Biogenesis of tight junctions: the C-terminal domain of occludin mediates basolateral targeting

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SUMMARY

Tight junctions form a morphological and physical border between the apical and the basolateral cell surface domains of epithelial cells; hence assembly of tight junctions could occur from both of the two plasma membrane domains. We show here that the C-terminal cytoplasmic domain of occludin, the only known transmembrane protein of tight junctions, was sufficient to mediate basolateral expression of a chimeric protein. Since this chimera was transported directly to the basolateral membrane during biosynthesis, the C-terminal domain of occludin contains a basolateral targeting signal. Additionally, the C-terminal domain of occludin was also able to mediate endocytosis. Thus, the C-terminal cytoplasmic domain appears to govern intracellular transport of occludin. To test whether the basolateral membrane is an obligatory intermediate in transport of occludin to tight junctions, we analyzed the expression of occludin molecules rendered unable to efficiently integrate into tight junctions by the introduction of N-linked glycosylation sites into the two extracellular loops. Indeed, glycosylated occludin accumulated in the basolateral membrane, supporting a model in which the biogenesis of tight junctions occurs from this cell-surface domain.

Key words: Tight junction, Epithelial cell, Polarized transport

INTRODUCTION

Polarized epithelial cells possess two functionally and biochemically distinct plasma membrane domains that are separated from each other by tight junctions. This intercellular junction forms a functional as well as morphological border between the apical and basolateral cell surfaces. Investigating the biogenesis of tight junctions has long been difficult due to the lack of knowledge of their molecular composition. Generally, investigators employed a calcium-switch protocol that allows the study of de novo assembly of tight junctions (Cereijido et al., 1978). It is not clear, however, how far this experimental protocol reflects the biogenesis of tight junctions as it occurs under steady state conditions.

Theoretically, one could think of four ways how the biogenesis of tight junctions occurs. The first possibility is that tight junctions are assembled from the basolateral cell surface domain. This is supported by their localization at the apical end of the lateral membrane and by the appearance of lateral intramembrane strands under certain experimental conditions (Farquhar and Palade, 1963; Bentzel et al., 1991; Polak-Charcon, 1991). The second possibility is that assembly of tight junctions occurs from the apical membrane. In cells grown in low calcium concentrations, apical membrane components localize to intracellular vacuolar structures (Vega-Salas et al., 1988). Since the tight junction protein ZO-1 also associates with these vacuoles, it is feasible that tight junction biogenesis is directly connected to apical membrane biogenesis. Alternatively, it is possible that membrane components of tight junctions are transported to both cell surface domains and are integrated into the junctions from two sides, or are targeted to tight junctions by a separate, direct pathway.

Almost all of the proteins associated with tight junctions are peripheral membrane proteins (e.g. ZO-1, ZO-2, cingulin, rab13) and are part of the submembrane plaque of tight junctions (Stevenson et al., 1986; Citi et al., 1988; Chapman and Eddy, 1989; Gumbiner et al., 1991; Zhong et al., 1993; Jesaitis and Goodenough, 1994; Zahraoui et al., 1994; Keon et al., 1996; Merzdorf and Goodenough, 1997). The only known transmembrane protein of tight junctions is called occludin (Furuse et al., 1993; Ando Akatsuka et al., 1996). It is a polytopic membrane protein that localizes directly to the intramembrane strands of tight junctions (Furuse et al., 1996). It cannot be the only component of these strands, however, since disruption of the continuous junctional distribution of occludin did not result in a disruption of the intramembrane strands (Balda et al., 1996). Nevertheless, occludin appears to be involved in all known functions of tight junctions (Balda et al., 1996; McCarthy et al., 1996; Chen et al., 1997; Wong and Gumbiner, 1997).

Sorting of plasma membrane proteins in epithelial cells appears to involve specific determinants. Targeting of several basolateral membrane proteins has been shown to depend on...
distinct sorting determinants in their cytoplasmic domains that are able to mediate basolateral sorting in the biosynthetic and endocytic pathways (for reviews, see Rodriguez-Boulan and Powell, 1992; Matter and Mellman, 1994). These determinants are often, but not always, co-linear with signals for clathrin-mediated endocytosis and are almost exclusively found in endocytic membrane proteins. Apical targeting of membrane proteins may involve sorting by partitioning to glycolipid-enriched subdomains (for reviews, see Rodriguez-Boulan and Powell, 1992; Simons and Ikonen, 1997). Such a model is supported by the finding that proteins with glycosylphosphatidylinositol anchors are generally expressed on the apical cell surface and by the description of a transmembrane domain that mediates apical sorting (Kundu et al., 1996).

