Identification and characterization of a novel human plant pathogenesis-related protein that localizes to lipid-enriched microdomains in the Golgi complex

Heike B. Eberle[1], Ramon L. Serrano[1], Joachim Füllekrug[2], Andreas Schlosser[3], Wolf D. Lehmann[3], Friedrich Lottspeich[4], Dora Kaloyanova[1], Felix T. Wieland[1] and J. Bernd Helms[1,*]

[1]Biochemie-Zentrum Heidelberg (BZH), University of Heidelberg, Im Neuenheimer Feld 328, 69120 Heidelberg, Germany
[2]Max-Planck-Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, D-01307 Dresden, Germany
[3]Central Spectroscopy Department, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany
[4]Max-Planck Institut für Biochemie, Martinsried, Germany
*Author for correspondence (e-mail: helms@uni-hd.de)

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Summary

Group 1 of plant pathogenesis-related proteins (PR-1) and a variety of related mammalian proteins constitute a superfamily of proteins that share structural similarities. Little is known about their function, but all the family members identified to date are co-translationally translocated to the lumen of the endoplasmic reticulum and are secreted as soluble proteins or are targeted to vacuoles. Here we report the identification of a novel family member that localizes to the cytosolic site of the endomembrane system in mammalian cells. After detergent solubilization of isolated Golgi membranes, a 17 kDa protein was found associated with a low-density detergent-insoluble fraction. The amino-acid sequence, determined by microsequencing and molecular cloning, revealed a significant homology with the superfamily of PR-1 proteins. Golgi-associated PR-1 protein (GAPR-1) showed a brefeldin-A-sensitive Golgi localization in immunofluorescence. Interestingly, the protein remained associated with the microdomain fraction in the presence of Brefeldin A.

By mass spectrometry, GAPR-1 was shown to be myristoylated. Immunoprecipitation of GAPR- 1 from Golgi membranes resulted in the coimmunoprecipitation of caveolin-1, indicating a direct interaction between these two proteins. Myristoylation, together with protein-protein or electrostatic interactions at physiological pH owing to the highly basic pI of GAPR-1 (pI 9.4) could explain the strong membrane association of GAPR-1.

Tissue screening revealed that GAPR-1 is not detectably expressed in liver, heart or adrenal glands. High expression was found in monocytes, leukocytes, lung, spleen and embryonic tissue. Consistent with the involvement of PR-1 proteins in the plant immune system, these data could indicate that GAPR-1 is involved in the immune system.

Key words: Golgi, Microdomains, Rafts, Plant pathogenesis-related protein, PR-1, Myristoylation, Caveolin

Introduction

Plant pathogenesis-related (PR) proteins were discovered by their induction in plants after infection with necrotizing viruses (Gianinazzi et al., 1970; van Loon and van Kammen, 1970). Plant PR proteins are classified into five groups (PR-1 to PR-5) on the basis of their structural homologies within groups but not between the different groups. PR-1 proteins are characterized by their often acidic or basic nature, their resistance to proteases and their extracellular secretion (Bol et al., 1990; Linthorst, 1991; van Loon, 1985). Catalytic functions of several plant PR groups have been described, but their mechanism of action is not clear. Although antifungal activity has been reported (Niderman et al., 1995), it is becoming increasingly clear that PR-1 proteins play an essential role in the defence of plants, for example during the manifestation of systemic required resistance (Klessig et al., 2000; Maleck et al., 2000; Zhang et al., 1999). The NMR solution structure of p14a, a basic PR-1 protein from tomato, revealed a possible active center containing two histidines and two glutamates that could function like Zn proteases, but so far metal ions have not been found associated with PR-1 proteins (Fernandez et al., 1997; Szyperski et al., 1998).

Since the identification of plant PR-1 proteins, secretory proteins with a significant sequence homology to PR-1 have been identified in various other organisms. Examples of PR-1-related proteins include fruiting body proteins in fungi that are expressed during infection (Schuren et al., 1993), insect allergens (Lu et al., 1993; Schreiber et al., 1997), mammalian CRISP proteins, which may be involved in sperm maturation or sperm-egg fusion (Kjeldsen et al., 1996; Kratzschmar et al., 1996), human GliPR/RTVP-1, which is specifically expressed in glial tumors (Murphy et al., 1995; Rich et al., 1996) and snake or lizard venoms, which are reported to block ryanodine receptors or cyclic nucleotide-gated ion channels (Brown et al., 1999; Morrissette et al., 1995). Together with plant PR-1 proteins, these proteins constitute a large PR-1 protein superfamily.

Here we describe a novel member of this family that was identified as a component of lipid-enriched microdomains at the Golgi complex. The lipid scaffold for this type of

microdomain is built mainly by sphingomyelin (SM) and cholesterol (Brown and London, 1998b; Simons and Ikonen, 1997). By differential participation of proteins and lipids, microdomains are believed to play an important role in signal transduction and intracellular membrane transport events such as protein sorting (Anderson, 1998; Brown and London, 2000; Simons and Toomre, 2000). Although the in vivo existence of microdomains in biological membranes has been difficult to prove, evidence is accumulating in favour of their presence. Important details such as their size and dynamics, however, are still intensely discussed (Brown and London, 1998a; Brown and London, 1998b; Brown and London, 2000; Simons and Toomre, 2000). A widely used approach to study microdomains has been their isolation as low-density detergent-insoluble complexes. We used this method for the isolation of lipid-enriched microdomains from isolated Golgi membranes (Gkantiragas et al., 2001). To determine the architecture and function of microdomains at the Golgi complex, we initiated a systematic identification of their proteins by microsequencing. Two peptide sequences derived from a protein with an apparent molecular weight of 17 kDa did not match any of the proteins identified to date. Here we report the cloning and biochemical characterization of this protein. On the basis of sequence homology, this novel protein belongs to the superfamily of plant pathogenesis-related proteins. Biochemical characterisation revealed some unique properties of this protein that have not yet been reported for other protein family members.

Materials and Methods

The monoclonal mouse antibody against KDEL receptor (Tang et al., 1993) was kindly donated by R. Pepperkok (EMBL, Germany) and provided by W. Hong (Singapore, Republic of Singapore). An antibody against the luminal domain of p23 (KAI2/3) is described by Sohn et al. (Sohn et al., 1996). A β-COP-specific antibody was used as previously described (Duden et al., 1991). Monoclonal antibodies against NSF were provided by Z. Elazar (Weizmann Institute of Science, Israel). Rabbit anti-peptide antibodies against GAPR-1 were generated and affinity purified according to standard procedures by coupling peptide #1852 (GFFEENVLPPKKCOOH), which corresponds to the C-terminal sequence of GAPR-1, to Keyhole Limpet hemacyanin (KLH). Brefeldin A (Roche Diagnostics GmbH, Germany) was stored at 2.5 mM in EtOH at –20°C. N-Hydroxylsulfosuccinimidyl-4-azidobenzoate was from Pierce (Rockford, IL, USA).

Subcellular fractions from CHO cells

CHO Golgi membranes and COPI-coated vesicles were isolated as described previously (Balch et al., 1984; Malhotra et al., 1989; Serafini et al., 1991). CHO cytosol was prepared by centrifugation of a CHO homogenate (prepared as described for the isolation of CHO Golgi membranes) for 1 hour at 100,000 ***g*** at 4°C. The supernatant was stored at –80°C before use.

Isolation of low-density detergent-insoluble fractions from Golgi membranes and from total cell lysates

For the identification of GAPR-1, low-density detergent-insoluble complexes were isolated from CHO Golgi membranes. This method is described in detail by Gkantiragas et al. (Gkantiragas et al., 2001). In short, 5 mg (protein) of CHO Golgi membranes (isolated as described above) were pelleted and resuspended in 2 ml of PEN buffer (25 mM Pipes, pH 6.5, 2 mM EDTA, 150 mM NaCl) containing 1% Triton X-100. The suspension was incubated for 30 minutes on ice, mixed with 2 ml of 80% (w/v) sucrose in PEN buffer and transferred to a SW41 rotor tube (Beckman). The 40% sucrose fraction was overlayed with subsequently 1.3 ml of each 30%, 25%, 20%, 15%, 10% and 5% sucrose in PEN buffer. The samples were centrifuged for 22 hours at 190,000 ***g*** at 4°C. After centrifugation, the Golgi-derived detergent-insoluble complexes (GICs) migrating as an opalescent band at the 10-15% sucrose interface was collected.

