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Membrane insertion and assembly of ductin: a polytopic channel with dual orientations

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Ductin is a highly conserved and polytopic transmembrane protein which is the subunit c component of the vacuolar H^+-ATPase (V-ATPase) and a component of a connexon channel of gap junctions. Previous studies have suggested that ductin in the V-ATPase has the opposite orientation of ductin in a connexon. Using an *in vitro* translation system coupled to microsomes derived from the endoplasmic reticulum, we show that ductin is co-translationally inserted into the membrane bilayer, suggesting a dependency on the signal recognition particle for synthesis. By attaching a C-terminal polypeptide derived from β-lactamase and by using cysteine replacement coupled to chemical labelling, we show that ductin is inserted into the microsomal membrane in both orientations in similar proportions. In contrast, squid rhodopsin appears to be inserted in a single orientation. Changing conserved charged residues at the N-terminus of ductin does not affect the ratio of the two orientations. Once in the microsomal membrane, ductin assembles into an oligomeric complex which contains a pore accessible to a water-soluble probe, reminiscent of the ductin complex found in the V-ATPase and a connexon.

Keywords: ductin/gap junctions/protein translocation/subunit c proteolipid/vacuolar H^+-ATPase

Introduction

Membrane proteins are generally thought to be inserted into the lipid bilayer at the endoplasmic recticulum (ER) in only one orientation, whether they possess a single transmembrane span or multiple spans. This has led to the classification of transmembrane-spanning proteins as either type 1 (N-terminus on the outside) or type 2 (N-terminus in the cytoplasm) (High and Dobberstein, 1992). These proteins are directed to the ER by a signal sequence, which in many instances is not cleaved and acts as a 'membrane anchor domain' that may form a functionally important part of the mature molecule. One protein that appears not to follow the general rule of having a single orientation is ductin.

Ductin is a hydrophobic and highly conserved eukaryotic polypeptide which varies in length between species from 155 to 165 residues (Finbow and Pitts, 1993; Finbow *et al.*, 1995). It was first found to be the major protein component of a connexon-like channel of gap junctions. These membrane domains form at points of contact between cells and are thought to provide pathways for cell–cell communication in the tissues of metazoan animals by allowing the movement of low molecular weight solutes and ions between adjoining cells (for review, see Finbow and Pitts, 1993). The ductin polypeptide forms a membrane–membrane channel independent of the connexin family of proteins which are thought to form similar channels that contribute to the pathway of cell–cell communication in vertebrates (for review, see Kumar and Gilula, 1992).

Ductin has also been identified as subunit c (proteolipid) of the membrane sector (V_0) of the vacuolar H^+-ATPase (V-ATPase) (Mandel *et al.*, 1988). This ATPase is a vital eukaryotic pump used for acidifying endomembrane compartments (e.g. lysosomes, Golgi, synaptic vesicles, etc.) or for proton translocation across the plasma membrane in certain cell types (for review, see Harvey and Nelson, 1992). The subunit c polypeptide is thought to provide the principal sites for proton translocation across the membrane (Noumi *et al.*, 1991). Sequencing, expression and genetic analyses show that a single gene product encoding ductin provides both a gap junction channel and a V-ATPase component (Holzenburg *et al.*, 1993; Finbow *et al.*, 1994; Harrison *et al.*, 1994).

Much is now known about ductin structure. It consists of a repeat of two domains, each of which has a primary structure similar to the 8 kDa subunit c (proteolipid) from the membrane-bound F_0 of the F_1F_0 ATPase (F-ATPase) (Mandel *et al.*, 1988). Ductin is thought to pass through the membrane as a four α-helix bundle (Finbow *et al.*, 1992; Holzenburg *et al.*, 1993). Such a disposition is consistent with the structure of the F_0 subunit c as a hairpin of two transmembrane α-helices (Girvin and Fillinghame, 1993, 1994). Six ductin polypeptides associate to form a channel complex which can be used for either the core structure of the V_0, the functional homologue of the F_0 (Arai *et al.*, 1988), or a connexon channel of gap junctions (Finbow *et al.*, 1992; Finbow and Pitts, 1993; Holzenburg *et al.*, 1993). Negative stain electron microscope images of gap junctions, containing ductin as the principal polypeptide, indicate the presence in each hexamer of a central aqueous pore that presumably acts as the conduit through which low molecular weight solutes pass between cells (Finbow *et al.*, 1992; Holzenburg *et al.*, 1993). A recent cysteine replacement analysis has identified residues on the first transmembrane α-helix which might line this central channel and also suggested that translocation of protons in the V_0 occurs through a pathway formed by each ductin polypeptide rather than through the central channel (Jones *et al.*, 1994; P.C.Jones *et al.*, in preparation).

From sequence conservation and orientation studies in

the gap junction form and the V-ATPase, ductin has been found in two orientations in the membrane bilayer. In one orientation two conserved extramembranous loops face the cytoplasm and are predicted to form the principle contact sites between the V_0 and the catalytic V_1 (Finbow *et al.*, 1992, 1993; Jones *et al.*, 1995). This orientation for ductin in the V-ATPase is similar to the observed orientation of the F_0 subunit c in the F-ATPase in which the N- and C-termini face the equivalent of the extracellular space (e.g. lumenal side; Fraga and Fillinghame, 1989). In gap junctions, however, proteinase and immunological studies indicate that these same extramembranous loops are on the extracellular face and may therefore form sites of end-to-end pairing across the extracellular space between cells (Finbow *et al.*, 1992, 1993). In this orientation, the N- and C-termini are located in the cytoplasm. Understanding how cells of metazoan animals achieve this apparent dual orientation of ductin is crucial to determining the assembly of two distinct and important membrane complexes.