We show here that the C-terminal domain of occludin was sufficient to mediate direct basolateral transport of a reporter protein as well as endocytosis. We then generated sites for N-linked glycosylation in the two extracellular loops of occludin in the hope that the carbohydrates would interfere with the integration into tight junctions and, therefore, that occludin would accumulate at a site which is earlier in its biosynthetic pathway. Indeed, glycosylated occludin accumulated in the lateral membrane, suggesting that biogenesis of tight junctions occurs from the basolateral membrane.

**MATERIALS AND METHODS**

**Cell culture**

MDCK (strain 2) cells were grown in DMEM (4.5 g/l glucose) and, for experiments, were plated on tissue-culture-treated polycarbonate filters (Costar Transwells) with a pore size of 0.4 μm and a diameter of 12 mm or 24 mm (Balda et al., 1996). MDCK cells stably expressing occludin, HAoccludin, HAoccludinCT3, FcLR5-22, FcLR5-50 and FcRII-B1 were previously described (Balda et al., 1996). The integrity of the junctions was analyzed by measuring transepithelial electrical resistance (Balda et al., 1996). The latter antibody or a polyclonal anti-FcRII antibody (Green et al., 1985) was used to stain the FcRII-occludin chimera. Rat monoclonal antibody R40.76 was used to localize ZO-1 (Anderson et al., 1988).

To label only chimeric proteins expressed at the cell surface, filter cultures were cooled on ice and incubated with anti-FcRII antibody diluted in PBS-BSA (PBS containing 0.5% BSA and 0.5 mM CaCl2). After 2 hours, cells were washed 4 times with the same solution and then fixed and permeabilized with paraformaldehyde and saponin as above. For endocytosis experiments, cells were grown on coverslips to about 50% confluency and were then labeled with the anti-FcRII antibody in PBS-BSA for 1 hour on ice. The cells were then washed as above and subsequently incubated at 37°C for 10 minutes. Thereafter, the cells were quickly cooled on ice and antibodies still at surface were visualized by incubating with an FITC-conjugated secondary antibody on ice before fixation. A second set of coverslips was fixed with paraformaldehyde and permeabilized with saponin before labeling with the fluorescent antibody to stain cell surface and internalized antibodies.

**Protein analysis**

MDCK cells were metabolically labeled with [35S]methionine/[35S]cysteine (Amersham, Corp., Arlington Heights, IL) (Matter et al., 1992). After completion of the pulse-chase protocol, the cells were blyophilized with Sulfo-NHS-LC-Biotin (Pierce, Rockford, IL), if the chimeric protein was analyzed (Matter et al., 1993), or with biotin-hydrazide (Pierce, Rockford, IL) if glycosylated occludin was studied (Braendli et al., 1990). Cells were then solubilized with extraction buffer (10 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium-deoxycholate, 0.2% sodium-dodecylsulfate and 40 μg/ml phenylmethylsulfonyl fluoride). For the co-immunoprecipitation, the cells were solubilized with extraction buffer with or without 1% Empigen BB (Calbiochem Corp., La Jolla, CA). Chimeras were then immunoprecipitated with the polyclonal anti-Fc receptor antibody bound to Protein A- sepharose (Balda et al., 1993). Endogenous and transfected full-length occludin were precipitated with the anti-occludin antibody specific for the C-terminal cytoplasmic domain of chicken occludin in MDCK cells (Balda et al., 1996).

The fixed cells were blocked, incubated with antibodies, mounted and analyzed as described (Balda et al., 1996). Transfected occludin was labeled with either a monoclonal antibody specific for the hemagglutinin (HA) epitope (Dar et al., 1996) or a polyclonal antibody specific for the C-terminal domain of chicken occludin that does not cross-react with the endogenous dog occludin (antibody B; Balda et al., 1996). The latter antibody or a polyclonal anti-FcRII antibody (Green et al., 1985) was used to stain the FcRII-occludin chimera. Rat monoclonal antibody R40.76 was used to localize ZO-1 (Anderson et al., 1988).

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**cDNAs, mutagenesis and transfection**

To generate the chimera containing the ecto- and transmembrane domain of FcRII and the C-terminal cytoplasmic domain of occludin (FcOCD), a PCR fragment was synthesized that contained the entire C-terminal cytoplasmic domain of chicken occludin (starting at lysine-254), with an AflII site on the 5’ end and an XbaI site following the stop codon on the 3’ end. The two restriction sites were used to clone the PCR fragment into the previously described FcRII cDNA that codes for a tail-minus receptor with an AflII site before the stop codon (Matter et al., 1992). To introduce a glycosylation site in the first predicted extracellular loop of occludin, methionine-203 was substituted by an asparagine, serine-108 was replaced with an alanine to engineer a glycosylation site in the first predicted extracellular loop. PCR fragments carrying the mutations were cloned into the cDNA of a hemagglutinin (HA) epitope-tagged full-length occludin (HAoccludin), resulting in HAoccludinGL1+2. All constructs were verified by dideoxy sequencing.

