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Linking microfilaments to intracellular membranes: the actin-binding and vesicle-associated protein comitin exhibits a mannose-specific lectin activity

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Comitin is a 24 kDa actin-binding protein from *Dictyostelium discoideum* that is located primarily on Golgi and vesicle membranes. We have probed the molecular basis of comitin's interaction with both actin and membranes using a series of truncation mutants obtained by expressing the appropriate cDNA in *Escherichia coli*. Comitin dimerizes in solution; its principle actin-binding activity is located between residues 90 and 135. The N-terminal 135 'core' residues of comitin contain a 3-fold sequence repeat that is homologous to several monocotyledon lectins and which retains key residues that determine these lectins' three-dimensional structure and mannose binding. These repeats of comitin appear to mediate its interaction with mannose residues in glycoproteins or glycolipids on the cytoplasmic surface of membrane vesicles from *D.discoideum*, and comitin can be released from membranes with mannose. Our data indicate that comitin binds to vesicle membranes via mannose residues and, by way of its interaction with actin, links these membranes to the cytoskeleton.

Keywords: actin-binding protein/lectin activity/mannose-specific lectin/membrane binding

Introduction

The actin cytoskeleton has been implicated in intracellular traffic and several aspects of endo- and exocytosis (Kelly, 1991). However, actin's role in these processes has been thought to be mainly to provide tracks for organelle and vesicle transport (Kuznetsov *et al.*, 1992) and to localize exocytotic and endocytotic vesicles in secretory and neuronal cells. Several actin-binding proteins, such as unconventional myosins (Adams and Pollard, 1986; Fath and Burgess, 1993; Mermall *et al.*, 1994), spectrin (Beck *et al.*, 1994; Tsakiridis *et al.*, 1994), synapsin (Valtorta *et al.*, 1992) and comitin (Weiner *et al.*, 1993) have been implicated in these processes.

Comitin is a 24 kDa actin-binding protein first identified in *Dictyostelium discoideum* (Stratford and Brown, 1985; Noegel *et al.*, 1990) which also appears to be present in mammalian cells (Weiner *et al.*, 1993). Bacterially expressed comitin cDNA produces a protein that pellets with F-actin in sedimentation assays and increases the viscosity of F-actin solutions. Immunofluorescence and immunoelectron microscopy suggest that comitin is located on the cytosolic site of the Golgi apparatus and on vesicles in cultured NIH 3T3 fibroblasts and in *D.discoideum* cells. As the localization of comitin, at least at the level of immunofluorescence microscopy, is disturbed by treatments that disrupt microfilaments, it has been proposed that comitin may help attach the Golgi and vesicles to this component of the cytoskeleton (Weiner *et al.*, 1993).

Comitin of *D.discoideum* is a 185 residue basic polypeptide chain (pI = 9.6) that is constructed from a 144 residue NH_2-terminal 'core' domain and a 41 residue COOH-terminal domain (Noegel *et al.*, 1990). The COOH-terminal domain has a characteristic GYP(P)Q repeat found also in octopus rhodopsin (Ovchinnikov *et al.*, 1988), annexins VII and XI (Burns *et al.*, 1989; Döring *et al.*, 1991; Towle and Treadwell, 1992), synaptophysin (Leube *et al.*, 1987) and a number of other proteins including mammalian lectins (Jia and Wang, 1988; Mehul *et al.*, 1994). Moreover, there is a 3-fold sequence repeat within the NH_2-terminal 'core' domain with residues 24–62, 63–96 and 97–127 being homologous. The comitin sequence does not show any homologies to other families of actin-binding proteins. Since it lacks any obvious transmembrane domain, the molecular basis for its association with membrane vesicles is not immediately apparent.

In this paper we have pursued the molecular basis of the interaction of comitin with both actin and Golgi or other membrane vesicles using a series of comitin truncation mutants. We show that the actin-binding activity is located primarily between residues 90 and 135, whereas the interaction with membranes appears to derive from a lectin-like activity that is associated with the three sequence repeats located in the NH_2-terminal 'core' of the molecule. These repeats are homologous to domains of plant lectins that are responsible for the strong binding of mannose. On the basis of these results we propose a model in which comitin links membrane vesicles to the actin cytoskeleton via mannose residues present on their cytoplasmic surface (Hirschberg and Snider, 1987; Vidugiriene and Menon, 1994). Such a linkage may have important consequences for the cell biology of these membrane systems.

Results

Purification and characterization of recombinant comitin proteins

Since previously only small quantities of comitin were expressed in *Escherichia coli* (Weiner *et al.*, 1993), we devised a superior protocol based on the pT7-7 system to

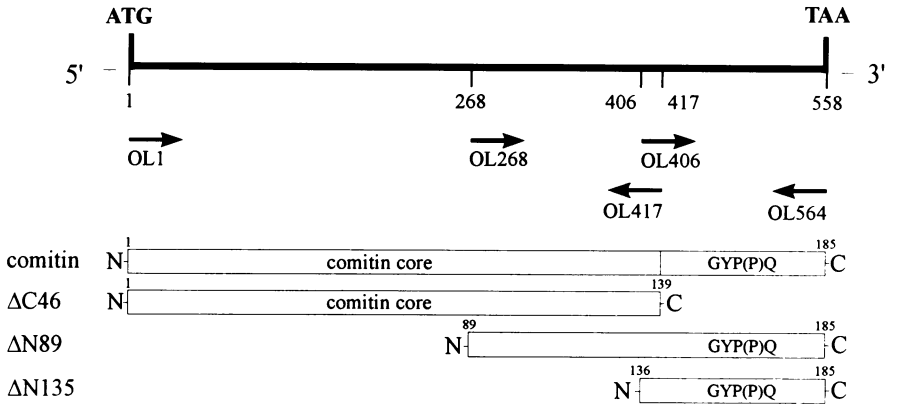

Fig. 1. Schematic presentation of the comitin cDNA clone cDRS17 and the resulting proteins (comitin, ΔC46, ΔN89 and ΔN135) obtained by PCR; the positions of the primers used are indicated (arrows).