Here we show, using an *in vitro* system for protein synthesis, that ductin is co-translationally inserted into microsomal membranes with dual orientations. The two orientations of ductin occur in roughly similar proportions. We also show that ductin assembles into an oligomeric complex soon after insertion into the membrane. The assembled ductin is labelled on the middle of the first putative transmembrane helix by a reagent that reacts with cysteine residues present in an aqueous environment. This indicates the presence of an aqueous pore within the newly assembled ductin complex, suggesting a structure that is similar to that of the mature ductin hexamer.

Results

In vitro synthesis of ductin

To examine the synthesis, insertion into the bilayer and orientation of ductin, as well as the assembly into a multimeric complex, we have used a cell-free system of rabbit reticulocyte lysate and canine pancreatic microsomes. Similar systems have been used by others to examine the synthesis and insertion of polytopic transmembrane proteins (Miao *et al.*, 1992; Falk *et al.*, 1994; Skach *et al.*, 1994; Tam *et al.*, 1994).

Translation of either *Nephrops norvegicus* (Norway lobster; Nductin) or bovine ductin (Bductin) mRNA gave rise to a 16 kDa polypeptide (Figures 1 and 5). With rabbit reticulocyte lysate, the efficient translation of ductin mRNA was found to be dependent on the presence of pancreatic microsomes (Figure 1, lanes 1 and 2). In contrast, ductin was synthesized in the absence of microsomes with wheat germ extract and its synthesis was depressed in the presence of microsomes (lanes 3 and 4). This is the expected pattern of synthesis of a signal recognition particle (SRP)-dependent pathway for ductin (Walter *et al.*, 1984; Wolin and Walter, 1989), consistent with ductin being a polytopic membrane polypeptide.

The location of newly synthesized ductin within the microsomes was examined by extraction with sodium carbonate and sensitivity to tryptic digestion. When the microsomes were extracted with sodium carbonate at pH 11 to separate microsomal contents from microsomal membranes, ductin was found in the membrane fraction

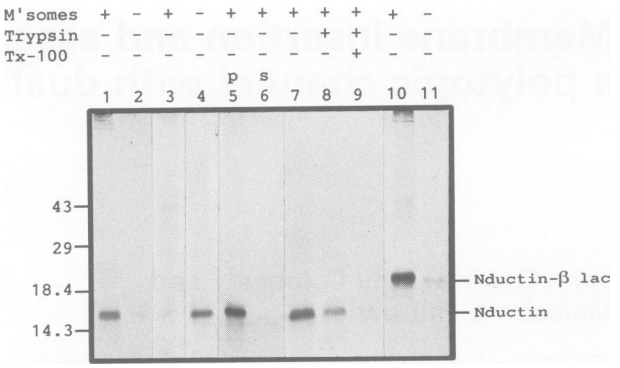

Fig. 1. Synthesis and insertion of ductin into microsomal membranes. Capped mRNA encoding Nductin (lanes 1–9) or the fusion protein Nductin–β lac (lanes 10 and 11) was translated in the presence (lanes 1, 3 and 5–10; shown as '+') or absence (lanes 2, 4 and 11; shown as '–') of microsomal membranes in rabbit reticulocyte lysate (lanes 1, 2 and 5–11), or in wheat germ extract (lanes 3 and 4), before immunoprecipitation with antibodies to Nductin. In lanes 5 and 6, microsomal membranes were extracted with sodium carbonate and centrifuged to pellet integral membrane protein (lane 5, 'p') from peripheral and lumenal protein (lane 6, 's'). Lanes 7–9 show a trypsin digestion experiment in which the membranes were treated with 0.025% trypsin in the absence (lane 8) or presence (lane 9) of 1% Triton X-100 (Tx-100), or with neither as a control (lane 7).

(Figure 1, lanes 5 and 6). Ductin was not degraded by the action of trypsin (Figure 1, lanes 6–9; Figure 2A) unless in the presence of detergents. These results show that ductin has been inserted into the microsomal membranes.

Orientation of ductin in the microsomal membranes

Ductin has no post-translational processing events (e.g. signal peptide cleavage or glycosylation sites) that can act as markers for the orientation of insertion. To overcome this, a fusion polypeptide was made by adding 60 amino acids from the N-terminus of β-lactamase to the C-terminus of Nductin (Nductin–β lac). This extension introduces eight potential trypsin cleavage sites, the closest being 12 amino acids downstream from the C-terminus of the ductin sequence. The synthesis of the fusion polypeptide retained the dependency on microsomes for synthesis in the reticulocyte lysate, giving a band of apparent size 22 kDa on SDS–PAGE (Figure 1, lanes 10 and 11). In addition, it is capable of forming complexes with ductin (see below), indicating that the ductin region is correctly folded in the microsomal membrane.