Low-density detergent-insoluble complexes from total-cell lysates (total DRM) were isolated according to the same procedure by solubilization of a subconfluent cell monolayer of NRK cells (5×175 cm^2 plates) in 2 ml of PEN buffer+1% TX-100.

Cloning, sequencing and sequence analysis of GAPR-1

Two peptide sequences, obtained from microsequening of p17 from CHO GICs, were used to search DNA databases. Several ESTs containing both peptide sequences in the same reading frame were identified (GenBank accession numbers T78145, aa339686, aa428123). EST44799 (obtained from ATCC (Rockville, USA)) was sequenced by TopLab, Martinsried, Germany, before being cloned into pBluescript SK- (GenBank accession number aa339686). The sequence revealed a complete open reading frame with 462 bases by use of vector primer M13 and primer walking. The corresponding sequence is accessible at the EMBL/GenBank database (NP_071738). Homologues of GAPR-1 were identified by searching the Swissprot database using the BLASTP2 search engine (EMBL, Heidelberg, Germany). The predicted coil-coil sequence (amino acid 29-49) was identified by the software described by Lupas et al. (Lupas et al., 1991) (www.ch.embnet.org/software/COILS_form.html).

In-gel tryptic digest

The protein band obtained after SDS-PAGE was excised, cut into small pieces, destained with 30% acetonitrile 0.1 M NH_4HCO_3, washed with water, dried in a centrifugal evaporator (vacuum concentrator, Bachofer, Reutlingen, Germany) and rehydrated with 10 μl of the digest solution. This solution contained 50 μg/ml trypsin in 0.1 M NH_4HCO_3. After incubation at 37°C over night, the tryptic fragments were extracted with 2×10 μl of 5% formic acid. The combined extracts were desalted using C-18 ZipTips (Millipore, Bedford, MA). The peptides were eluted in 3 μl 50% acetonitrile, 2% formic acid and transferred into a nanoESI capillary.

Mass spectrometry

Mass spectra were recorded using a quadrupole time-of-flight instrument Q-TOF 2 (Micromass, Manchester, UK) equipped with a nanoESI source and operated with a resolution of about 8000 (FWHM). Spray capillaries were prepared in-house using a micropipette puller P-87 (Sutter Instruments, Novato, CA), and subsequently surface coated with a semitransparent film of gold using a gold sputter unit. The spray voltage used was about 1200 V, and argon was used as a collision gas.

Immunoprecipitation

10 μl of polyclonal serum against the C-terminus of GAPR-1 (#1852) was incubated with 50 μl of protein A Sepharose (Fast Flow, Amersham Pharmacia Biotech, Freiburg, Germany) and 50 μl of PBS containing 0.5% milk for 90 minutes at RT. The beads were washed twice with PBS and twice with IP buffer (PEN+1% TX-100) before use. Golgi membranes (500 μg) were centrifuged (100,000 ***g*** for 30 minutes at 4°C), and the pellet was resuspended in 100 μl 1%SDS and incubated for 5 minutes at 95°C. The sample containing denatured proteins was diluted with 900 μl of PEN+1%TX- 100 and used for

immunoprecipitation of GAPR-1 by incubation overnight at 4°C with the protein A beads. The beads were subsequently washed twice in PEN+1%TX-100 and four times in PEN before analysis. For western blot analysis, a protein A-HRP conjugate (Biorad Laboratories GmbH, München, Germany) was used that does not recognize the denatured IgGs on the blot that were eluted from the immunoprecipitation beads.

Immunofluorescence

Vero cells were cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 10% FCS to subconfluency and prepared for indirect immunofluorescence according to standard procedures. Cells were fixed either in MeOH (1-2 minutes at –20°C) or fixed in 3% (w/v) paraformaldehyde for 20 minutes and permeabilized for 5 minutes in 0.1% saponin. After incubation of first and secondary antibodies, cells were washed and embedded in Fluoromount G (biozol, Eching, Germany). Images were taken using the Leica TCS-NT confocal laser scan microscope (Leica Lasertechnique, Heidelberg, Germany). Confocal images of double labelling experiments were obtained simultaneously to exclude any artefacts from sequential acquisition. Both channels were adjusted to ensure that the maximum fluorescence intensity was still in the recording range. Only one focal plane was analysed. Stainings shifted against each other were confirmed by series of z sections and repeated simultaneous scans. Micrographs were arranged with Adobe Photoshop and Illustrator.

Viral infection

Vero cells were maintained and infected with tsO45 vesicular stomatitis virus (VSV; Indiana Serotype) as described previously (Kreis, 1986). Incubation of cells at 15°C was performed under conditions as described in Fullekrug et al. (Fullekrug et al., 1999). In short, cell culture medium was replaced by DMEM containing 20 mM Hepes, pH 7.2 and 200 μM cyclohexamide. After incubation, the cells were fixed with methanol for 4 minutes at –20°C. A mouse mAb that recognizes an exoplasmic epitope of VSV-G (Pepperkok et al., 1993) was used to detect the presence of tsO45-G protein, followed by Cy3-labelled antimouse secondary antibody (Alexis Corporation, CA). Cells were mounted in Fluoromount G (Biozol) and analysed using a Zeiss Axiovert 35 microscope equipped with the appropriate filters for Cy3-derived fluorescence.

Protease digestion of Golgi membranes

Isolated CHO Golgi membranes (10 μg) were incubated with Trypsin (2.5 μg) in 25 mM Hepes/KOH, pH 7.2, 20 mM KCl, 2.5 mM MgOAc for 30 minutes at 30°C. Where indicated, Trypsin inhibitor (25 μg) or Triton X-100 (1% final concentration) were added to the assay. The Golgi membranes were pelleted through a sucrose cushion (15% sucrose w/v) at 14,000 ***g*** for 30 minutes. The proteins were analysed by SDS-PAGE and subsequent western blot analysis (ECL) using a GAPR-1-specific antibody (#1852) and a p23-specific antibody raised against the N-terminus of the protein (KAI2/3 (Sohn et al., 1996)).

Preparation of a total membrane fraction from various tissues

Isolated tissues (heart, brain, testis, lung, liver, spleen, muscle, kidney, adrenal gland and pancreas) from male rats were homogenized in 0.2 M sucrose in PEN buffer (tissue:buffer 1:4 (w/w)) in the presence of protease inhibitors. The homogenate was centrifuged for 10 minutes at 650 ***g*** at 4°C to obtain a postnuclear supernatant (PNS). A membrane fraction was isolated from the PNS by centrifugation for 1 hour at 100,000 ***g*** at 4°C. Leukocytes were prepared from rat blood by extensive washing of 4 ml of rat blood in lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2) until all erythrocytes were lysed. The white cell pellet is enriched in leukocytes and a total membrane fraction was obtained as described above.

The embryo, uterus and placenta from rats were kindly provided by Celina Cziploch (ATV, Heidelberg). Monocytes (from human blood) were kindly provided by Anne Große Wilde (DKFZ, Heidelberg).

Miscellaneous

Protein-determination was performed according to Lowry (Lowry et al., 1951). Quantitation of phosphatidylcholine in CHO Golgi membranes and COPI-coated vesicles was performed by nano-electrospray ionization tandem mass spectrometry (Brügger et al., 1997; Helms et al., 1998).

Results

Cloning of GAPR-1

Isolation of detergent-insoluble complexes from isolated CHO Golgi membranes resulted in the identification of a protein complex containing 10 major polypeptides. The proteins were identified by microsequencing, and from one protein with an apparent molecular weight of 17 kDa, two peptides were obtained and sequenced that did not correspond to a known protein (Gkantiragas et al., 2001) (Fig. 1A). This information was used to search the protein and DNA databases. Several ESTs were found that contained both peptide sequences in the same reading frame. Sequencing of EST44799 (GenBank AA339686, derived from human fetal brain) revealed a complete cDNA sequence, and the corresponding protein sequence is shown in Fig. 1B. Except for one conservative exchange, the peptide sequences derived from chinese hamster are identical to their human homologue. A hydrophobicity plot of the protein sequence revealed an extremely hydrophilic protein with an isoelectric point of 9.4, without a potential transmembrane spanning domain (data not shown).