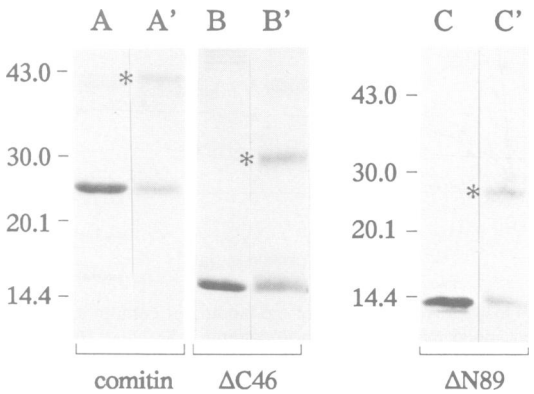

Fig. 2. Chemical cross-linking of comitin, ΔC46 and ΔN89. Purified comitin proteins at a concentration of 0.1 mg/ml were cross-linked for 20 min at room temperature in the presence of 0.02% glutaraldehyde and separated by SDS–PAGE (15% acrylamide). A and B show Coomassie Blue-stained gels. (A) comitin control, (A') comitin after cross-linking, (B) ΔC46 control, (B') ΔC46 after chemical cross-linking. In (C) ΔN89 was detected by Western blot analysis using mAb 190-68-1. (C) ΔN89 control, (C') ΔN89 after chemical cross-linking. The dimer is indicated by an asterisk.

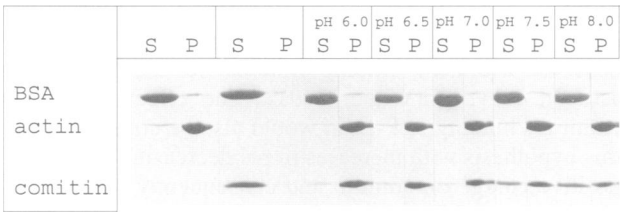

Fig. 3. Co-sedimentation of comitin with F-actin in the presence of BSA at varying pH values. G-actin (5 μM) was mixed with 5 μM comitin and 3 μM BSA in polymerization buffer at varying pH values and incubated for 1 h at room temperature. The samples were sedimented in an airfuge at 120 000 *g* for 1 h, aliquots of the supernatants (S) and pellets (P) were subjected to SDS–PAGE (15% acrylamide), and the gels stained with Coomassie Blue. The first four lanes show the actin and comitin controls.

express comitin and a series of truncated constructs (ΔN89, ΔN135 and ΔC46) (Figure 1). Using a lower expression temperature (28°C) and inducing the T7 RNA-polymerase by adding 0.2 mM IPTG to growing cells, instead of induction by heat shock, a product was obtained that could be solubilized in 4 M urea and purified to homogeneity by using a single cation exchange column. This was a significant advantage over previous methods that had employed harsher denaturing conditions, such as 8 M urea or 6 M guanidinium hydrochloride (Stratford and Brown, 1985; Weiner *et al.*, 1993). The material obtained after cation exchange was essentially homogenous. After dialysis it could be used directly for actin-binding studies.

Comitin forms dimers in solution

Chemical cross-linking of comitin with 0.02% (v/v) glutaraldehyde produced a prominent band with a molecular mass of ~42 kDa in SDS–PAGE (Figure 2), indicating the presence of a dimer. Higher molecular weight bands were not formed readily and so we conclude that the comitin molecule exists as a homodimer in solution. Both the comitin core domain (ΔC46) and the N-terminally shortened comitin (ΔN89) showed cross-linking behaviour (Figure 2), indicating that they too had dimerized. So residues responsible for dimerization were located between residues 90 and 135. Because it lacked lysine residues, we were not able to determine whether the COOH-terminal GYP(P)Q domain (ΔN135) also dimerized.

Binding of comitin to F-actin is pH-dependent and saturable

The F-actin binding activity of comitin was analysed using actin sedimentation assays in which purified comitin and G-actin were mixed and the actin polymerized by addition of 2 mM Mg^{2+} in imidazole buffer. These experiments confirmed previous *in vitro* binding studies which showed that comitin had actin binding activity (Stratford and Brown, 1985; Weiner *et al.*, 1993). We extended these studies by examining the role of pH over the range 6–8 and showed that comitin co-sedimented with the F-actin in a pH-dependent manner: between pH 6.0 and 7.0 comitin was almost completely pelleted under the conditions used. At pH 7.5 and above, comitin was also recovered in the supernatant (Figure 3). The sedimentation of F-actin was unaffected under these conditions. Controls for actin and comitin alone showed that actin sedimented quantitatively, whereas comitin remained completely in the supernatant (Figure 3). It did not sediment without actin under any of the conditions used in this or the following experiments. Ca^{2+} did not influence F-actin binding by comitin (data not shown). The results of these co-sedimentation assays indicate that binding of comitin did not prevent actin

polymerization. Thus, comitin is neither an F-actin capping or severing protein, nor does it act like profilin in preventing G-actin from polymerization.

For determination of the affinity of comitin for F-actin the binding was measured at varying molar ratios of actin to comitin by densitometric analysis of supernatants and pellets of Coomassie Blue-stained SDS–polyacrylamide gels. Figure 4 illustrates the binding curves obtained. The quantity of comitin in the pellet increased up to a ratio of 1:1 (comitin per actin subunit). Half-maximal binding was estimated to be at 0.2 μM. The specificity of the interaction was assayed by the addition of increasing amounts of salt (50–150 mM KCl) and BSA. Addition of BSA showed no effect on the actin-binding activity of comitin (Figure 3), in the presence of KCl the actin-binding activity was reduced, which indicated ionic interactions of the negatively charged actin (pI 5.6) and the positively charged comitin (pI 9.6). Sensitivity of the actin interaction towards high salt concentrations is a phenomenon quite frequently observed with actin-binding proteins, i.e. α-actinin, when assayed *in vitro* (Witke, 1991). The effect of pH on comitin's binding to F-actin would also be consistent with this hypothesis with increases in pH decreasing the overall positive charge on comitin and consequently reducing its affinity for actin.

Localization of the comitin actin-binding domain

To identify putative actin-binding regions in comitin, we probed the actin-binding ability of three fragments obtained by cloning and expressing parts of comitin cDNA in *E.coli*. Fragment ΔC46 consisted only of the comitin N-terminal core domain and showed unequivocal but markedly reduced actin-binding activity in sedimentation assays. Figure 4 shows a plot of the amount of bound ΔC46 to F-actin. Binding was relatively weak and saturated at a molar ratio of 1 mol ΔC46 to 5 mol F-actin.