A trypsin time course digestion of Nductin–β lac in microsomes results in the appearance of a 17 kDa band along with the 22 kDa band (Figure 2A). This slightly larger size of the product (17 kDa compared with 16 kDa) is expected from the nearest trypsin cleavage site in the β-lactamase tail. The double band pattern is present within 5 min and is stable for at least 60 min. An intermediate sized band is also seen after immunoprecipitation, which can be accounted for by cleavage at one of the other tryptic cleavage sites in the β-lactamase tail. The cleavage pattern of Nductin–β lac can be explained if it is inserted in similar amounts in both orientations in the microsomal membranes. However, it could also be explained if the fusion polypeptide had been inserted in a single orientation with the C-terminal tag being inside the lumen of the microsomes, but a population of the microsomes had

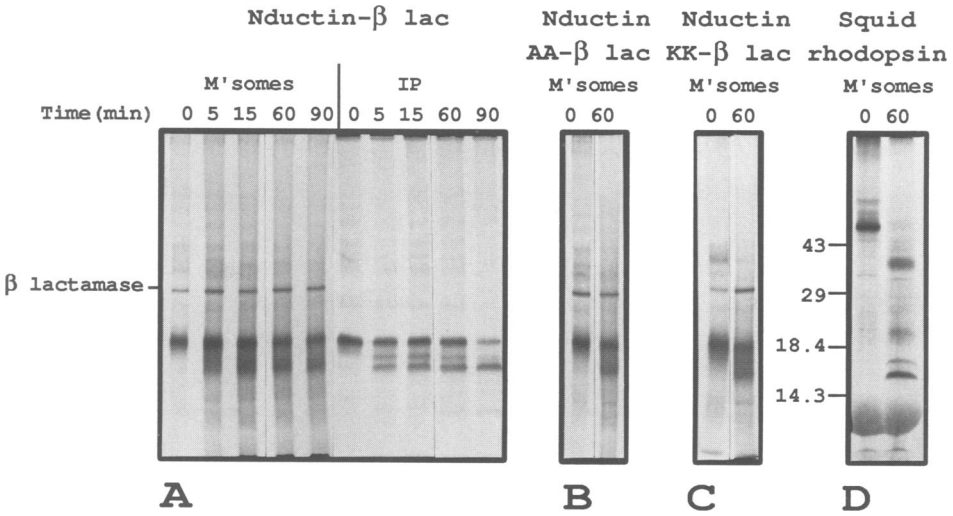

Fig. 2. Time course of trypsin digestion of Nductin–β lac in microsomal membranes. (**A**) Microsomes containing the fusion protein, Nductin–β lac, and a control protein β-lactamase, were treated with trypsin over a 90 min period and, at the designated time points, samples were analysed by SDS–PAGE. The gel on the left shows the pelleted microsome membranes and the gel on the right after immunoprecipitation with antibodies to Nductin. (**B**) As in (A) but with the glutamic acid residues at positions 4 and 5 exchanged for alanine residues (NductinAA–β lac). (**C**) As in (A) but with the glutamic acid residues at positions 4 and 5 exchanged for lysine residues (NductinKK–β lac). (**D**) Microsomes containing squid rhodopsin (M_r 45 000) were treated for the times shown with endoproteinase glu-C.

become leaky and trypsin had, in consequence, gained access to the microsomal contents. As a control for this, β-lactamase was co-translated and inserted into the same microsomes. Over the time course there is no change in the intensity of the mature β-lactamase, showing that trypsin has not gained access to the microsomal contents during the digestion period (Figure 2A). In the presence of Triton X-100, the mature β-lactamase is degraded rapidly by trypsin under the same conditions (results not shown).

To show further the dual orientation, we used a second strategy based upon cysteine substitution. A cysteine-free form of Nductin has been prepared previously (Jones *et al.*, 1994) which restores V-ATPase activity when expressed in a mutant strain of *Saccharomyces cerevisiae* in which the *VMA*3 gene that encodes the subunit c of the V-ATPase has been inactivated (Noumi *et al.*, 1991; Harrison *et al.*, 1994). Replacement of the serine residues at position 6 (N-terminus of helix 1) and 44 (loop between helices 1 and 2; Figure 3) by cysteine had no deleterious effects on V-ATPase activity. These two residues are in extramembranous regions of ductin and react to the water-soluble reagent fluorescein-5-maleimide (FM), which causes a shift in the mobility of the polypeptide on SDS–PAGE (P.C.Jones *et al.*, in preparation). Because these two residues are on opposite sides of the membrane (Figure 3), they act as markers for the orientation of ductin. Both residues will be labelled at half maximum if there are two orientations, whilst in a single orientation only one residue will be labelled.

The two cysteine-substituted mutants were analysed for reaction to FM over a range of FM concentrations (Figure 4). Both mutations exhibited partial labelling at 0.25, 0.5 and 1.0 mM concentrations of FM but, at 3.0 mM FM, a much higher proportion of the polypeptide reacts with FM. That the partial labelling is not due to limiting amounts of FM was shown by the near complete labelling of the mutant forms of ductin in the presence of 1% SDS

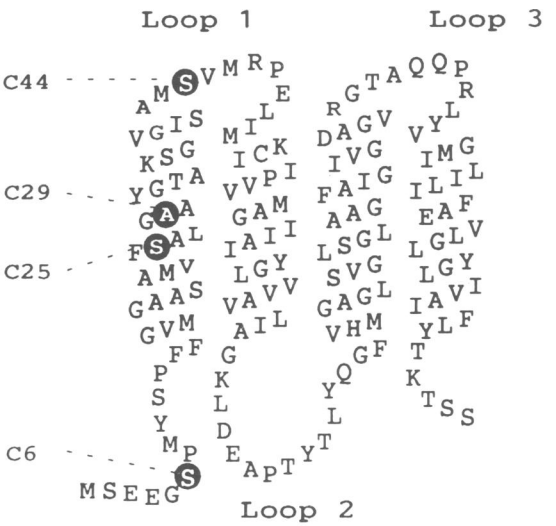

Fig. 3. Disposition of Nductin in the bilayer showing the positions of the residues replaced by cysteine (see Finbow *et al.*, 1992; Jones *et al.*, 1994).