Sequence analysis revealed the presence of a consensus sequence for myristoylation (Met-Gly-X-X-X-Ser/Thr) (Resh, 1999) at the N-terminus of GAPR-1 and a coiled-coil region (Lupas, 1996) between amino acids 29-49, which might be involved in protein-protein interactions. A caveolin-binding motive (Okamoto et al., 1998) (-X-XXXX-, where - is aromatic amino acid Trp, Phe or Tyr) is found between amino acids 90-97.

GAPR-1 belongs to a superfamily of proteins

No homologous proteins have been identified to date, although several expressed sequence tags from various mammalian sources (including bovine, pig, mouse, and zebrafish) have been reported to contain fragments with identical sequences. GAPR-1 belongs to a superfamily of proteins that include human GliPR (Murphy et al., 1995), mammalian cysteine-rich secretory proteins (Kratzschmar et al., 1996), plant pathogenesis-related proteins group 1 (plant PR-1 proteins) (Bol et al., 1990), allergens of insect venoms (Lu et al., 1993) and snake or lizard venoms (Morrissette et al., 1995). The members within these subfamilies that share the highest homology to GAPR-1 are shown in Fig. 1C. The identity of GAPR-1 to these proteins varies between 30% (TPX1) and 50% (*Drosophila* CG2337), and the homology varies between 46% (AG5) and 63%

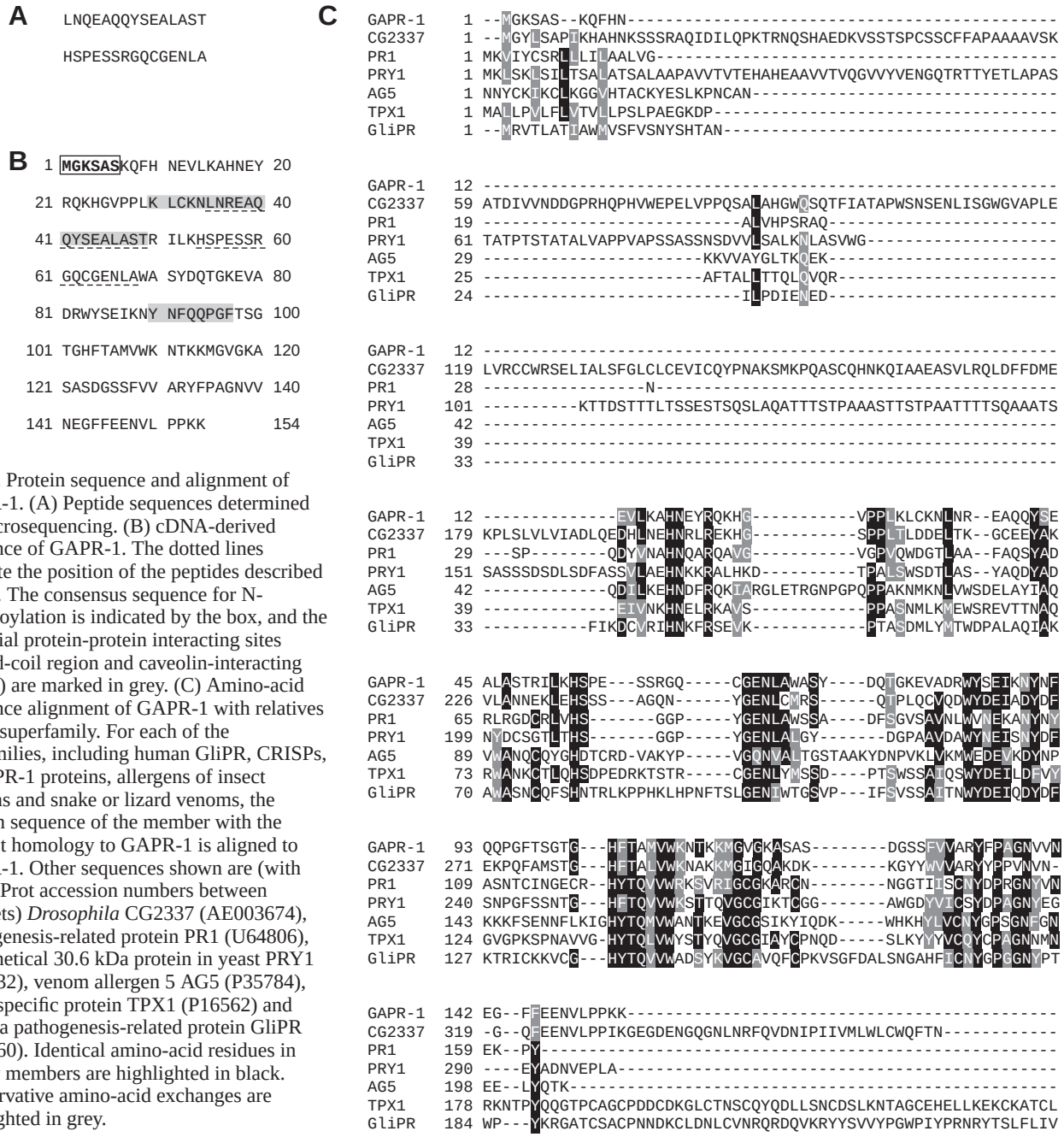

Fig. 1. Protein sequence and alignment of GAPR-1. (A) Peptide sequences determined by microsequencing. (B) cDNA-derived sequence of GAPR-1. The dotted lines indicate the position of the peptides described in (A). The consensus sequence for N-myristoylation is indicated by the box, and the potential protein-protein interacting sites (coiled-coil region and caveolin-interacting region) are marked in grey. (C) Amino-acid sequence alignment of GAPR-1 with relatives of the superfamily. For each of the subfamilies, including human GliPR, CRISPs, plant PR-1 proteins, allergens of insect venoms and snake or lizard venoms, the protein sequence of the member with the highest homology to GAPR-1 is aligned to GAPR-1. Other sequences shown are (with Swiss Prot accession numbers between brackets) *Drosophila* CG2337 (AE003674), pathogenesis-related protein PR1 (U64806), hypothetical 30.6 kDa protein in yeast PRY1 (P47032), venom allergen 5 AG5 (P35784), testis-specific protein TPX1 (P16562) and Glioma pathogenesis-related protein GliPR (P48060). Identical amino-acid residues in family members are highlighted in black. Conservative amino-acid exchanges are highlighted in grey.

(*Drosophila* CG2337). In yeast there are three hypothetical proteins (PRY1-3) that have a domain with a similar level of identity (35-41%) and homology (51-53%) to GAPR-1. The functions of this protein superfamily are presently unknown. GAPR-1 contains two histidine (His54 and His103) and two glutamate residues (Glu65 and Glu86) that are highly conserved throughout the protein family. In the NMR structure of p14a, a plant PR-1 protein, these four amino-acid residues are located in a cleft that might represent an active site of the protein (Fernandez et al., 1997; Szyperski et al., 1998).

GAPR-1 is a Golgi localized peripheral membrane protein

GAPR-1 was originally identified in a low-density detergent-insoluble fraction from a Golgi-enriched fraction named GICs (Gkantiragas et al., 2001). To confirm the identity of the cloned protein, enrichment of GAPR-1 in Golgi membranes was determined by use of a peptide antibody that was generated from the sequence of the cloned protein. As shown in Fig. 2A, GAPR-1 is enriched (30-50 fold) in an isolated Golgi fraction, which corresponds well with the enrichment of Golgi markers in this fraction (Brügger et al., 2000). In addition, GAPR-1 is highly enriched in a GIC fraction (~65 fold compared to Golgi membranes, as evaluated by quantitation of Fig. 2A, lanes 3 and 4). Quantitative analysis showed that GAPR-1 represents ~0.1% of total Golgi proteins (data not shown). GAPR-1 could not be detected in the cytosolic fraction (see also Fig. 4).