Deletions from the NH_2-terminus of comitin produced several effects. ΔN135 showed no binding to F-actin (data not shown) whereas ΔN89 bound strongly and its binding curve reached saturation at 1 mol ΔN89/mol F-actin, similar to whole comitin (Figure 4). The dissociation constant of ΔN89 for actin was ~0.1 μM which was lower than that for the intact molecule. The Scatchard analysis of the interaction between whole comitin and actin, however, suggests that the complete molecule harbors a high-affinity binding site which, like ΔN89, saturates roughly at a 1:1 molar ratio. The additional low-affinity binding site might be due to ionic interactions. These results show that the NH_2-terminal 89 residues of comitin are not required for actin-binding. The principle actin-binding domain in comitin therefore seems to be located in the transition zone of the comitin core domain and the GYP(P)Q repeat between residues 90 and 135.

Comitin bundles actin filaments in vitro

When G-actin was polymerized in the presence of varying amounts of comitin and truncated comitin proteins, the viscosity of the actin solution increased markedly as would be expected if the actin filaments formed a cross-linked network (Figure 5). Consistent with its high affinity for F-actin, ΔN89 increased the viscosity of the F-actin solution even more than comitin. ΔC46 also produced an increase in viscosity of the F-actin solution, even though

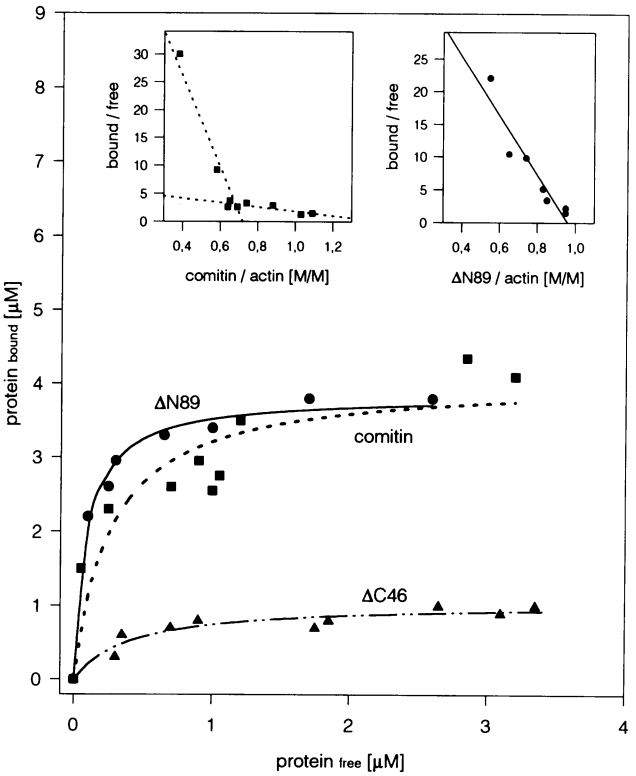

Fig. 4. Quantitative evaluation of the binding of comitin to F-actin. Different molar ratios of G-actin to comitin, ΔN89 and ΔC46, with the actin concentration kept constant, were co-polymerized, centrifuged at 120 000 *g*, the pellets solubilized in the original volume, and the proteins from the supernatants and pellets subjected to SDS–PAGE. The Coomassie Blue-stained gels were scanned, and the amounts of free comitin, ΔN89 or ΔC46 were plotted versus the bound protein per actin. The insets show a Scatchard plot of comitin and ΔN89, where the ratios of the respective protein to actin were plotted versus bound protein over free protein.

it bound comparatively weakly to F-actin in sedimentation experiments. This is not unusual and indeed many proteins show a similar effect which is thought to be due to the high rate at which the proteins exchange with actin (Pollard and Cooper, 1986). Under these circumstances, less of the binding protein is sedimented, but the large number of transient interactions that occur in solution still produce a marked increase in viscosity.

In the presence of comitin, F-actin could be sedimented in low-speed centrifugations at 15 000 *g* which was analogous to the bundling of filaments observed with the actin-cross-linking protein α-actinin (Meyer and Aebi, 1990) and the severin domain 2+3, which is a genetically engineered polypeptide (Eichinger and Schleicher, 1992). Addition of comitin to actin at a molar ratio of 1:1 rendered the polymerized actin completely pelletable at 15 000 *g* (Figure 6).

Electron microscopy confirmed that comitin was forming bundles of F-actin (Figure 7). Addition of comitin to a polymerizing actin solution produced thick bundles that were easily seen by negative staining. These bundles have considerable paracrystalline order and it was often possible to observe distinctive 'zig-zag' patterns along them that derived from the 5.1 nm short-pitch actin genetic helices (Figure 7A). A distinctive feature of these paracrystalline comitin-induced actin bundles was their frequent breaking

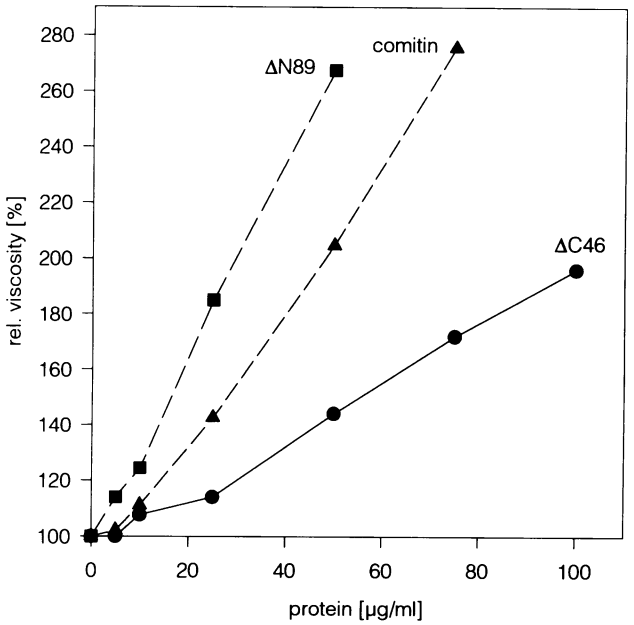

Fig. 5. Enhancement of the viscosity of an F-actin solution in the presence of comitin, ΔC46 and ΔN89 respectively. Skeletal muscle actin (0.25 mg/ml) was polymerized with increasing amounts of purified, recombinant comitin proteins and analysed by low shear viscometry assays. The data shown are the mean values of three independent experiments.