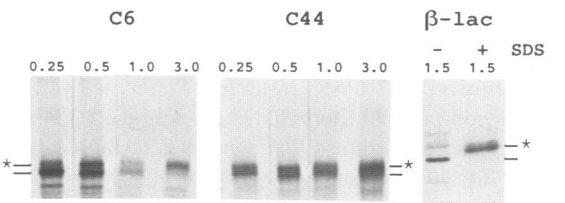

Fig. 4. Labelling of the Cys6 and Cys44 substitutions of Nductin by fluorescein-5-maleimide (FM). Microsomal membranes containing the Cys6 and Cys44 substitutions of Nductin were labelled for 60 min with different concentrations of FM shown above each lane as mM. Approximately half-labelling is achieved at 1 mM FM, and near full labelling is achieved at 3 mM. As a control for the labelling of lumenal sides of the microsomes, β-lactamase is used and shows no labelling except in the presence of 1% SDS.

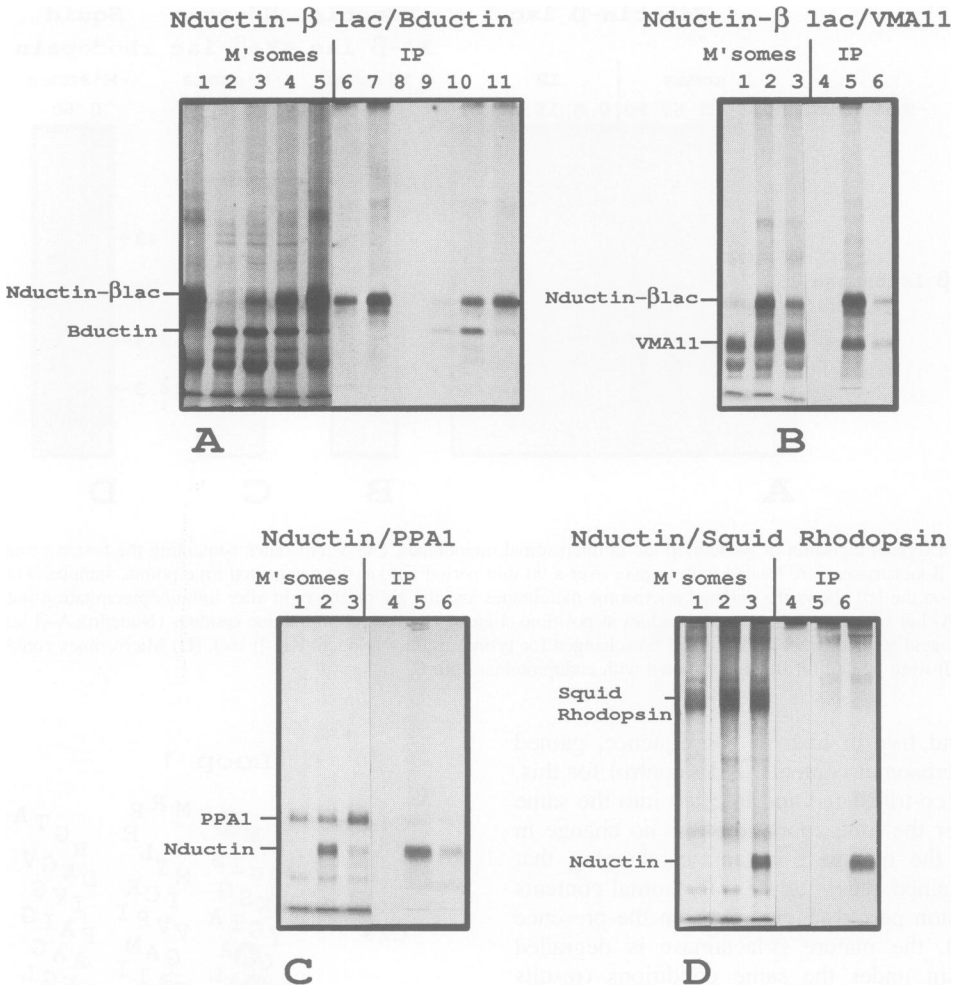

Fig. 5. Assembly of ductin complexes. Capped mRNA encoding Nductin or the fusion protein Nductin–β lac was co-translated in the presence of microsomes with mRNA encoding bovine ductin (Bductin) (**A**), *S.cerevisiae* VMA11 polypeptide (**B**), *S.cerevisiae* PPA1 polypeptide (**C**) or squid rhodopsin (**D**). On the left in each is shown the microsomal fraction and on the right the corresponding immunoprecipitation with antibodies to Nductin. In (A), lanes 1 and 2 show the translation products of mRNA encoding for Nductin–β lac and Bductin translated separately. Lanes 3–5 show a co-translation of the above mRNAs in approximate ratios of 1:3, 1:1 and 3:1 respectively. Lanes 7 and 8 show the immunoprecipitation (IP) from the microsomes from lanes 1 and 2 respectively and lane 6 shows the IP from an equal mix of lanes 1 and 2 after first solubilizing the microsomal membranes in RIPA buffer. Lanes 9–11 show the IP of the microsomes shown in lanes 3–5. In (B), lane 1 shows the translation product in the microsomes from mRNA encoding the VMA11 polypeptide and lanes 2 and 3 the products of 1:1 (lane 2) or 3:1 mixtures of mRNA encoding respectively VMA11 and Nductin. Lanes 4–6 show the IP from lanes 1–3 respectively. In (C), lane 1 shows the translation product in the microsomes from mRNA encoding the PPA1 polypeptide and lanes 2 and 3 the products of 1:1 (lane 2) or 3:1 mixtures of mRNA encoding respectively PPA1 and Nductin. Lanes 4–6 show the IP from lanes 1–3 respectively. In (D), lane 1 shows the translation product in the microsomes from mRNA encoding squid rhodopsin and lanes 2 and 3 the products of 1:1 (lane 2) or 3:1 mixtures of mRNA encoding respectively squid rhodopsin and Nductin. Lanes 4–6 show the IP from lanes 1–3 respectively.

(Figure 6). The labelling at the highest concentration can be explained by the FM crossing the membrane and gaining access to the lumenal contents. Mature β-lactamase contains two cysteines residues and likewise migrates more slowly on SDS–PAGE after reaction with FM (Figure 4). At concentrations where there was only partial labelling of the two ductin mutants, there was no detectable labelling of β-lactamase. However, concentrations of FM which shifted the proportion of labelling of ductin also resulted in near full labelling of β-lactamase (results not shown). The partial-labelling of both Cys6 and Cys44, which are on opposites sides of the ductin polypeptide in the membrane and when the microsomal membranes retain their integrity, confirms that ductin is indeed inserted into the microsomal membranes in approximately similar amounts in opposite orientations.

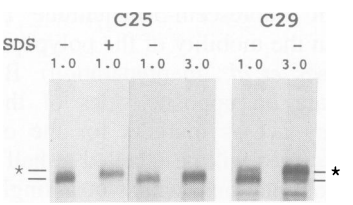

Fig. 6. Labelling of the Cys25 and Cys29 substitutions of Nductin by FM. The experiment was carried out as in Figure 4 except the Cys25 and Cys29 substitutions of Nductin were used. Partial labelling of Cys29 is achieved at 1 mM FM except when SDS is added as marked ('+' or '–'). Near full labelling is achieved at the highest concentration of 3 mM. There is no labelling of Cys25 except in the presence of SDS.