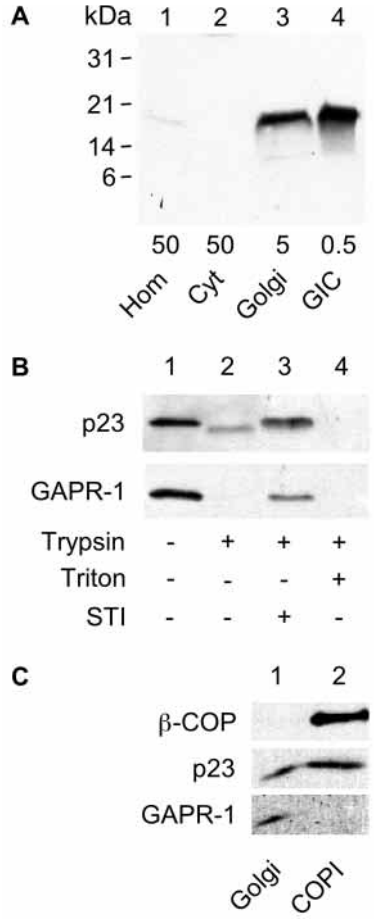

Fig. 2. GAPR-1 is a Golgi-localized peripheral membrane protein. (A) The enrichment of GAPR-1 in CHO Golgi membranes and CHO GIC was determined by comparing the amounts of GAPR-1 in CHO homogenate (lane 1, 50 μg), CHO cytosol (lane 2, 50 μg), CHO Golgi membranes (lane 3, 5 μg) and CHO GIC (lane 4, 0.5 μg) by use of SDS-PAGE and western blotting. (B) The topology of GAPR-1 at Golgi membranes was determined by protease digestion of Golgi membranes (lanes 2-4) in the absence or presence of Trypsin inhibitor (lane 3) or Triton X-100 (lane 4). After incubation (as described in Materials and Methods), the proteins were separated by SDS-PAGE and GAPR-1 and p23 were visualized by western-blotting. (C) Exclusion of GAPR-1 from COPI-coated vesicles. Equivalent amounts of isolated Golgi membranes and COPI-coated vesicles (10.8 μg total phospholipid) were analysed by SDS-PAGE and western blotting for the presence of GAPR-1. As positive controls for COPI-coated vesicles, the blots were probed with antibodies against β-COP and p23. Golgi membranes and COPI-coated vesicles were isolated as described in Materials and Methods.

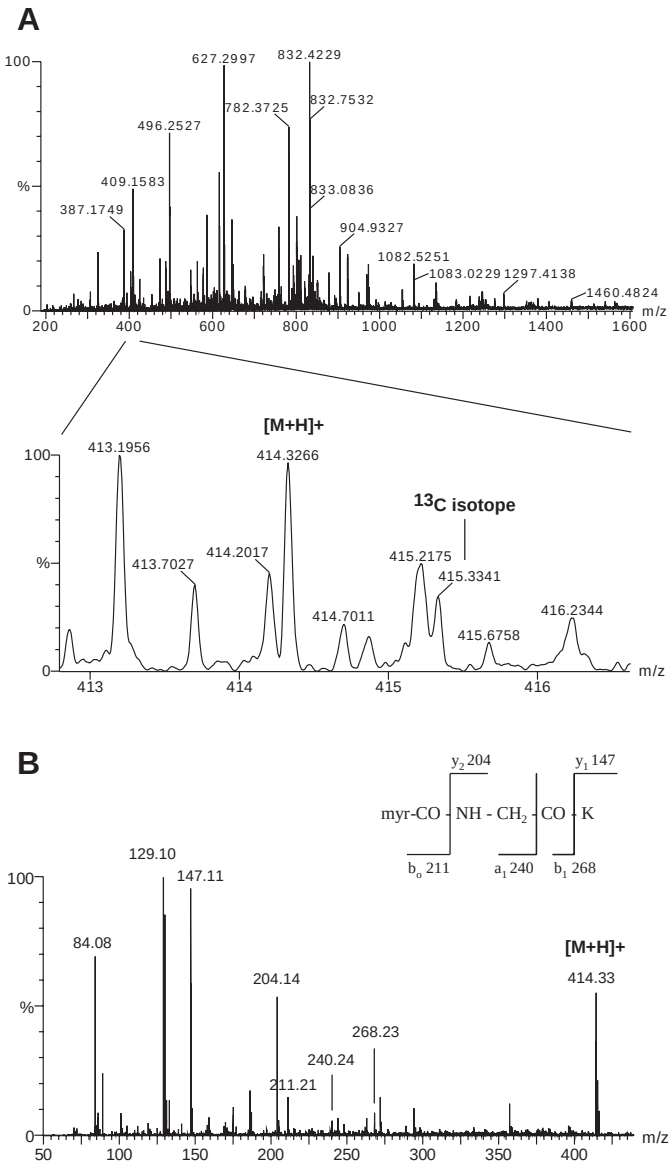

Fig. 3. GAPR-1 is myristoylated in vivo. (A) Positive nanoESI spectrum of the in-gel digest of native GAPR-1. The top panel shows the complete survey spectrum, and the lower panel shows the expanded view from m/z 413 to 417 showing the singly protonated molecular ion of the T1 fragment including the 13C isotope peak. (B) Positive nanoESI product ion spectrum of m/z 414.33. The spectrum shows the key fragments for the myrG structure at m/z 211, 240 and 268 and sequence-specific fragment ions, which identify the peptide as the T1 fragment myrGK of GAPR-1.

The topological orientation of GAPR-1 was determined by trypsin treatment of Golgi membranes. As shown in Fig. 2B, GAPR-1 was completely digested by trypsin in the absence of detergent. As a control, only the C-terminal fragment (~1 kDa) was removed from p23, a Golgi-localized type I membrane protein with a short cytoplasmic tail, showing that its large luminal domain was not accessible to trypsin and thus the membranes are sealed under these conditions. In a control experiment it was shown that the luminal domain of p23 can in principle be digested by trypsin in the presence of detergent (Fig. 2B, lane 4). Thus, with intact membranes, GAPR-1 is accessible to trypsin and therefore has a cytosolic orientation.

As GAPR-1 is localized to Golgi membranes, we determined whether GAPR-1 is also present in COPI vesicles, which mediate transport of proteins between different Golgi cisternae. COPI-coated vesicles were isolated from a large-scale cell-free incubation of Golgi membranes in the presence of GTPγS. The vesicles were quantified on the basis of their phosphatidylcholine (PC) composition. This lipid is present in relatively high amounts in membranes and is not expected to be sorted within the Golgi Stack (Brügger et al., 2000; van Meer, 1989). Fig. 2C shows a comparison of equal amounts of donor Golgi membranes and COPI-coated vesicles, on the basis of their phosphatidylcholine content. They are characterized by p23, a Golgi-localized protein that is enriched in COPI-coated vesicles, in agreement with previous observations (Sohn et al., 1996). In addition, β′-COP, a subunit of the coatomer complex, is enriched in the vesicle fraction, as expected. Antibodies against GAPR-1 were then used to identify the presence of GAPR-1 on the vesicles. Whereas GAPR-1 is present on Golgi membranes, GAPR-1 could not be detected in the purified COPI-vesicle fraction derived from the donor Golgi membranes. This is in agreement with previous observations, where we showed that some of the GIC

proteins were found to be excluded from COPI vesicles (Gkantiragas et al., 2001).