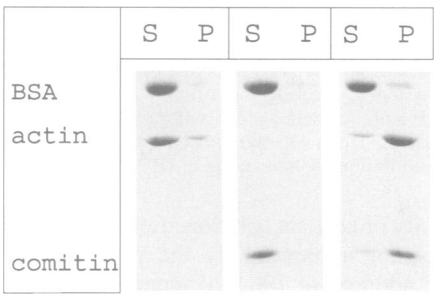

Fig. 6. Low speed sedimentation of F-actin in the presence of comitin. G-actin (4 μM) and comitin (4 μM) were co-polymerized for 1 h at room temperature in the presence of 3 μM BSA in polymerization buffer, centrifuged at 15 000 *g* for 60 min, and the supernatants (S) and pellets (P) analysed by SDS–PAGE. On the left is a control for actin, in the middle a control for comitin, treated as described above.

to produce unusual square ends (Figure 7B). This observation indicated that the binding of comitin to actin made the paracrystals unusually stiff and brittle. The highly ordered nature of these bundles was consistent with the tight binding observed. In summary, both low shear viscometry and electron microscopy indicated that comitin interacts with actin to form networks and bundles.

Comitin is a mannose-specific lectin

Previous studies (Noegel *et al.*, 1990) indicated that comitin is a member of a multigene family and its COOH-terminal repeat has a homology to the COOH-terminal repeat sequences of octopus rhodopsin, synaptophysin and annexin VII (Matsushima *et al.*, 1990). Recently, the sequence of several new plant lectins has been reported (van Damme *et al.*, 1991, 1992, 1993a,b) which show a significant homology to the 3-fold repeat in the comitin NH_2-terminus (Figure 8). A common feature of the homologous plant lectins is their specificity for mannose. Accordingly, we investigated the interaction of comitin with mannose and the effect of this sugar on comitin's molecular interactions.

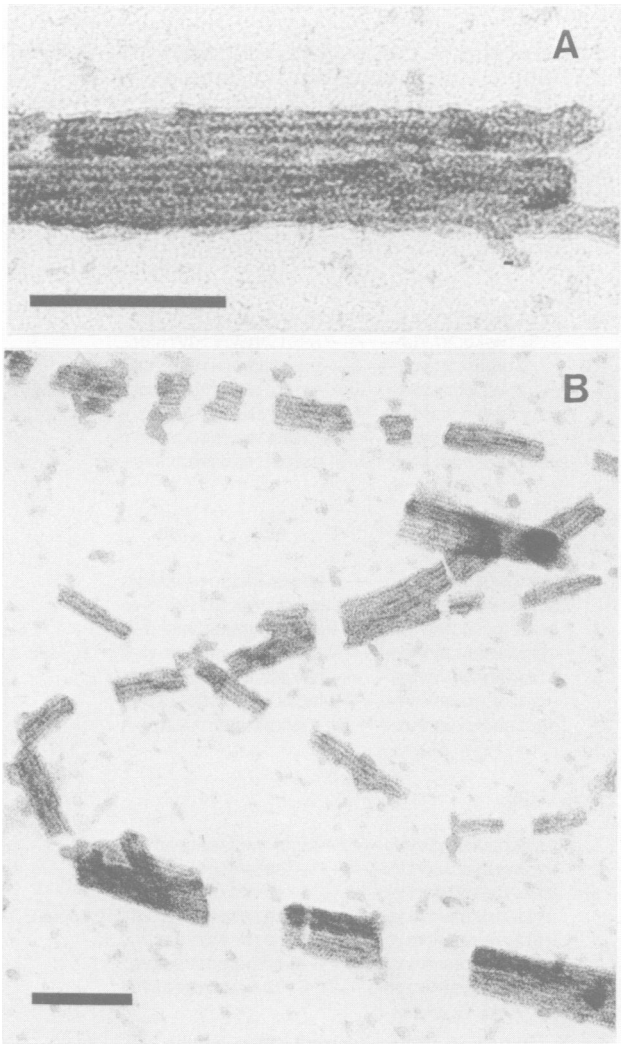

Fig. 7. Electron micrographs of negatively stained actin filament bundles (1% uranyl acetate). Actin filament bundles were formed by mixing purified bacterially expressed comitin and G-actin in polymerization buffer, negative staining was performed after 2 h of incubation at room temperature. (**A**) A single bundle at higher magnification. (**B**) An overview. Bar = 100 nm.

Haemagglutination assays indicated that comitin, at a concentration of 0.2 mg/ml, agglutinated rabbit erythrocytes and that this activity is specifically inhibited by 100 mM mannose. The agglutination activity did not depend on Ca^{2+} ions and was insensitive to the addition of sugars other than mannose. For instance, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine and fucose had no effect on the haemagglutination activity of comitin (Figure 9). The ΔN89 truncation mutant that lacked most of the sequence homologous to plant lectins (Figure 8) had no haemagglutinating activity, whereas the comitin core domain construct ΔC46 showed the same haemagglutination activity as comitin (Figure 9). Overall, therefore, the haemagglutination data was strongly indicative of an interaction between comitin and mannose that involved the NH_2-terminal 135 residues of the molecule.

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                          10          20
                          |           |
1 comitin ΔC46      MELLRQGEHLHSTSRSTLESKCRR
2 A.ursinum         RNLLGNEEGL--YADQSLEEG---
3 A.sativum          NILMNDEGL--YAGQSLDVE---
4 A.ascolonicum     RNVLVNNEGL--YAGQSLVEE---
5 A.cepa            RNVLVNNEGL--YAGQSLVVE---
6 N.spec.           DNILYSGDTL--STGQFLSYG---
7 G.nivalis         DNILYSGETL--STGEFLNYG---
                       *     *     *

          30          40          50          60
          |           |           |           |
1   KYKLIMQNDGNLVLYIGSLKSQSDEYCLWSSASCGKGHG
2   PYKLIMQKDCNLVLF-------EHSTVVWATKTGVTGRN
3   PYHLIMQEDCNLVLY-------DHSTAVWTSNTDIPGKK
4   QYTFIMQDDCNLVLY-------EYSTPIWASNTGITGKN    repeat I
5   QYTFIMQDDCNLVLY-------EYSTPIWASNTGVTGKN
6   SYVFIMQEDCNLVLY-------DVDKPIWATNTGGLSSD
7   SFVFIMQEDCNLVLY-------DVDKPIWATNTGGLSRS
       * *Δ Δ Δ*Δ*Δ              *