Although microsomal membranes contain all that is required for insertion and integration of polytopic membrane proteins, it is possible that orientation signals are not correctly interpreted, and this might account for the dual orientation of ductin. To explore this possibility further, the orientation of squid rhodopsin was examined (Hall *et al.*, 1991). This 47 kDa polypeptide is a member of the seven transmembrane α-helical receptor family in which the N-terminus is extracellular and the C-terminus is cytoplasmic. In isolated photoreceptor membrane sheets, the C-terminus of squid rhodopsin is removed by endoproteinase glu-C to give an N-terminal 36 kDa fragment and various smaller fragments from the C-terminal cytoplasmic region (Ryba *et al.*, 1993). Therefore, if squid rhodopsin is inserted into the microsomal membranes in the correct orientation, then a similar digestion pattern should be obtained with endoproteinase glu-C. If not, then the polypeptide should be insensitive to the action of the proteinase. Endoproteinase glu-C treatment of squid rhodopsin in the microsomal membranes results in complete digestion of the mature polypeptide of which a major product migrates at the expected position of 36 kDa. This indicates a single orientation for this polypeptide (Figure 2D), showing that *in vitro* synthesis coupled to microsome membranes is capable of inserting squid rhodopsin in the correct orientation.

The charge of the N-terminus has been implicated as a possible determinant of orientation (Parks and Lamb, 1993; Gafvelin and von Heijne, 1994). All forms of ductin sequenced so far have been found to have acidic residues present at their N-termini. For Nductin, there are glutamic acid residues at positions 3 and 4 (Figure 3). These were exchanged for either lysine (NductinKK–β lac) or alanine (NductinAA–β lac) residues. Both N-terminal changes had no detectable effect on shifting the proportion of ductin in the two orientations (Figure 2B and C).

The assembly of ductin complexes

The membrane complexes in both the V_0 of the V-ATPase and in connexon channels contain six ductin polypeptides. We therefore examined whether ductin in the microsomal membrane could self-associate. This was investigated by the co-immunoprecipitation of Nductin and Bductin with antibodies which were specific to the *Nephrops* polypeptide. The Nductin–β lac fusion polypeptide was used to allow a clear distinction between the migration of the *Nephrops* and bovine forms.

After co-expression of Bductin with Nductin–β lac, the Bductin was found to be co-immunoprecipitated with Nductin–β lac (Figure 5A, lanes 9–11). This association was not a result of binding after the microsomes had been solubilized in the RIPA buffer since mixing of Bductin and Nductin–β lac after solubilization in RIPA buffer did not lead to any association between the two forms of ductin (Figure 5A, lane 6).

The specificity of this association was tested by co-expressing ductin with a number of other polypeptides of varying homology with ductin. Three polypeptides were used; VMA11 (70% identity; Umemoto *et al.*, 1991), PPA1 (20% identity; Apperson *et al.*, 1990) and squid rhodopsin (no identity). No binding between Nductin and rhodopsin was observed. PPA1 was found to bind poorly or not at all, whereas VMA11 bound to Nductin–β lac in a similar fashion to Bductin (Figure 6B, C and D).