N-myristoylation of GAPR-1

To determine whether the consensus sequence for N-myristoylation results in in vivo myristoylation of GAPR-1, proteins from a large-scale preparation of Golgi-derived detergent-insoluble complexes were separated by SDS-PAGE. The protein band at 17 kDa was excised from the gel and digested with trypsin. The resulting peptides were analysed by electrospray mass spectrometry (Fig. 3A, top panel). If native GAPR-1 is myristoylated, trypsinization should generate the myristoylated dipeptide myrGK with an $[M+H]^+$ signal at m/z 414.33. The survey spectrum of the tryptic digest of GAPR-1 showed a signal at m/z 414.327 (Fig. 3A, lower panel), which is consistent with the calculated m/z value for the myristoylated T1-fragment (within the accuracy of our mass determination). The ion at m/z 414.327 was further analysed by ESI tandem mass spectrometry, and the product obtained from the ion spectrum is displayed in Fig. 3B. All major fragment ions in this spectrum can be assigned to the myrGK sequence, as indicated in Fig. 3B. In particular the fragment ion triplet at 211, 240 and 268 is indicative of the myrG structure. In summary, the mass spectroscopic data show that native GAPR-1 is N-terminally myristoylated. It remains to be established whether GAPR-1 is completely or only partially myristoylated.

Association of GAPR-1 with Golgi membranes

GAPR-1 is tightly associated with Golgi membranes, which is reflected in the absence of GAPR-1 from the cytosolic fraction (Fig. 2). In agreement with this, GAPR-1 could not be detected by immunoprecipitation from large amounts of cytosol (Fig. 4A). GAPR-1 could, however, be efficiently immunoprecipitated from Golgi membranes and the total membrane fraction. This indicates that GAPR-1 is completely absent from the cytosol and that GAPR-1 is not likely to cycle on and off Golgi membranes like other myristoylated Golgi-localized proteins such as ARFs. The characteristics of this tight membrane association of GAPR-1 were further investigated by incubation of isolated Golgi membranes under various conditions. As shown in Fig. 4B, treatment of Golgi membranes with 1 M KCl does not strip GAPR-1 off the membranes, whereas NSF, a peripheral Golgi membrane protein (Block et al., 1988), and exogenous GAPR-1 (see below) are affected by this treatment. Alkaline treatment of the membranes causes the dissociation of most peripheral membrane proteins, including NSF and GAPR-1 (Fig. 4B). In contrast, p23, a type I transmembrane protein of the Golgi complex (Sohn et al., 1996), remains present in salt or alkaline-

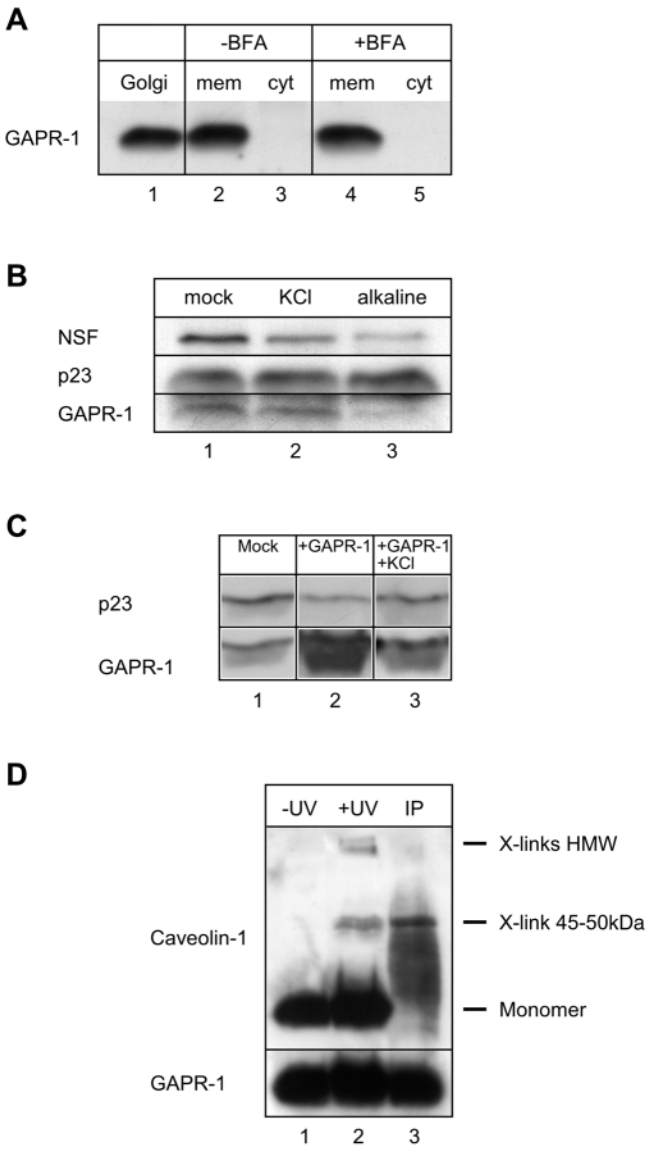

Fig. 4. Strong interaction of GAPR-1 with Golgi membranes. In all panels, the incubations were analysed by SDS-PAGE and western blotting for the presence of the indicated proteins. (A) CHO cells were incubated for 30 minutes in the absence (lane 2 and 3) or presence of 5 μM Brefeldin A (lane 4 and 5). The BFA-induced redistribution of GAPR-1 into tubulo-vesicular structures was confirmed by concomitant immunofluorescence (data not shown). After homogenisation, the homogenate was centrifuged for 1hour at 100,000 ***g*** to yield a total membrane (lanes 2 and 4) and cytosolic fraction (lanes 3 and 5). GAPR-1 was immunoprecipitated from the membrane fraction (2 mg) or from the cytosolic fraction (2 mg) as described in the Materials and Methods. As a control, GAPR-1 was immunoprecipitated from isolated CHO Golgi membranes (50 μg) (lane 1). (B) 50 μg of CHO Golgi membranes (lanes 1-3) was incubated with 1 M KCl (lane 2) or with 0.1 M Na_2CO_3, pH 11 (lane 3) for 30 minutes on ice. After centrifugation through a 15% (w/v) sucrose cushion, equal amounts of membrane (29 nmol phospholipid) were analysed. (C) CHO Golgi membranes (25 μg) were incubated for 30 minues at 4°C in the absence (lane 1) or presence (lanes 2 an 3) of 3 μl of bacterially expressed, purified and non-myristoylated GAPR-1 (5.3 mg/ml) in 25 mM Hepes/KOH, pH 7.2, 20 mM Kcl, 2.5 mM magnesium acetate, 0.1 M sucrose, 1 mg/ml ovalbumine and 10 mM DTT. KCl (1 M final concentration) was added to one incubation (lane 3). Golgi membranes were re-isolated by centrifugation through a 15% (w/v) sucrose cushion. (D) CHO Golgi membranes (50 μg) were incubated with Hydroxylsulfosuccinimidyl-4-azidobenzoate (5 mM) in PBS for 30 minutes at RT and left on ice (lane 1) or irradiated for 10 minutes at 254 nm (lane 2 and 3) and analysed for crosslinked products. For immunoprecipitation (lane 3), 500 μg of Golgi membranes was used, and GAPR-1 was immunoprecipitated as described in Materials and Methods.

treated membranes (Fig. 4B). To determine whether the myristoyl moiety of GAPR-1 could contribute to the salt-resistant membrane binding, purified non-myristoylated GAPR-1 was bound to isolated Golgi membranes (Fig. 4C). Upon salt treatment of the membranes, most of the non-myristoylated GAPR-1 is stripped from the membranes. These data indicate that native GAPR-1 is bound to Golgi membranes not only by ionic interactions but also through the myristoyl moiety, which might affect the membrane anchoring of the protein.

GAPR-1 contains a caveolin-binding motive, which might contribute to its strong membrane-binding characteristics. A direct interaction between caveolin-1 and GAPR-1 was investigated by coimmunoprecipitation studies. Under native conditions using various detergents, caveolin-1 could not be coimmunoprecipitated with GAPR-1 (data not shown). Therefore, coimmunoprecipitation studies were performed after chemical crosslinking of Golgi membranes. As shown in Fig. 4D (lane 1 and 2), crosslinking with NHydroxylsulfosuccinimidyl-4-azidobenzoate results in irradiation-dependent crosslink products of caveolin-1 at 45-50 kDa (Fig. 4D, X-link 45-50 kDa) and at high molecular weights (Fig. 4D, X-links HMW). At 45-50 kDa, a similar crosslink product was observed for GAPR-1 (data not shown). When GAPR-1 was immunoprecipitated from these incubations, caveolin-1 was found to coimmunoprecipitate with GAPR-1 in a crosslink product of 45-50 kDa (Fig. 4D, lane 3). These results indicate a direct interaction of GAPR-1 with caveolin-1. The crosslink products at high molecular weights did not coimmunoprecipitate with GAPR-1. These crosslink products thus probably reflect an interaction of caveolin-1 with other proteins or with other caveolin molecules, as caveolins are known to form stable high molecular weight oligomers (Anderson, 1998).