            70                  80          90
            |                   |           |
1   PYRLSMQEDGNLVIYD------SRNSAIWASGTMGHGVRG
2   GCKAVMQADGNFVVED------SNGRIVWASNSIKG-N-G
3   GCKAVLQSDGNFVVYD------AEGRSLWASHSVRG-N-G
4   GCRAVMQPDGNFVVYN------VKGRAVWASNSRRG-N-G   repeat II
5   GCRAVMQADGNFVVYD------VKGRAVWASNSRRG-N-G
6   -CHLSMQTDGNLVVYS------PQNKAIWASNTD-GEN-G
7   -CFLSMQTDGNLVVYN------PSNKPIWASNTG-GQN-G
       * *Δ Δ Δ*Δ*Δ              *

        100         110                 120
        |           |                   |
1   HYSMKLRSSGQIVVYD------KYKQILYSSKPCTRDHLL
2   NYILVLRNNGNVVIYG---------SDIWSTGTHIRGVKG
3   NYVLVLQEDGNVVIYG---------SDIWSTNTYKGSARG
4   NYILVLQKDRNVVIYG---------SDIWSTGTYRKKVGG   repeat III
5   NYILVLQKDRNVVIYG---------SDIWSTGTYRKKVGG
6   HFVCILQKDRNVVIYG---------TDRWATGTYTGAVGI
7   NYVCILQKDRNVVIYG---------TDRWATGTHTGLVGI
       * *Δ Δ Δ*Δ*Δ              *
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Fig. 8. Multiple sequence alignment of the comitin core domain (ΔC46) with mannose-specific monocotyledon lectins. Key residues in the three-dimensional structure of snowdrop (*Galanthus nivalis*) lectin (Hester *et al.*, 1995) are shown in bold. Residues that make up the hydrophobic core of the domain are indicated by asterisks whereas those surface residues that constitute the mannose-binding site are indicated by triangles. Clearly the comitin repeats have retained the key hydrophobic core residues as well as those involved in mannose binding. The numbering of the sequences corresponds to comitin. Interestingly the comitin sequence has insertions between residues 38 and 44 (in repeat I) and 113–115 (in repeat III). These insertions are close together in the three-dimensional structure of snowdrop lectin (Hester *et al.*, 1995) and may form part of the comitin actin binding site. Accession numbers for the GenBank/EMBL and references are as follows: *Allium ursinum*, S38258 (van Damme *et al.*, 1993a), *Allium sativum*, S23491 (van Damme *et al.*, 1992), *Allium ascolonicum*, S39488 (van Damme *et al.*, 1993b), *Allium cepa*, S39487 (van Damme *et al.*, 1993b), *Narcissus* spp., M88117 (unpublished) and *Galanthus nivalis*, S19735 (van Damme *et al.*, 1991).

Mannose also influenced the binding of comitin to membranes in *D.discoideum* homogenates. Thus, comitin was removed from these membranes by phosphate buffer containing mannose, whereas it remained fully bound when mannose was omitted or was substituted for by galactose or *N*-acetylglucosamine (Figure 10).

Mannose inhibits the comitin–F-actin interaction

The effect of mannose on the comitin–actin interaction was investigated by low shear viscometry assays in the presence of 100 mM mannose. In control experiments,

protein \ sugar	-	Man	GlcNAc	L-Fuc	Gal	GalNAc
KP	–	–	–	–	–	–
comitin	+	–	+	+	+	+
ΔC46	+	–	+	+	+	+
ΔN89	–	–	–	–	–	–

Fig. 9. Determination of carbohydrate specificities of comitin proteins by inhibition of haemagglutination of erythrocytes in the presence of varying sugars (final concentration: 100 mM). The assay was carried out as described in Materials and methods, in the control only KP buffer was added; (+) haemagglutination positive, (–) no haemagglutination.

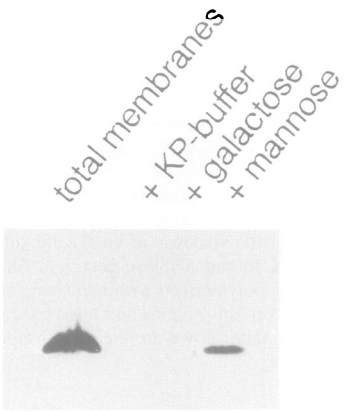

Fig. 10. Selective release of comitin from membranes by addition of mannose. Membranes prepared using the polyethylene glycol/dextrane two-phase system of Brunette and Till (1971) were extracted with KP buffer or KP buffer containing 200 mM galactose or mannose. After centrifugation for 1 h at 120 000 *g*, equal amounts of the supernatants were analysed in immunoblots using mAB 190-68-1.

the viscosity of an actin solution after 30 min of polymerization was not influenced by the presence of mannose. However, addition of 100 mM mannose to the polymerization buffer resulted in a strong decrease of the cross-linking activity of comitin; addition of *N*-acetylglucosamine at the same concentration showed no effect (Figure 11). The activity of ΔN89 was not influenced by mannose (Figure 11), whereas the activity of the ΔC46 polypeptide was virtually completely inhibited by mannose (Figure 11). In accord with these results, in sedimentation assays with F-actin more of the comitin remained in the supernatant in the presence of mannose than without mannose (Table I), and electron microscopy of F-actin–comitin in the presence of 100 mM mannose showed primarily single filaments (Figure 12).

Discussion

Molecular basis for the interaction between comitin and actin

Comitin, a protein originally isolated from *D.discoideum* based on its actin-binding activity, was shown by immunogold EM in mammalian cells to be present on cisternal and associated elements of the Golgi apparatus. It was also found on the cytoplasmic side of rough ER membranes as well as early and late endosomes. Treatment with agents that affected the actin cytoskeleton or the Golgi apparatus led to a reversible redistribution of comitin (Weiner *et al.*,