The cDNAs were cloned into pCB6 and MDCK cells were transfected and screened for expression as described (Matter et al., 1992; Balda et al., 1996). The integrity of the junctions was analyzed by measuring transepithelial electrical resistance (Balda et al., 1996) and by immunofluorescence with anti-ZO-1 antibodies (see below).

**Immunofluorescence**

Wild-type or transfected MDCK cells were grown on glass coverslips for screening of transfections, or on Transwell filters for localization studies. All constructs were localized using different fixation and permeabilization methods (Balda et al., 1996) and all of them gave comparable results. Cells were fixed with 3% paraformaldehyde in PBS for 20 minutes and subsequently permeabilized with 0.1% saponin (Fig. 3A,C, Fig. 5). We also used cells permeabilized for 2 minutes on ice with Triton X-100 (0.2% in 100 mM KCl, 3 mM MgCl2, 1 mM CaCl2, 200 mM sucrose and 10 mM Hepes, pH 7.1), which were then immediately fixed for 30 minutes with 95% ethanol on ice (Figs 3B, 6). Thereafter, the samples were rehydrated with phosphate-buffered saline (PBS). This method was shown previously to result in good junctional staining of endogenous and transfected chicken occludin in MDCK cells (Balda et al., 1996).

The fixed cells were blocked, incubated with antibodies, mounted and analyzed as described (Balda et al., 1996). Transfected occludin was labeled with either a monoclonal antibody specific for the hemagglutinin (HA) epitope (Dar et al., 1996) or a polyclonal antibody specific for the C-terminal domain of chicken occludin that does not cross-react with the endogenous dog occludin (antibody B; Balda et al., 1996). The latter antibody or a polyclonal anti-FcRII antibody (Green et al., 1985) was used to stain the FcRII-occludin chimera. Rat monoclonal antibody R40.76 was used to localize ZO-1 (Anderson et al., 1988).

To label only chimeric proteins expressed at the cell surface, filter cultures were cooled on ice and incubated with anti-FcRII antibody diluted in PBS-BSA (PBS containing 0.5% BSA and 0.5 mM CaCl2). After 2 hours, cells were washed 4 times with the same solution and then fixed and permeabilized with paraformaldehyde and saponin as above.

**RESULTS**

**The C-terminal cytoplasmic domain of occludin mediates basolateral expression**

If transport of occludin to tight junctions involves prior insertion into either the apical or the basolateral cell surface...
Targeting of occludin domain, one would expect that occludin contains a corresponding targeting determinant. For several membrane proteins, sorting to the basolateral cell surface domain has been shown to be mediated by distinct targeting determinants in their cytoplasmic domains (for reviews, see Rodriguez-Boulan and Powell, 1992; Matter and Mellman, 1994). The homologies among known targeting signals are not sufficient, however, to make predictions based on the sequence of protein.

Basolateral targeting determinants are generally transferable to a reporter protein without losing the capability to mediate basolateral expression. We used the mouse Fc receptor for IgG (FcRII) as a reporter protein since this protein is expressed in the apical membrane without a functional basolateral sorting signal in its cytoplasmic domain (Hunziker et al., 1991; Matter et al., 1992). To test whether the C-terminal cytoplasmic domain of occludin contains a basolateral sorting signal, we constructed a chimera consisting of the ecto- and transmembrane domains of the Fc receptor and the C-terminal cytoplasmic domain of occludin (Fig. 1, FcOCD). For all constructs in this paper, we made use of a cDNA coding for chicken occludin. Chicken occludin has already been expressed in MDCK and other mammalian epithelial cells and, in all cases, was found to be efficiently integrated into tight junctions (Furuse et al., 1994; Balda et al., 1996; McCarthy et al., 1996). Thus, chicken occludin has everything that is necessary to become properly targeted to tight junctions of MDCK cells.