GAPR-1 localizes to early Golgi compartments

The Golgi localization of GAPR-1 was confirmed by immunofluorescence. In CHO cells, NRK cells and Hela cells, GAPR-1 colocalizes with established Golgi markers such as coatomer and TGN38 (data not shown). As shown in Fig. 5, GAPR-1 also colocalizes with the KDEL receptor in Vero cells, which cycles through the early secretory pathway but has a predominant Golgi localization under steady-state conditions (Tang et al., 1993). In the presence of brefeldin A (BFA), the Golgi structure is disrupted, and Golgi enzymes are transferred to the endoplasmic reticulum (reviewed in (Klausner et al., 1992)). However, certain cycling membrane proteins of the early secretory pathway such as ERGIC-53, KDEL receptor and p24 family proteins accumulate instead in tubulo-vesicular clusters scattered in the cytoplasm (Fullekrug et al., 1999; Lippincott-Schwartz et al., 1990; Tang et al., 1995; Tang et al., 1993). As shown in Fig. 5, GAPR-1 also has a BFA-sensitive Golgi

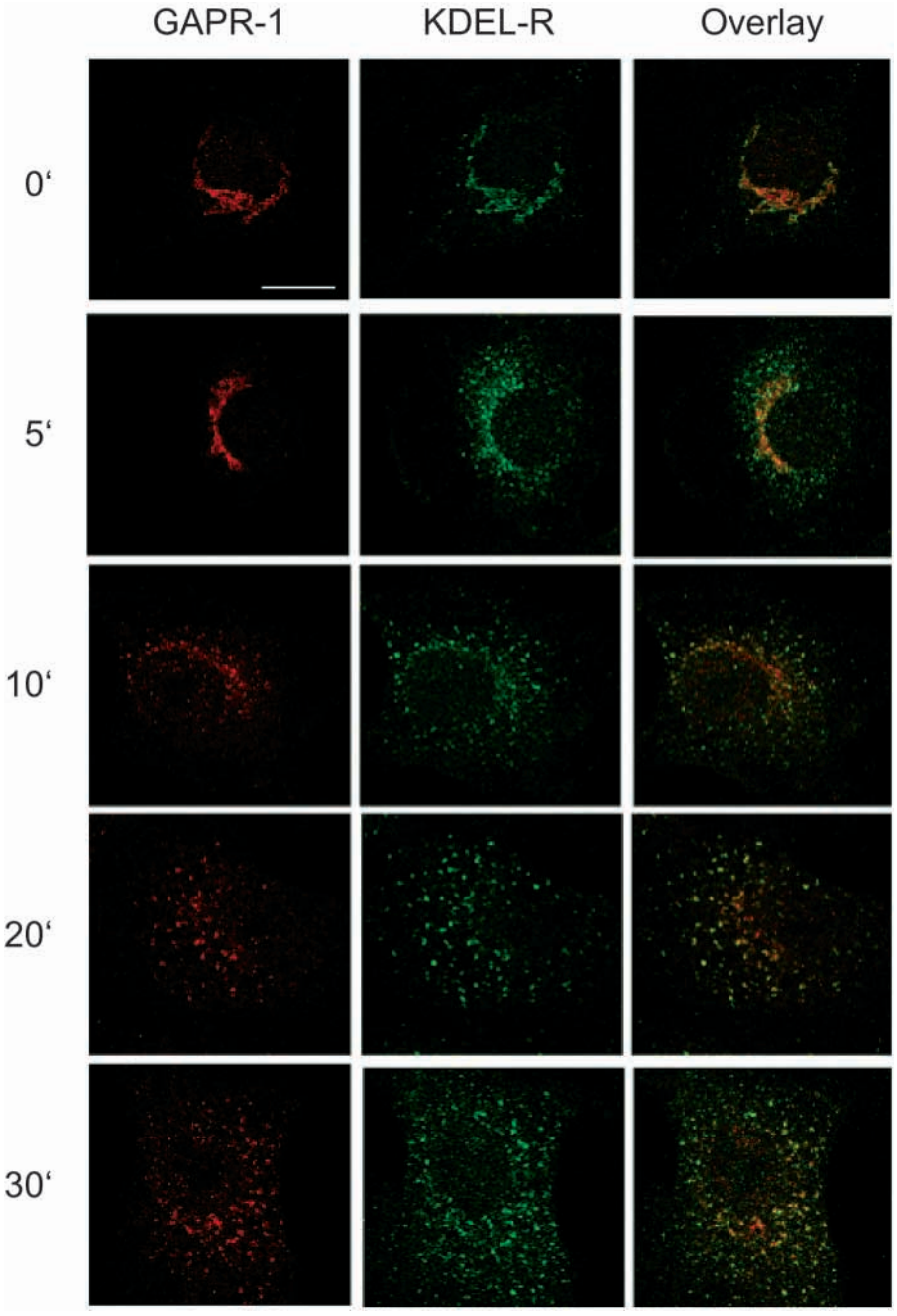

Fig. 5. Immunolocalization of GAPR-1 to the Golgi complex. Vero cells were incubated in the absence (top panels) or presence of BFA (5mM) for the indicated times (5-30 minutes) and processed for double immunofluorescence. Left and middle panels represent immunofluorescent labelling of the cells with an antibody against GAPR-1 and the KDEL receptor, respectively. The right panels shows the colocalization of GAPR-1 and KDEL receptor by merging the two panels. Bar, 20μm.

localization and colocalizes with the KDEL receptor in a punctuate pattern scattered throughout the cytosol. Interestingly, the kinetics by which the Golgi localization of GAPR-1 is disrupted is different from that of the KDEL receptor. The localization of the KDEL receptor is already affected by a 5 minute treatment of the cells with BFA. In contrast to the KDEL receptor, the Golgi localization of GAPR-1 is affected only after a 10 minute incubation of the cells with BFA. After prolonged incubation with BFA, GAPR-1 and KDEL receptor show an increased colocalization to tubulo-vesicular clusters. These data indicate that GAPR-1 is localized to the early secretory pathway and behaves like other cycling proteins. During the BFA-induced redistribution of GAPR-1 from Golgi membranes to tubulo-vesicular structures, GAPR-1 remains associated with membranes. GAPR-1 could not be detected in the cytosol after BFA-treatment of the cells (Fig. 4A).

To further corroborate this finding, we analysed the localization of GAPR-1 relative to the anterograde cargo accumulating at 15°C in the intermediate compartment between the ER and the cis Golgi (Saraste and Kuismanen, 1984; Schweizer et al., 1990). A temperature-sensitive mutant of the membrane glycoprotein VSV-G is misfolded at the restricted temperature of 39.5°C and accumulates in the ER (Fig. 6). At 15°C, VSV-G accumulates in punctuate structures that are scattered throughout the cytoplasm and probably represent the tubulo-vesicular structures of the intermediate compartment. Double immunofluorescence of GAPR-1 and VSV-G protein at the restricted temperature shows an ER-localization pattern for VSV-G, whereas GAPR-1 is localized to the perinuclear region (Fig. 6). After incubation at 15°C in the presence of the protein synthesis inhibitor cyclohexamide, VSV-G accumulates in punctuate structures. Under these conditions, some GAPR-1 colocalizes to these structures, resulting in a partial colocalization of GAPR-1 with VSV-G. The colocalization of GAPR-1 with VSV-G is not as pronounced as other cycling proteins such as the KDEL receptor (Tang et al., 1993) and VIP36 (Fullekrug et al., 1999). This indicates that the cycling of GAPR-1 through the early secretory might be slow, in accordance with the slow redistribution induced by BFA as compared to the cycling protein KDEL receptor.