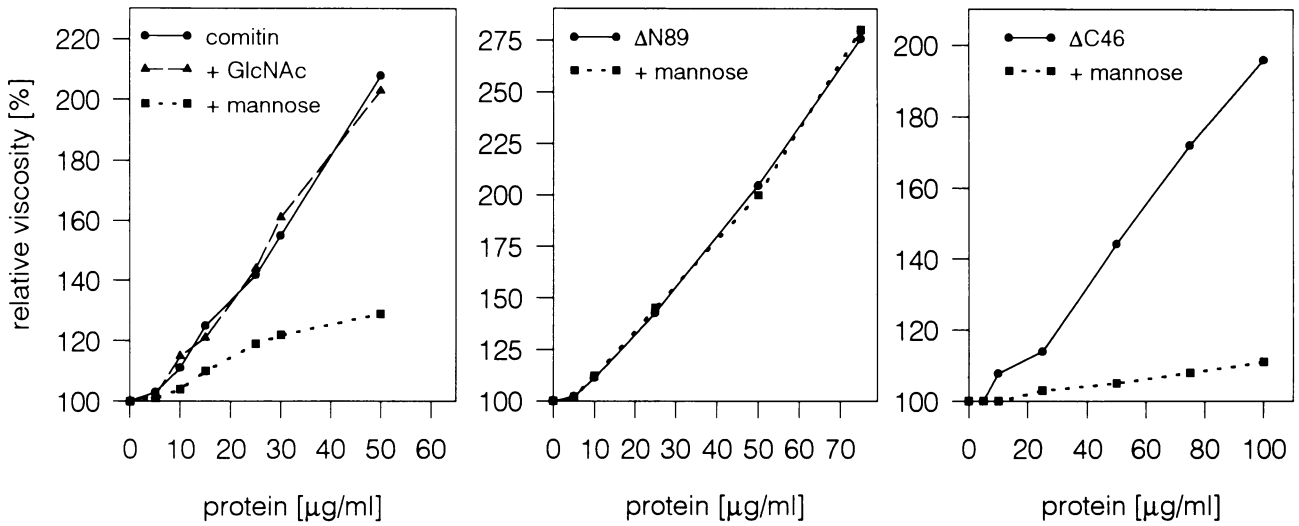

Fig. 11. Low shear viscometry assays in the presence and absence of sugars. The effects of *N*-acetylglucosamine or mannose on the cross-linking activity of comitin, and the cross-linking activities of ΔN89 and ΔC46 in the presence and absence of mannose are shown. The data are the mean values of three independent experiments.

Table I. Influence of mannose on the binding of comitin to F actin

Com:Act	(%S+M)–(%S–M)	M:(Com:Act)
0.08	58.00	1250.00
0.30	38.00	330.00
0.60	20.00	166.00
1.20	19.00	83.00

G-actin (6 μM) was mixed with different concentrations of comitin at pH 7.6 in the presence of 100 mM mannose and incubated for 1 h at room temperature. The samples were sedimented in an airfuge for 1 h and aliquots of the supernatants and pellets were subjected to SDS–PAGE (15% acrylamide). The Coomassie Blue-stained gels were scanned and the amount of unbound comitin determined. Com:Act is the comitin:actin ratio used in the assay, M:(Com:Act) is the calculated ratio of mannose to the comitin:actin ratio (%S+M)–(%S–M) shows the difference between amount of comitin in the supernatant when the experiment was done in the presence of mannose (%S+M) and the amount of comitin in the supernatant when the experiment was done in the absence of mannose (%S–M).

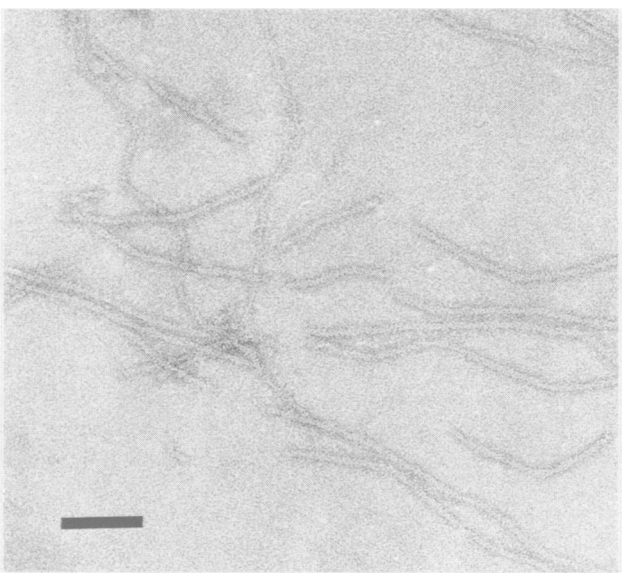

Fig. 12. Electron micrograph of a field of F-actin–comitin in the presence of 100 mM mannose (negatively stained with 1% uranyl acetate). Note that, in contrast to the bundles formed in the absence of mannose (see Figure 7), most of the F-actin is now in the form of isolated filaments, consistent with mannose inhibiting the actin-binding activity of comitin. Bar = 100 nm.

1993). We have now identified the actin-binding region of comitin and the mechanism of its binding to membranes.

Studies with a series of truncation mutants indicated that a site responsible for the strong binding to actin was located between residues 90 and 135 of the polypeptide chain. However, we also found a low affinity of the ΔC46 polypeptide for actin that indicated regions not present in ΔN89 might also influence actin-binding. Glycine- and proline-rich domains, as they are found in ΔN89, have been implicated in actin-binding of myosin IB and IC of *D.discoideum* (Jung and Hammer, 1994; Rosenfeld and Renner, 1994) and of vinculin (Menkel *et al.*, 1994). Whereas in the myosins the actin-binding site seems to reside entirely within these sequences, in vinculin as in comitin these sequences appear only to enhance the binding or be part of the binding region.

To be able to cross-link actin filaments into bundles, the comitin molecule must have two actin-binding sites. Probably each polypeptide chain has only a single actin-binding site and the bifunctional character of the molecule is supplied by its dimerization as is commonly observed with other actin-bundling proteins such as α-actinin (Matsudaira, 1991; Otto, 1994). The 1:1 stoichiometry of comitin's binding to actin and the evidence for its dimerization in solution favour such a mechanism.