The question of whether the assembled ductin is in a form similar to that found in the V-ATPase and connexon channel was examined by using cysteine-substituted mutants. Two single cysteine-substituted mutants were used, Cys25 (serine) and Cys29 (alanine), each of which is located in the middle of the first transmembrane domain (Figure 3) but on different faces of the putative helix (Finbow *et al.*, 1992). These two cysteine mutants have been used previously in a yeast expression system to determine their environment in the fully assembled V-ATPase complex (Jones *et al.*, 1994). Cys25 was not labelled by FM in the whole V-ATPase, but Cys29 was.

The concentration range of FM was the same as that used for the other two cysteine-substituted mutants. Cys25 was not labelled at any concentration unless the protein had first been denatured in SDS (Figure 6; results shown only for 1 and 3 mM). However, Cys29 was labelled and, as with the Cys6 and Cys44 mutants, only partial labelling was achieved at the lower FM concentrations at which there was no access of FM from the lumenal side. The ability of the hydrophilic FM to react with the cysteine residues at position 29 of helix 1 indicates that this residue is exposed to the aqueous environment, even though it lies in the central region of the bilayer.

Discussion

As would be expected from its location and disposition in the membrane in the cell, the synthesis of ductin is dependent on the involvement of SRP and it therefore seems likely that, in eukaryotic cells, ductin is synthesized on bound ribosomes and inserted directly into the ER. Such a site of synthesis and insertion is consistent with the functions of ductin in membrane transport.

By two independent approaches, we have shown that ductin is inserted into microsomal membranes in both orientations. Trypsin cleavage of the fusion polypeptide Nductin–β lac and the chemical modification of single cysteine residues show that ductin is inserted in similar amounts in each of the two orientations. The two orientations can be related to the orientation of ductin in connexons of gap junctions and in the V_0 of the V-ATPase.

Biochemical and immunological evidence (Finbow *et al.*, 1992, 1993) shows that the N-terminus of ductin in gap junctions is cytoplasmic, suggesting that the two extramembranous loops between the first and second and between the third and fourth putative transmembrane α-helices are on the extracellular face. However, in the V-ATPase, mutagenesis studies and the retention of V-ATPase activity after addition of a C-terminal polyhistidine tail, suggest that these two loops face the catalytic V_1 in the cytoplasm (Harrison *et al.*, 1994; Jones *et al.*, 1994; Dunlop and Finbow, unpublished results). These loops are homologous to the loop in subunit c of F_0, which likewise faces the cytoplasm in the F-ATPase (Fraga and Fillinghame, 1989). Therefore, the insertion of ductin into microsomal membranes in both orientations may provide a mechanism for segregating the assembly of the V_0 from the assembly of a connexon of gap junctions (Figure 7).

The signals which might direct the dual orientation of

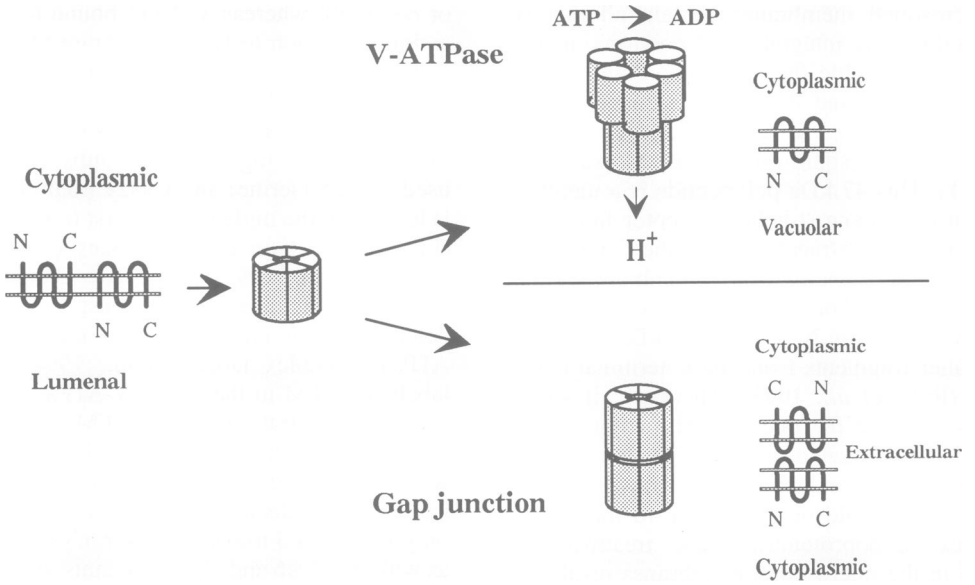

Fig. 7. Proposed scheme for the insertion of ductin leading to dual orientations and the segregation of the assembly of the V_0 of the V-ATPase from a connexon of gap junctions.

ductin have not yet been identified. The relative disposition of charged residues in extramembranous domains has been thought to determine orientation in membranes (Parks and Lamb, 1993; Gafvelin and von Heijne, 1994). Switching conserved acidic residues at the N-terminus of ductin for basic or neutral residues appears to have no effect on the ratio of the two orientations. A truncated form of ductin containing the first three transmembrane segments also appeared to be inserted in both orientations (Dunlop, unpublished results). It is possible that the signal for the dual orientation might be an intrinsic property of the transmembrane domains.