As the early compartments of the secretory pathway contain strikingly lower levels of sphingomyelin and cholesterol than the Golgi (van Meer, 1998), we determined whether the characteristics of detergent insolubility of GAPR-1 would change when GAPR-1 is present in the tubulo-vesicular structures. To this end, cells were treated with BFA, and low-density detergent-insoluble complexes were isolated from these cells by isopycnic sucrose density centrifugation. As shown in Fig. 7, GAPR-1 remains in a low-density detergent-insoluble fraction, indicating that once these microdomains have formed, they are stable enough to survive in an altered membrane environment such as tubulo-vesicular structures. This is consistent with the behaviour of other GIC proteins (Gkantiragas et al., 2001).

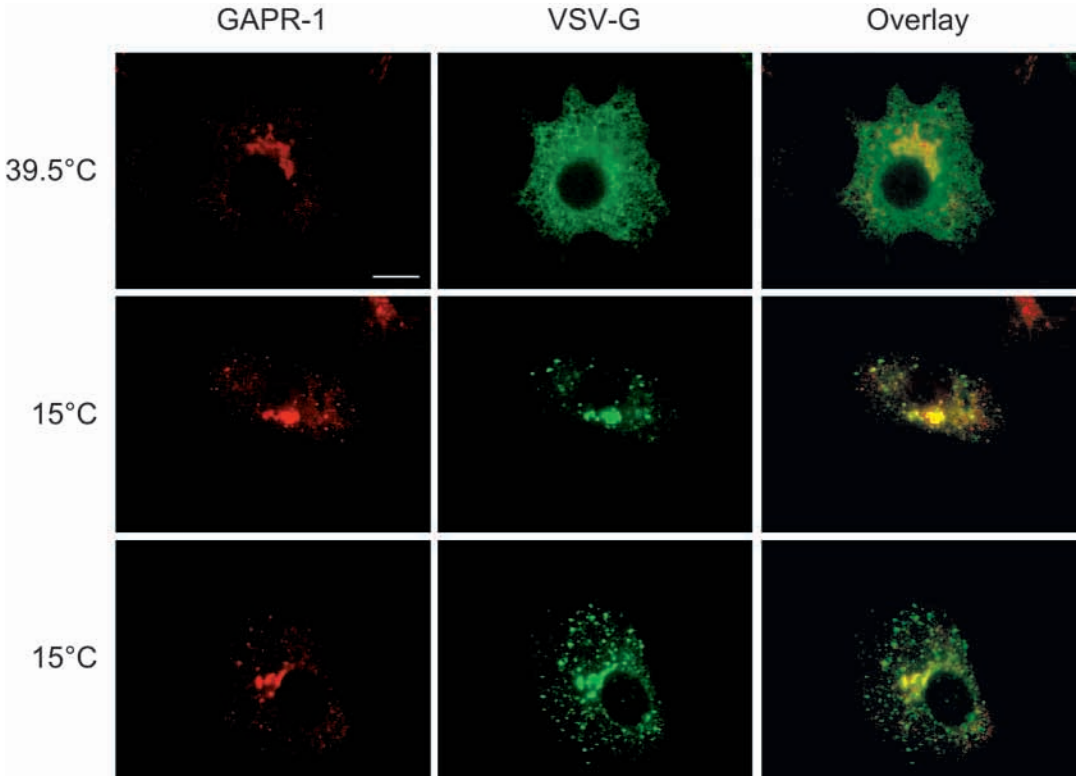

Fig. 6. Colocalization of GAPR-1 with anterograde cargo in the intermediate compartment. Vero cells were infected with vesicular stomatitis virus tsO45 (VSV-G tsO45) expressing a glycoprotein with temperature-sensitive folding properties. After infection, the cells were incubated at the non-permissive temperature of 39.5°C (upper panels). Two representative examples are shown of the subsequent incubation of the cells at 15°C (middle and lower panels). The cells were analysed for the localization of GAPR-1 and VSV-G by double immunofluorescence as described in the Materials and Methods. Bar, 20 μm.

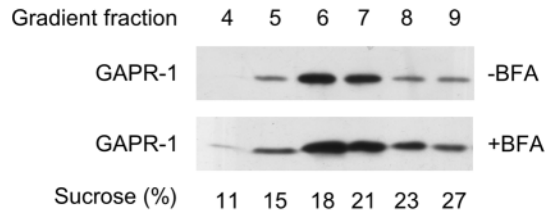

Fig. 7. Detergent-insolubility of GAPR-1 is not sensitive to BFA. NRK cells were incubated for 15 minutes at 37°C in the absence or presence of BFA (5 μM). The BFA-induced redistribution of GAPR-1 into tubulo-vesicular structures was confirmed by concomitant immunofluorescence (data not shown). After incubation, the cells were directly solubilized in PEN+1% TX-100 and incubated for 30 minutes at 0°C. The lysate was analysed for the presence or absence of low-density detergent insoluble complexes by isopycnic sucrose density centrifugation as described in the Materials and Methods. The gradients were fractionated and the fractions analysed by SDS-PAGE and western blotting for the presence of GAPR-1.

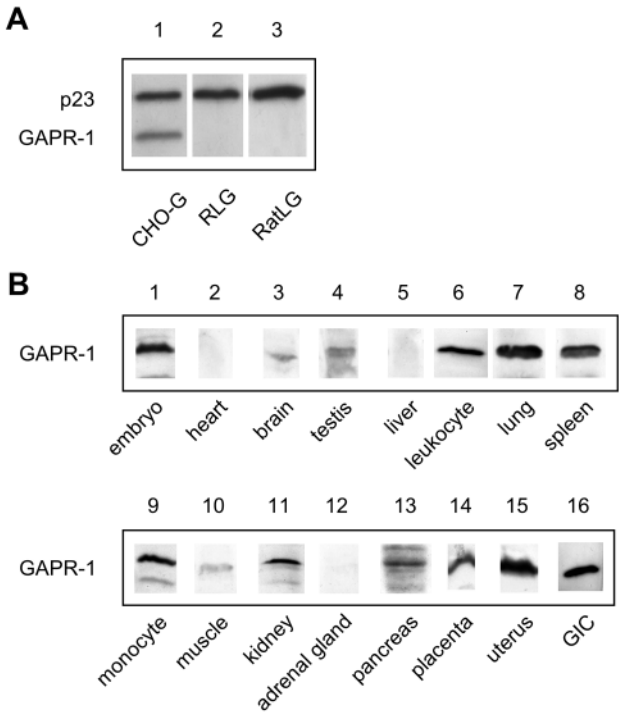

Fig. 8. Differential expression of GAPR-1. After homogenization of the various tissues and preparation of a postnuclear fraction, a total membrane fraction was prepared, and 100 μg of each membrane fraction was analyzed for the presence of GAPR-1 by SDS-PAGE and western blotting. All membrane fractions were isolated from rat tissues, except monocytes, which were isolated from human blood. As a positive control, CHO GIC (0.5 μg) was loaded in lane 16.

Differential expression of GAPR-1

During the course of these studies, we found that in contrast to isolated Golgi membranes from CHO cells, Golgi membranes from other sources such as from rat and rabbit liver did not contain any GAPR-1 (Fig. 8A). This prompted us to determine the expression levels of GAPR-1 in various rat tissues. As shown in Fig. 8B, GAPR-1 is differentially expressed and could not be detected in liver, heart and adrenal glands. High expression levels were found in monocytes, the lung, spleen, kidney lymphocytes, uterus and embryonic tissue. Thus, high expression levels are found in immunocompetent cells and organs (Klein and Horejs'i, 1999) and might indicate a function for GAPR-1 in the immune system.