Comitin as an intracellular lectin

Lectins are a diverse group of carbohydrate binding proteins that can be grouped into the monocotyledon, leguminous and cereal families in plants and into the pentraxins and C-, S- and P-type lectins in animals (Drickamer and Taylor, 1993; Sharon, 1993). The three sequence repeats in the N-terminal domain of comitin show very clearly homology to a group of monocotyledon lectins which do not belong to the cereal lectin family (van Damme *et al.*, 1987, 1991, 1992, 1993a,b) and do

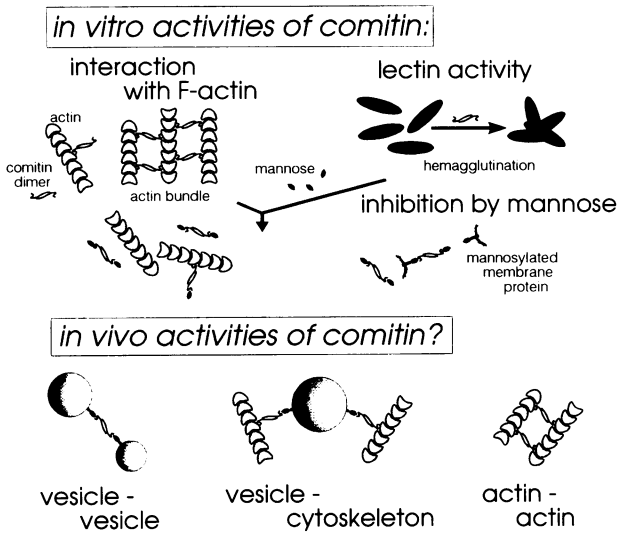

Fig. 13. Summary of comitin interactions. Two activities were identified for comitin, an actin-binding activity and a mannose-specific lectin activity. Due to its presence as a dimer in solution it can cross-link F-actin and bundle the filaments, and it can bind to vesicles and connect them (haemagglutination). Its properties would allow comitin to connect vesicles to filaments *in vivo*.

not contain the characteristic mannose recognition domain found in, for example, the human mannose receptor (Taylor *et al.*, 1990). X-ray crystallography studies (Hester *et al.*, 1995) have shown that in the monocotyledon *Galanthus nivalis* (snowdrop) lectin, this repeat corresponds to an antiparallel four-stranded beta sheet subdomain and that the 3-fold sequence repeat produces a 12-stranded beta-barrel in the molecule. Significantly, the region of sequence homology shared by the monocotyledon lectins and comitin contains both the key hydrophobic core residues of this structure as well as those surface residues involved in binding to mannose (see Figure 8). Overall, the sequence homology observed, together with the clear evidence for mannose binding, indicates that the N-terminal 3-fold sequence repeat in comitin will form a structure closely paralleling that found for the monocotyledon lectins. However, there are two interesting differences between the comitin and lectin sequences. Comitin has an insertion between residues 36 and 44 in repeat I, which is between strands 3 and 4 of subdomain III (using the numbering convention of Hester *et al.*, 1995) and, in addition, comitin repeat III, (which corresponds to subdomain I in the three-dimensional structure) has three extra lysine residues compared with subdomains II and III in both comitin and the lectins. These changes are all concentrated in the same region of the three-dimensional structure of the lectin molecule (see Figure 2 of Hester *et al.*, 1995) and would produce a positively-charged projection from comitin that could act as one of its actin-binding sites. Moreover, the monocotyledon lectin forms stable dimers through C-terminal strand exchange (Hester *et al.*, 1995) which would be consistent with the dimerizing function we have proposed for the comitin C-terminus.

Cellular role of comitin

Comitin interacts with both actin and vesicles and may function to link these membranes to the cytoskeleton. The release of comitin from membrane vesicles following treatment with mannose indicated that comitin bound to the membranes via mannose residues on either glycoproteins or glycolipids. Although research has focussed on the glycosylation of proteins within the lumen of the ER and Golgi, several cytoplasmic proteins are known to be modified by *N*-acetyl-glucosamine (Hart *et al.*, 1994; Hayes and Hart, 1994). There is also evidence for the incorporation of mannose into oligosaccharides in the cytoplasm. In the synthesis of $Glc_3Man_9GlcNAc_2$-PP-dolichol (Hirschberg and Snider, 1987), the precursor $Man_5GlcNAc_2$-PP-dolichol is formed at the cytoplasmic side of the ER, then translocated across the ER membrane and used at the lumenal side for the *N*-glycosylation of proteins. Another example for glycosylation in the cytoplasmic compartment is provided by the GPI anchor (Vidugiriene and Menon, 1994). The use of membrane-impermeant probes and *Trypanosoma* microsomal vesicles has shown that the formation of GlcN-PI carrying three mannose residues occurs on the cytoplasmic face of the ER. The glycolipid is then translocated across the ER membrane and, after completion of synthesis, transferred to protein.

Our data are consistent with comitin's having a role in coupling Golgi and membrane vesicles to the actin cytoskeleton. Comitin's lectin-like activity enables it to bind to intracellular membranes carrying glycoconjugates on their cytoplasmic faces and link them to the cytoskeleton (Figure 13). This way it could function by inhibiting their diffusion and so maintain them in the appropriate cellular location. Modulating such an attachment would, of course, have considerable potential for regulating aspects of the biosynthetic machinery associated with these membranous compartments.

Materials and methods

Construction, expression and purification of comitin and truncated comitin molecules

To clone comitin cDNA into the ATG-expression vector pT7-7 (Tabor, 1990) an *Nde*I restriction site was introduced into the full-length cDNA clone cDRS17 (Noegel *et al.*, 1990) by PCR mutagenesis (Weiner *et al.*, 1993). The ATG represents the authentic start codon for comitin. Additionally, three truncated cDNAs were amplified via PCR using the following primers (Figure 1): OL1: d (5′-CGC CAT ATG GAA TTA TTA AGA CAA GGT-3′); OL268: d (5′-GCG CAT ATG GGT CAT GGA GTT AGA G-3′); OL406: d (5′-GCG CAT ATG AAA CCA TCA GGA CAT CCA-3′); OL417: d (5′-GCG AAT TCT TAT CCT GAT GGT TTG GCA CA-3′); OL564: d (5′-GCG GAA TTC ATT ACT TTA ATA ATA TCC TGG-3′). The amplified DNAs were cloned into pT7-7 and their sequences confirmed using the pT7-7-specific primers. Recombinant *E.coli* BL21 (DE) (Studier and Moffat, 1986) were grown at 37°C to an OD_{600} of 1.0, expression was induced by addition of 0.2 mM IPTG, and the cells were further incubated for 8 h at 28°C and then harvested by low-speed centrifugation (10 min, 5000 *g*, 4°C) and washed once in TE-buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA). The cells were then resuspended in KP buffer (20 mM KH_2PO_4, pH 7.5, 3 mM DTT) and lysed by addition of lysozyme (0.1 mg/ml) followed by ultrasonication. After centrifugation (30 min, 30 000 *g*, 4°C) all comitin proteins except ΔN135 remained in the pellet, which was washed with KP buffer. The pellets were homogenized three times with KP buffer containing 4 M urea to solubilize comitin, ΔN89 and ΔC46 respectively. The comitin-containing supernatants were applied to a cation exchange column (CM52 cellulose, Whatman) equilibrated in the same buffer. Comitin and the truncated molecules were eluted by a linear gradient of the same buffer containing 0–500 mM NaCl. Comitin and ΔC46 were eluted at a molarity of 150–200 mM NaCl, ΔN89 at 180–220 mM NaCl. With the soluble ΔN135 protein, the cation-exchange chromatography was carried out in a KP buffer at pH 6.8, the protein

was eluted at a molarity of 100–150 mM NaCl. The proteins were essentially pure after this step. The comitin-containing fractions were pooled and dialysed three times against KP buffer. After dialysis, comitin proteins were centrifuged at 100 000 *g* for 1 h at 4°C whereby only properly folded and soluble comitin proteins remained in the supernatant. If necessary, the protein solution was concentrated in centriprep 10 or centriprep 3 concentrators (Amicon, Beverly, MA, USA).

