 Q72R were purified from recombinant *E. coli*. Increasing amounts of rab6 wt or rab6 Q72R protein were added to reticulocyte lysate in the presence of [^{3}H]mevalonolactone (20–30 Ci/mmol) and incubated for 1 h. The amount of modified rab6 protein was determined by TCA precipitation. The insoluble material was filtered onto glass fiber filters and the incorporated radioactivity messuased by scintillation counting. (A) Determination of the K_m for rab6 wt. Increasing amounts of rab6 protein were incubated in the in vitro prenylation assay. (B) Comparison of the isoprenylation of rab6 wt and rab6 Q72R. Equal amounts of both proteins (0.3 μg) were added to the reticulocyte lysate and the amount of modified rab6 protein was determined.

Rab6 wt and rab6 Q72R proteins were expressed and purified from *E. coli* and incubated with reticulocyte lysate in the presence of [^{3}H]mevalonolactone as described by Hori et al. [26]. Fig. 5A shows that rab6 wt is an efficient substrate for the rab geranylgeranyl transferase from the reticulocyte lysate. Increasing amounts of rab6 protein were added to determine the K_m value for the rab6 wt protein and a K_m value of 1.3 μM could be calculated. This is similar to the K_m of 1.42 μM which was determined by Beranger et al. for the reaction of rab6 and purified rab geranylgeranyl transferase [17]. While rab6 wt protein is modified in the mammalian system, only a negligable incorporation of radioactive geranylgeranyl could be detected for the rab6 Q72R protein as shown in Fig. 5B.

To assess the membrane-binding ability of different rab6 polypeptides, we separated proteins from infected cells into cytosolic and membrane fractions. To discriminate between loosely associated and tightly bound rab proteins, membranes were extracted with 1 M KCl, since rab6 protein is bound to the membranes in a salt-resistant manner. Individual fractions were assayed for presences of rab proteins by Western blotting. The results are summarized in Fig. 6. A substantial amount of rab6 wt protein became associated with membranes as demonstrated in Fig. 4A, lanes 1 and 2. At later time points after

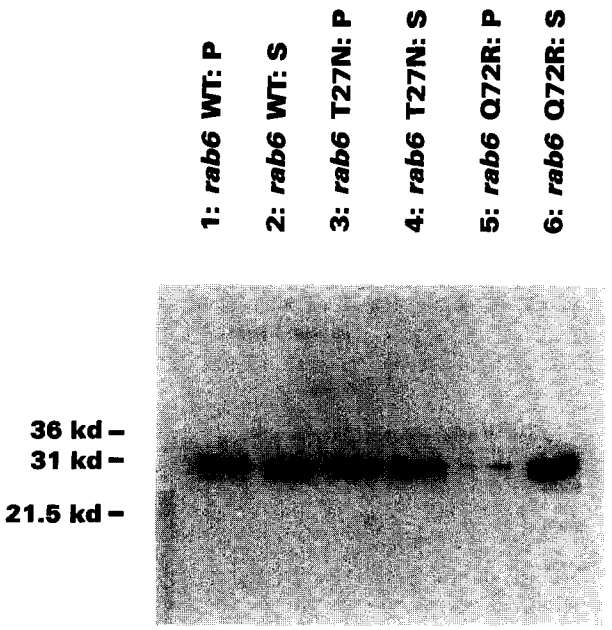

Fig. 6. Membrane association of rab6 proteins in infected Sf9 cells. Sf9 cells were infected and harvested as described in Fig. 2. Cell lysates were adjusted to 1 M KCl and separated into a cytosolic fraction (S) and a membrane fraction (P) by centrifugation at 100,000 × *g* for 1 h at 4°C. Corresponding aliquots of the fractions were separated on a 15% SDS-gel and blotted onto nitrocellulose. Rab6 was detected by the epitope tag specific mab 9E10. Blots were developed as described in Fig. 2.

infection, the majority of the rab proteins is detected within the cytosolic phase (data not shown). The binding of the human recombinant rab6 protein to Sf9-derived insect membranes suggests that the putative membrane receptor of insect cells may associate with the primate rab6 protein, since the rab proteins become attached to the membrane in a salt-resistant manner. The same principle must apply for the GDI and GDS proteins, which guide the different rab family members to their target membranes [9,10]. Membrane binding characteristics of rab6 T27N protein mimics those of wt rab6 (Fig. 6, lanes 3 and 4). The rab6 Q72R protein remains entirely within the cytosolic fraction. This was due to the fact that Q72R protein did not become isoprenylated, as shown in Fig. 4B. These results demonstrate that isoprenylation is a strict prerequisite for membrane binding.