Once inserted into the membrane, ductin can self-associate. This association suggests that assembly of membrane complexes containing ductin takes place in the ER where assembly of other oligomeric complexes is thought to occur (Hurtley and Helenius, 1989). The association is specific, with little or no binding of ductin to polytopic membrane proteins of limited (PPA1) or no homology (squid rhodopsin). Ductin, however, can bind to the VMA11 polypeptide with which it has 70% sequence identity (Umemoto *et al.*, 1991). This polypeptide is of unknown function but is required for the assembly of the V-ATPase, although it has not been detected in the isolated V-ATPase complex in *S.cerevisiae*. Given that it can bind to ductin, the reason for its apparent absence in the V-ATPase is not clear unless it is present in much lower amounts than the ductin polypeptide which forms the subunit c of the V_0.

The extramembranous domains of the VMA11 polypeptide have no homology to ductin, including the highly conserved loop regions that form the apparent sites of connexon pairing and V_1 attachment. This suggests that binding between ductin polypeptides occurs primarily within transmembrane domains, as might be expected. The binding suggests that ductin made in the *in vitro* system folds with the correct secondary and tertiary structure. Whether folding is spontaneous or somehow directed by chaperones is not known. However, the small amount of ductin exposed as extramembranous regions would indicate that there is minimal opportunity for the involvement of BiP and other ER chaperones.

The labelling of Cys29 in the centre of the first putative transmembrane domain by FM suggests the presence of an aqueous pore in the oligomeric complex. Such a pore can been detected in the connexons of gap junction by negative stain (Holzenburg *et al.*, 1993). Of interest is the observation that FM only partially labels the ductin polypeptide until the reagent is able to cross the microsomal membrane, when near full labelling can be achieved. This suggests that there is some kind of occlusion in the nascent oligomeric complex that prevents movement of the FM from one side of the oligomeric complex. The presence of an occlusion is consistent with the assumption that the V_0 is impermeable to protons and larger molecules after removal of the V_1 or after reconstitution in liposomes (Harrison *et al.*, 1994). Closed connexons have also been predicted from studies examining gap junction assembly (Johnson *et al.*, 1974).

In summary, the dual orientations of ductin allow the separation of ductin's two known transport functions, as a V-ATPase component and as a connexon component of gap junctions. Its ability to self-associate reflects the oligomeric construction of the V_0 and connexon channel. The direct insertion of ductin into microsomal membrane in an apparent native conformation, which allows self-association in these membranes, is distinctly different from the connexin family of proteins which are also thought to form a similar connexon channel in gap junctions. Connexins contain a cryptic signal sequence and are inappropriately incorporated into microsomes (Falk *et al.*, 1994). They also do not assemble into multimeric complexes in the ER (Musil and Goodenough, 1993).

Materials and methods

Plasmids and PCR constructs

The Nductin–β lac RNA was constructed using PCR methodology. Two overlapping PCR products were first prepared, *T7pol*ductin and

β-lac*term*. *T7pol*ductin contained a T7 polymerase site, the open reading frame for *Nephrops* ductin and an overlap with the second PCR product. This was generated with the standard primer oligonucleotide for pBluescript (5′-GTTTTCCCAGTCACGAC) which is 5′ of the T7 polymerase site in this vector, and a reverse primer oligonucleotide (5′-GTGAGCAAAAACAGGAAGGCTGGAGGTCTTGGTG) which encoded the 5′ C-terminal residues of *Nephrops* ductin plus one extra base (underlined), and residues 18–23 of β-lactamase (*Escherichia coli*). β-lac*term* was generated using the complimentary oligonucleotide to the reverse oligonucleotide used to generate *T7pol*ductin (5′-CACCAAGACCTCCAGCCTTCCTGTTTTTGCTCAC), and a reverse oligonucleotide primer (5′-GCGAGCTCGTTTACCCGGCGTCAACACGGG) which encoded residues 80–85 plus two extra bases together with an in-frame termination codon (underlined) and an *Xho*I restriction site. After 'Wizard' spin column purification (Promega) of the PCR products, they were hybridized and used as a template for PCR to generate Nductin–β lac DNA using the standard primer oligonucleotide with the reverse oligonucleotide used to generate β-lac*term*.

The VMA11 gene (Umemoto *et al.*, 1991) was cloned by PCR from yeast genomic DNA (Promega) into the TA cloning vector (Invitrogen) with the following oligonucleotides, 5′-CCGGATCCGTAAACATGTCAACGCAACTCG (forward) and 3′-CGACTCGAGATCAACTTTTGACTCATTCAG (reverse). The PPA1 gene (Apperson *et al.*, 1990) was cloned in the same manner using the following oligonucleotides, 5′-CCTCTAGAATAATGAACAAGGAATCTAAAGAT (forward) and 3′-CCGAATTCTAAAAACGGATTACTGAAATTCAGAAGC (reverse). Both genes were sequenced after cloning and had complete identity with the published sequences.

The N-terminal mutants were constructed by PCR using the same oligonucleotides as were used for Nductin–β lac, except for the 5′ oligonucleotide, which contained a T7 promoter site upstream of the ATG start site, and were as follows; NductinAA–β lac, 5′-GGATCCTAATACGACTCACTATAAGGAGGCTCAAA**ATG**TCTGCAGCGGGTAGTCCT, and NductinKK–β lac, 5′-GGATCCTAATACGACTCACTATAGGGAGGCTCAAA**ATG**TCTAAAAAGGGTAGTCCT. The initiation ATG sequence is shown in bold and the underlined sequences are the sites of glutamic acid substitutions. The PCR products were used directly without purification for *in vitro* transcription.