Chemical cross-linking

0.1 to 0.3 mg/ml of protein in 20 mM KP buffer, pH 6.8, were incubated with 0.02% (v/v) glutaraldehyde at room temperature for 30 min. The reaction was stopped by the addition of glycine (50 mM final concentration) and the products separated by SDS–PAGE.

Low-shear viscometry

Low-shear viscometry was carried out after 30 min polymerization of G-actin at 25°C in a falling ball viscometer (MacLean-Fletcher and Pollard, 1980). The reaction mix (160 µl) contained 0.25 mg/ml gel filtered rabbit-muscle actin (purified according to Spudich and Watt, 1971) and comitin proteins at concentrations of 0–100 µg/ml. Polymerization was initiated by addition of G-actin to buffered $MgCl_2$ (final concentration: 2 mM $MgCl_2$, 10 mM imidazole, pH 7.2, 1 mM ATP, 0.2 mM $CaCl_2$ or 1 mM EGTA). The data shown are the mean values of four independent experiments.

Sedimentation assays

F-actin binding of comitin and truncated comitin molecules was investigated in sedimentation assays at 120 000 *g*. G-actin and comitin protein solutions were pre-centrifuged at 120 000 *g* for 30 min at 4°C in an airfuge (Beckman, Palo Alto, CA, USA). G-actin (final concentration 4 µM) was polymerized in a 10 mM imidazole buffer (pH 7.2) containing 2 mM $MgCl_2$, 0.2 mM $CaCl_2$ or 1 mM EGTA in the presence of varying amounts of purified comitin proteins for 1 h at room temperature. The pH of the polymerizing mixture was varied over a range of pH 6 to 8 by adding the appropriate MOPS buffer to a final concentration of 17 mM. After airfuge centrifugation (1 h, 120 000 *g*, 4°C), supernatants and pellets were resuspended in equal amounts and analysed by SDS–PAGE and Coomassie Blue staining. To quantify the co-sedimentation of comitin proteins with F-actin, the protein bands were scanned (Elscript 400, Hirschmann Co., FRG) and calculated relative to co-sedimented F-actin. Low speed sedimentation assays (15 000 *g*) at room temperature were also performed using the same conditions.

Electron microscopy

G-actin and comitin solutions were pre-centrifuged at 120 000 *g* for 30 min at 4°C. After polymerization of 2.5 µM G-actin in polymerization buffer in the presence of 2.5 µM comitin for 2 h at room temperature, the sample was diluted 1:10 in polymerization buffer. Controls without comitin were prepared in the same way. Samples were applied to formvar-coated copper grids, stained with an aqueous solution of 1% uranyl acetate and examined at 80 kV in either Jeol 100X (JOEL, Analytical Instruments Division, Cranford, NJ, USA) or Philips EM301 or EM420 (Philips, Eindhoven, Netherlands) electron microscopes. Micrographs were recorded on Kodak SO-163 film at nominal magnifications between ×30 000 and ×60 000.

Haemagglutination assay

The ability of comitin proteins to agglutinate rabbit erythrocytes was investigated in haemagglutination assays. The assay was carried out essentially as described by Sharon and Lis (1989) in microtiter wells (Greiner, Nürtingen, FRG) using 10 µl of a 10% suspension of erythrocytes and varying concentrations of protein up to 0.3 mg/ml. The samples were incubated for 2 h at room temperature and then examined. The agglutinated erythrocytes form a carpet that covers the whole well; where no agglutination occurred, the cells form a button at the bottom of the well. To determine the carbohydrate specificity of comitin we inhibited the agglutination by addition of 100 mM of different sugars.

Cell fractionation

Dictyostelium discoideum cells were grown at 21°C to ~2×10^6 cells/ml in liquid nutrient medium (Claviez *et al.*, 1982), harvested and washed in 17 mM Soerensen phosphate buffer (pH 6.0). The cells were resuspended to a density of 5×10^7 cells/ml in KP buffer and lysed in a Parr bomb by nitrogen cavitation after equilibrium for 10 min at 1000 psi. Microscopic examination revealed that essentially no intact cells remained in the lysate. The following steps were carried out at 4°C. The lysate was centrifuged for 1 h at 120 000 *g*, the comitin containing pellet (equivalent of ~3×10^6 cells) was homogenized in KP buffer and centrifuged for a second time in an airfuge at 120 000 *g* for 1 h. After this step no comitin was detectable in the 120 000 *g* supernatant. Aliquots of the pellet were homogenized in KP buffer, in KP buffer containing 200 mM *N*-acetylglucosamine (or 200 mM galactose), and in KP buffer containing 200 mM mannose. All samples were centrifuged in an airfuge at 120 000 *g* for 1 h and supernatants and pellets were analysed by SDS–PAGE and by Western blots using the comitin specific mAb 190-68-1 or mAB 190-23-5 (Weiner *et al.*, 1993). Bound antibodies were detected using the enhanced chemiluminescence method (ECL kit, Amersham Corp.). Alternatively membranes were prepared using the polyethylene glycol/dextrane two-phase system of Brunette and Till (1971). The membranes were washed with KP buffer and extracted with KP buffer or KP buffer containing 200 mM mannose or galactose. Samples were centrifuged for 1 h at 120 000 *g* and pellets and supernatants analysed as described above.

Miscellaneous methods

Protein concentrations were determined by the method of Bradford (1976) and Lowry *et al.* (1951) using BSA as a standard, and by amino acid analysis. All methods gave congruous results for comitin. SDS–PAGE was done according to Laemmli (1970) and Western blotting according to Towbin *et al.* (1979). All DNA manipulations were done as described in Sambrook *et al.* (1992).

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