4. Discussion

Proteins belonging to the ras superfamily are believed to operate in a cyclic manner which involves hydrolysis of bound nucleotide and cytosolic and membrane bound forms of the proteins [29]. The association and dissociation of rab proteins with their target membranes appear to be results of multi-step processes [29,30]. Posttranslational modification of rab proteins by the rab geranylgeranyl-transferase at the two C-terminal cysteines initiate the rab protein life cycle [13,31]. Rab apoprotein is bound by REP protein, the component A of rab GGTase and then presented to the catalytic α and β subunits of this enzyme [15]. In addition parts of the rab proteins other than the C-terminal sequences are required for the rab's isoprenylation [32]. Recently Beranger et al. reported that loop 3 and the hypervariable region of rab6 are structural requirements for isoprenylation [17]. In addition, certain point mutations in the effector domain of rab1B inhibit lipidation [32]. There is currently no consensus about the nucleotide requirement for lipidation. Our results clearly prove that the conformation of rab6 induced by bound GDP is required for isoprenylation. While the wildtype and a GDP-bound form of rab6 was modified in insect cells, rab6 Q72R protein did not display geranylgeranylation. Since the rab-GGTase acts on newly synthesized rab proteins it has to be concluded that cytosolic and thus GDP-bound rab protein is the preferential substrate of this enzyme. In vitro isoprenylation assays using the non hydrolysable GTP-analogues GTPγS or GppNHp led to ambiguous results [17,18]. While Sanford et al. reported reduction of prenylation of rab 5 preloaded with GTPγS, Beranger et al. observed normal processing of GTPγS bound rab6 [17,18]. To study the posttranslational modification of rab6 in vivo we used insect Sf9 cells. Yang and colleagues have shown previously that the rab6 wt protein becomes modified with geranylgeranyl residues after expression in Sf9 cells, thus demonstrating that the insect cell system is a suitable tool to study isoprenylation [28].

Our results clearly show that rab6 wt and rab6 T27N undergo isoprenylation in insect cells. For the rab6 Q72R protein we were unable to detect any efficient lipid modification using both Triton X-114 separation and metabolic labeling with [^{3}H]mevalonolactone. In the living cell rab proteins shuttle between the cytosolic GDP-bound form, and the membrane associated GTP-bound form. Two explanations are possible for the lack of rab6 Q72R prenylation.

(1) This protein is not bound to the REP-protein. Alexandrow et al. reported that REP-1 is associated with the GDP-bound form of the rab proteins [33]. After accompanying the rab–GDP complex to the target membrane the REP-protein is released and the GDP is exchanged to GTP. This behavior indicates that the REP protein similar to the GDI protein forms complexes predominantly with the GDP-bound form of rab proteins.

(2) The catalytic α and β subunits have a strong preference for rab proteins in the GDP conformation. In contrast to the GGTase I, which modifies rho proteins and even accepts short peptides as substrates, the rab GGTase needs to interact not only with the C-terminus of rab proteins but also with remote parts of the polypeptide [17,32]. This fact emphasius that the three-dimensional structure seems to be an important prerequisite for the rab6/rabGGTase interaction. The effector loop is a domain in ras-like proteins showing a major conformational change upon GTP/GDP switch. For the rab1B protein Wilson and Maltese have shown that two point mutations in the effector loop, I41N and D44N, reduce prenylation of the protein after translation in a reticulocyte lysate [32]. The same rab6 region is affected by the amino acid change in rab6 Q72R. An alternative explanation for the lack of rab6 Q72R isoprenylation could be that not the change of the overall conformation but the point substitution of Gln^{72} to Arg in loop 4 abolishes isoprenylation. However, this explanation seems unlikely since it was recently reported that loop 4 is not necessary for the modification of rab6 [17]. The fact that rab6 bound to GTPγS is prenylated in vitro by the purified rabGGTase as shown by Beranger et al. remains in contrast to our findings that rab6 Q72R was not modified in insect cells. In support of our findings, the regulatory factors interacting with rab proteins are known to be conserved in all eukaryotic cells, from man to

yeast, thus making a different specificity of the rab GGTase in the insect cell unlikely. On the other hand, it is not known whether rab GGTase isolates have different requirements for the enzyme than in its physiological cellular enviroment. Both in the living cell as well as in reticulocyte lysate a whole array of different rab proteins compete as substrates for the GGTase. In such a competitive environment, rab proteins showing a less favorable conformation may be not modified.

Further experiments are needed to prove whether the nucleotide-dependent geranylgeranylation is a general behavior of rab proteins or a specific characteristic of rab6.

Acknowledgments: We thank Dr. Montenarh (Universität des Saarlandes, Homburg) for the Sf9 cells and Annette Janning for technical assistance. This work is part of the Ph.D. thesis of A.S.; A.S. is a fellow of the Graduiertenförderung of NRW. The project was supported by a grant of the Deutsche Forschungsgemeinschaft (SFB 310, project A9) to A.B. and T.M. and a grant by the Fond Chemischer Industrie.