Discussion

Localization of GAPR-1 to microdomains in the Golgi complex

Here we have identified and characterized a novel human protein that localizes to lipid-enriched microdomains in the Golgi complex of mammalian cells. The amino-acid sequence of GAPR-1 does not predict the presence of a transmembrane-spanning domain. Instead, at physiological pH, GAPR-1 is highly charged (pI 9.4). Since GAPR-1 is tightly bound to membranes and not present in the cytosol, this raises the question of how this protein is anchored to membranes and to microdomains in particular. GAPR-1 contains the consensus sequence for N-myristoylation, and by mass spectroscopic analysis, native GAPR-1 was found to be myristoylated. Fatty acid modification could provide a mechanism to anchor this protein to the membrane. It is, however, unlikely that N-myristoylation is the only anchor for membrane binding, as it has been shown that the binding energy of myristate is not sufficient to stably anchor a protein to a membrane (Peitzsch and McLaughlin, 1993). In support of this, several myristoylated proteins do not show an exclusive membrane localization, and a second interaction is required for efficient membrane-binding (reviewed in Resh, 1999; Taniguchi, 1999). For GAPR-1, additional membrane-binding interactions could be provided by protein-protein interactions. We have shown that GAPR-1 interacts directly with caveolin-1, possibly via a potential caveolin-binding motif. In addition, a predicted coiled-coil region might be involved in membrane anchoring. As GAPR-1 is expected to be highly charged at physiological pH (pI=9.4), electrostatic interactions could provide an additional anchoring signal as has been shown for MARCKS proteins (McLaughlin and Aderem, 1995).

The mechanisms of microdomain localization of GAPR-1 remain to be determined. We previously showed that several proteins that localize to microdomains in the Golgi complex (GICs), including the B subunit of the v-ATPase, flotillin-1, caveolin-1 and p17 (GAPR-1), interact with each other, even after disruption of the microdomain scaffold (Gkantiragas et al., 2001). This indicates that the lipid scaffold is not absolutely required for interactions between these proteins. We have now extended these findings by the demonstration of a direct interaction between GAPR-1 and caveolin-1. The interaction of GAPR-1 with caveolin-1 and/or other GIC proteins might provide a means to localize GAPR-1 to microdomains.

Cycling of GAPR-1 through the early secretory pathway

As shown in Fig. 5, GAPR-1 redistributes into tubulo-vesicular clusters that are scattered in the cytoplasm upon treatment of cells with BFA. BFA inhibits anterograde but not retrograde transport of proteins, causing disassembly of the Golgi complex. Under these conditions, Golgi-resident proteins such as mannosidase II are transported back to the endoplasmic reticulum. Other proteins that cycle through the early secretory pathway, such as ERGIC-53, KDEL receptor and p24 family

proteins, have been reported to localize to tubulo-vesicular clusters scattered in the cytoplasm (Fullekrug et al., 1999; Hendricks et al., 1992; Lippincott-Schwartz et al., 1990; Tang et al., 1993). Not much is known about the nature of these dispersed tubulo-vesicular clusters, also termed Golgi remnants. Recently, Warren and co-workers suggested that Golgi remnants may be the substrates for the synthesis of new Golgi cisternae (Seemann et al., 2000). In contrast to, for example, the KDEL receptor and p24 family proteins, GAPR-1 is unlikely to be transported by COPI-coated vesicles, as it is excluded from these carriers (Fig. 2C). In support of this, we find that the kinetics of BFA-induced redistribution of GAPR-1 are different from proteins that are transported by COPI-coated vesicles such as the KDEL-receptor. COPI-independent pathways through the early secretory pathway have been described (Girod et al., 1999; White et al., 1999). It remains to be established, however, whether the slower kinetics observed are due to an alternative pathway or due to an effect on sorting (e.g. due to the presence of GAPR-1 in microdomains).

GAPR-1 is a new member of the protein superfamily of PR proteins

As shown in Fig. 1, GAPR-1 shares significant amino-acid sequence homology and identity with a large variety of proteins derived from the three kingdoms of animals, plants and fungi. These proteins share several distinctive features unique to this superfamily and present in most family members: (i) two highly conserved histidines (AA 54 and 103 in GAPR-1) and glutamates (amino acids 65 and 86), which might represent an active center in a cleft, as observed in the NMR structure of family member p14a (Fernandez et al., 1997; Szyperski et al., 1998); and (ii) two motifs, GENL(A) and gHyTQvVW, are conserved in all family members (corresponding to amino acid 64-68 and 102-109 in GAPR-1).

Despite the diversity within this superfamily, very little is known about the function of the individual members. The first identified and most intensely studied family members are the plant pathogenesis-related (PR) proteins (Bol et al., 1990). There are five groups of PR proteins (PR-1 to PR-5) that do not share structural or functional similarities except for the induction of expression of PR proteins after infection of the plants by bacteria, fungi and viruses. More recently, the induction of PR proteins is used as an early marker for systemic acquired resistance, a long lasting defence mechanism of the plants against pathogens (Klessig et al., 2000; Maleck et al., 2000; Zhang et al., 1999). Of all the mammalian homologues identified to date, GAPR-1 has the highest level of identity and homology to PR-1 proteins. In addition, the molecular mass of GAPR-1 (17 kDa) is very similar to that of PR-1 proteins (16-19 kDa). The size of all other family members ranges between 23 and 89 kDa, reflecting the presence of a PR domain in a larger protein sequence.

As mentioned above, the plant PR-1 protein family is involved in systemic acquired resistance, which has similarities to the innate immune system in animals (Kitajima and Sato, 1999; Klessig et al., 2000; Maleck et al., 2000; Zhang et al., 1999). The innate immune system is the first line of defence and is similar to the plant immune system; a primary challenge is the discrimination of pathogens from self with the use of a restricted number of receptors. We find high levels of GAPR-1 expression in immunocompetent cells and organs. Consistent with a possible role in the innate immune system, we find high levels of expression in leukocytes, monocytes, lung and spleen. Another interesting finding is the high expression level of GAPR-1 in embryonic tissue. This is also consistent with its homology to plant PR-1 proteins, which are highly expressed during cell division and differentiation events in the development of plant roots and flowering (Fraser, 1981; Kitajima and Sato, 1999; Memelink et al., 1990).

Another human family member of PR-1 proteins, glioma pathogenesis-related protein or GLIPR, is highly expressed in glial tumors and glioma-derived cell lines. On the basis of these findings and induced expression upon phorbol ester treatment in macrophages, Murphy et al. speculated about a function for PR family members in the human immune response (Murphy et al., 1995).

Unique features of GAPR-1 within the superfamily of PR-proteins

On the basis of sequence homology, GAPR-1 belongs to the superfamily of plant pathogenesis-related proteins. Biochemical analysis of this protein, however, revealed properties that are unique to GAPR-1 and not found in other family members. The most prominent difference is that, in contrast to GAPR-1, all other family members studied so far are encoded by DNA that contains a signal sequence for translocation across the endoplasmic reticulum during protein synthesis, resulting in their secretion, storage in secretory granules, or accumulation in vacuoles (Bol et al., 1990; Kjeldsen et al., 1996; Kratzschmar et al., 1996; Linthorst, 1991; Lu et al., 1993; Magdaleno et al., 1997; Schreiber et al., 1997; Schuren et al., 1993). The other human family member, named GLIPR or RTVP-1 (Murphy et al., 1995; Rich et al., 1996), is preferentially expressed in glial tumours and also contains a probable signal sequence (Rich et al., 1996).

GAPR-1 is the first family member that does not have a signal sequence, and as a result has an intracellular cytoplasmic localization. In addition, GAPR-1 is the first family member shown to bind to membranes. The involvement of myristoylation in membrane binding of GAPR-1 has been discussed above. Acylation of other members of the protein superfamily has not been reported. Finally, the association of GAPR-1 with microdomains is an unexpected finding, which provides new possibilities to study a function of GAPR-1, in particular with respect to interactions with other proteins localized to these microdomains. An important role of microdomains is their function in signal transduction by clustering of specific proteins (Brown and London, 1998a; Simons and Ikonen, 1997). The immune response is also regulated by the association of proteins with microdomains (reviewed in Brown and London, 2000; Simons and Toomre, 2000). Studies on the association of GAPR-1 with microdomains and the possible link with the immune response might provide valuable information on the function of this protein and probably of other members of the superfamily as well.

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