The single cysteine replacement mutants Cys6, Cys25, Cys29 and Cys44 were those used in a previous study (Jones *et al.*, 1995). A T7 RNA polymerase site was placed immediately upstream of the ATG codon by a PCR-based strategy. The 5′ oligonucleotide was essentially the same as for the N-terminal mutants, except that there were no substitutions at the glutamic acids at residues 3 and 4, and was as follows: GGATCCTAATACGACTCACTATAGGGAGGCTCAAAATGTCTGAAGAGGG. The 3′ oligonucleotide was directed against the stop codon of the Nductin and was as follows: CCCTCGAGTCCATTTAGCTGGAGGTCTTGG. The PCR products were used directly in the *in vitro* transcription reaction without any purification.

In vitro transcription, translation and translocation

Capped RNAs for *Nephrops* ductin, NductinAA–β lac, NductinKK–β lac, Cys6, Cys25, Cys29 and Cys44 were transcribed directly from the PCR product, while VMA11 and PPA1 were transcribed from *Xba*I-linearized plasmid, and for all the above the T7 mMessage mMachine kit (Ambion) was used. Bductin (Goldstein *et al.*, 1991) was transcribed from pBluescript (Stratagene) using the Sp6 mMessage mMachine kit (Ambion). Squid rhodopsin RNA was transcribed using T3 polymerase (Promega) from *Bam*HI-linearized pSqRho plasmid (courtesy of Professor J.B.Findlay). The transcribed capped RNA was stored at −70°C in 20 μl aliquots. The capped RNA (1–2 μg) was translated in a reaction mix which contained nuclease-treated rabbit reticulocyte lysate (Promega), [^{35}S]methionine (0.74 MBq; Amersham p.l.c.) and, where indicated, canine pancreatic microsomal membranes (2 μl; Promega). The final volume was 25 μl and was incubated at 30°C for 1 h.

Immunoprecipitation

Samples were immunoprecipitated by first solubilizing 10 μl of translation reaction mixture in 200 μl of RIPA buffer (25 mM Tris–HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 1 μg/ml aprotinin). Rabbit polyclonal antiserum to *Nephrops* gap junctions (Leitch and Finbow, 1990) at 1:100 dilution was then added and incubated at 4°C for 1 h with agitation. Ten μl of a Protein A–Sepharose bead (Sigma) slurry in phosphate-buffered saline (PBS) was subsequently added, followed by a further incubation at 4°C for 1 h with agitation. The beads were collected by centrifugation in a microfuge and washed three times in 200 μl of RIPA buffer. Bound protein was stripped off the beads by resuspending the beads in 10 μl of 2× SDS–PAGE loading buffer. The labelled polypeptides were visualized by SDS–PAGE analysis on a 12.5% gel, followed by autoradiography.

Alkali extraction of microsomal membranes

The Na_2CO_3 extraction was performed on microsomal membranes that had been pelleted using a Beckman Benchtop Ultracentrifuge at 50 000 r.p.m. for 1 h. The pellet was resuspended in 200 μl of 100 mM Na_2CO_3, pH 11.5 and incubated at 4°C for 1 h. The membrane fraction was separated from the carbonate-released fraction by centrifugation as above.

Proteolysis reactions

Following translation, the reactions were cooled on ice and tetracaine HCl was added to a final concentration of 2 mM. Trypsin digests were carried out using 2.5% Gibco BRL as a stock solution. Prior to the digest, the trypsin was diluted 10× in PBS. One μl of this diluted trypsin solution was added per 10 μl of translation mix. Digests were carried out on ice for the times indicated. The proteolysis was stopped either by adding 200 μl of RIPA buffer, if the samples were to be immunoprecipitated, or by adding 150 μl of MP buffer (0.25 M sucrose, 80 mM KCl, 3 mM $MgCl_2$, 10 mM HEPES–KOH, pH 7.5) containing aprotinin to a final concentration of 1 μg/ml. Once the MP was added, the microsomes were separated from the reaction mix by ultracentrifugation at 50 000 r.p.m. for 1 h at 4°C. The pellet was resuspended in an equal volume of MP buffer then analysed by SDS–PAGE.

Rhodopsin was digested using endoproteinase glu-C (Boehringer Mannheim) added to 10 μl of translation mix for 1 h at room temperature at a final concentration of 0.01 mg/ml. The digestion mixtures were loaded immediately onto a 12.5% SDS–PAGE gel after addition of SDS loading buffer.

Chemical modification of cysteine residues

The cysteine-specific maleimide reagent FM was purchased from Molecular Probes (Cambridge Bioscience, Cambridge). The FM was prepared in dimethylformamide to a stock concentration of 0.25 M and stored at −20°C. Prior to each cysteine labelling reaction, 7.5 μl of the stock FM was diluted into 500 μl of MP buffer to give a 3.75 mM FM solution. To 12.5 μl of translation mix, varying volumes of the 3.75 mM FM solution were added to give the desired final concentration of FM after being made up to 104 μl with MP buffer. The reaction mix was incubated at 30°C for 1 h, then stopped by adding β-mercaptoethanol to a final concentration of 1%. The microsomes were separated from the reaction mix by ultracentrifugation at 50 000 r.p.m. for 1 h at 4°C. The pellet fraction was resuspended in 12.5 μl of MP buffer and 2× SDS loading buffer.

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