References

[1] Rothman, J.E. (1994) Nature 372, 55–63.
[2] Söllner, T., Whiteheart, S.W., Brunner, M., Erdjument-Bromage, H., Geroamanos, S., Tempst, P. and Rothman J.E. (1993) Nature 362, 318–324.
[3] Sogaard, M., Tani, K., Ye, RR., Geromanos, S., Tempst, P., Kirchhausen., T., Rothman, J.E. and Söllner, T. (1994) Cell 78, 937–948.
[4] Pfeffer, S.R. (1994) Curr. Opin. Cell Biol. 6, 522–526.
[5] Elazar, Z., Orci, L., Ostermann, J., Amherdt, M., Tanigawa, G. and Rothman, J.E. (1994) J. Cell. Biol. 415–424.
[6] Stow, J.L., de-Almeida, J.B., Narula, N., Holtzman, J.E., Ercolani, L. and Ausiello, D.A. (1991) J. Cell Biol. 114, 1113–1124.
[7] Takai, Y., Kaibuchi, K., Kikuchi, A., Sasaki, T. and Shirataki, H. (1993) CIBA Found. Symp. 176, 128–138.
[8] Ullrich, O., Stenmark, H., Alexandrov, K., Huber, L.A., Kaibuchi, K., Sasaki, T., Takai, Y. and Zerial, M. (1993) J. Biol. Chem. 268, 18143–18150.
[9] Ullrich, O., Horiuchi, H., Bucci, C. and Zerial, M. (1994) Nature 368, 157–160.
[10] Soldati, T., Shapiro, A.D., Svejstrup, A.B. and Pfeffer, S.R. (1994) Nature 369, 76–78.
[11] Elazar, Z., Mayer, T. and Rothman, J.E. (1994) J. Biol. Chem. 269, 794–797.
[12] Barbacid, M. (1987) Annu. Rev. Biochem. 56, 779–827.
[13] Farnsworth, C.C., Seabra, M.C., Ericsson, L.H., Gelb, M.H. and Glomset, J.A. (1994) Proc. Natl. Acad. Sci. USA 91, 11963–11967.
[14] Araki, S., Kaibuchi, K., Sasaki, T., Hata, Y. and Takai, Y. (1991) Mol. Cell Biol. 11, 1438–1447.
[15] Seabra, M.C., Goldstein, J.L., Südhof, T.C. and Brown, M.S. (1992) J. Biol. Chem. 267, 14497–14503.
[16] Andres, D.A., Seabra, M.C., Brown, M.S., Armstrong, S.A., Smeland, T.E., Cremers, F.P. and Goldstein, J.L. (1993) Cell 73, 1091–1099.
[17] Beranger, F., Cadwallader, K., Porfiri, E., Powers, S., Evans, T., de-Gunzburg, J. and Hancock, J.F. (1994) J. Biol. Chem. 269, 13637–13643.
[18] Sanford, J.C., Pan, Y. and Wessling-Resnick, M. (1993) J. Biol. Chem. 268, 23773–23776.
[19] Munro, S. and Pelham, H.R.B. (1987) Cell 48, 899–907.
[20] Zahraoui, A., Touchot, N., Chardin, P. and Taviatian, A. (1989) J. Biol. Chem. 264, 12394–12401.
[21] Taylor, J.W., Ott, J. and Eckstein, F. (1985) Nucleic Acids Res. 13, 8764–8785.
[22] Guarino, L.A. and Summers, M.D. (1986) J. Virol. 57, 563–771.
[23] Bordier, C. (1980) J. Biol. Chem. 256 16041–607.
[24] Huber, L.A., Ullrich, O., Takai, Y., Lütcke, A., Dupree, P., Olkkonen, V., Virta, H., de-Hoop, M.J., Alexandrov, K., Peter, M., Zerial, M. and Simons, K. (1994) Proc. Natl. Acad. Sci. USA 91, 7874–7878.
[25] Tavitian, A. and Zahraoui, A. (1992) Methods Enzymol. 219, 387–407.
[26] Hori, Y., Kikuchi, A., Isomura, M., Katayama, M., Miura, Y., Fujioka, H., Kaibuchi, K. and Takai, Y. (1991) Oncogene 6, 515–522.
[27] Khosravi-Far, R., Lutz, R.J., Cox, A.D., Conroy, L., Bourne, J.R., Sinensky, M., Balch, W.E., Buss, J.E. and Der, C.J. (1991) Proc. Natl. Acad. Sci. USA 88, 6264–6268.
[28] Yang, C., Slepnev, V.I. and Goud, B. (1994) J. Biol. Chem. 269, 31891–31899.
[29] Chavrier, P., Gorvel, J.P. and Bertoglio, J. (1993) Immunol. Today 14, 440–444.
[30] Novick, P. and Brennwald, P. (1993) Cell 75, 597–601.
[31] Newman, C.M., Giannakouros, T., Hancock, J.F., Fawell, E.H., Armstrong, J. and Magee, A.I. (1992) J. Biol. Chem. 297, 11329–11336.
[32] Wilson, A.L. and Maltese, W.A. (1993) J. Biol. Chem. 298, 14561–14564.
[33] Alexandrov, A., Horiuchi, H., Steele-Mortimer, O., Seabra, M.C. and Zerial, M. (1994) EMBO J. 13, 5292–5273.