To test for expression in stably transfected MDCK cells, total cell extracts of wild-type and transfected MDCK cells were tested with either anti-FcRII antibodies or anti-occludin antibodies on immunoblots. Fig. 2 shows that the antibody specific for the C-terminal domain of chicken occludin (antibody B; Balda et al., 1996) only recognized a band in lanes corresponding to transfected cells, and expression of the chimera resulted in a higher molecular weight band (lanes 1-6). Expression of the chimeric protein could also be confirmed with an antibody directed against the ectodomain of FcRII (Green et al., 1985), which recognized a band of the same molecular weight as the anti-occludin antibody (lanes 7 and 8). In all cases, the recognized band was stronger in lanes derived from cells that had been treated with sodium butyrate; this treatment is known to result in higher expression of cDNAs that are controlled by a cytomegalovirus-derived promoter (Brown et al., 1989).

We next asked whether the chimeric protein is specifically expressed on one of the two cell surface domains or in tight junctions. Therefore, we plated cells expressing FcOCD or control chimeras on filters and then processed the cells for immunofluorescence with either anti-FcRII or anti-chicken occludin antibodies. Using biochemical assays, we have previously shown that FcLR5-50, a chimera consisting of the ecto- and transmembrane domains of FcRII and the cytoplasmic tail of human LDL receptor that contains two basolateral targeting domains, is basolaterally expressed (Matter et al., 1992). In contrast, FcLR5-22 is a protein that is preferentially expressed apically, since it contains only the clathrin-coated pit domain of the LDL receptor and neither of the two basolateral sorting signals. Fig. 3A shows confocal sections of cells expressing these chimeras, which were labeled with anti-FcRII antibody on only the cell surface, that confirm the previously described polarized expression of FcLR5-50 and FcLR5-22. Thus, this immunofluorescence assay can detect basolateral sorting signals in C-terminal cytosolic domains connected to the ecto- and transmembrane domain of FcRII.

We next analyzed the expression of FcOCD in filter-grown MDCK cells by staining cells that had been fixed and permeabilized with the Triton X-100/ethanol procedure, with

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**Fig. 1.** Schematic diagram of wild-type occludin, epitope-tagged full-length occludin (HAoccludin), a deletion mutant that lacks the C-terminal domain (HAoccludinCT3) and chimeric occludin (FcOCD). Glycopolysylated HAoccludin (HAoccludinGL1+2) is also shown.

**Fig. 2.** Expression of mutant and chimeric occludin. Filter-grown wild-type and transfected MDCK cells that had been preincubated without (−) or with (+) sodium butyrate were lysed by boiling in SDS-PAGE sample buffer. The samples were then separated on 6% to 15% gradient gels and subsequently transferred to nitrocellulose. The blots were incubated with either anti-FcRII antibodies or anti-occludin antibodies on immunoblots. Fig. 2 shows that the antibody specific for the C-terminal domain of chicken occludin (antibody B; Balda et al., 1996) only recognized a band in lanes corresponding to transfected cells, and expression of the chimera resulted in a higher molecular weight band (lanes 1-6). Expression of the chimeric protein could also be confirmed with an antibody directed against the ectodomain of FcRII (Green et al., 1985), which recognized a band of the same molecular weight as the anti-occludin antibody (lanes 7 and 8). In all cases, the recognized band was stronger in lanes derived from cells that had been treated with sodium butyrate; this treatment is known to result in higher expression of cDNAs that are controlled by a cytomegalovirus-derived promoter (Brown et al., 1989).
anti-chicken occludin antibody to visualize the chimera and with anti-ZO-1 antibody to mark the position of tight junctions. Optical sections obtained from the lateral part of the cells showed the typical reticular staining pattern that had also been observed with FcLR5-50 in Fig. 3A, while no staining could be observed in sections derived from the apical region of the monolayers and very little in the part of the lateral membrane that contains tight junctions (Fig. 3B). This indicates that FcOCD was primarily expressed at the basolateral cell surface. This was confirmed by staining non-permeabilized cells with the anti-FcRII antibody as described above for the FcRII-LDL receptor chimera (Z-section in Fig. 3C). Furthermore, the inside parts of the permeabilized cells in Fig. 3B also showed some punctate staining, indicating that a fraction of the chimeric protein was in an intracellular compartment, possibly endosomes or lysosomes. Thus, the C-terminal domain of occludin is capable of mediating basolateral expression of a membrane protein.

The ZO-1 staining in Fig. 3B shows that the distribution of the peripheral membrane protein ZO-1 was not affected by the expression of the chimeric FcOCD protein even though the C-terminal domain of occludin contains a binding site for ZO-1 (Furuse et al., 1994), nor could we observe any effects on the distribution of ZO-1 when the chimera was locally concentrated by crosslinking with immunocomplexes (not shown). This suggests that the distribution of ZO-1 is not primarily determined by the availability of binding sites provided by the C-terminal domain of occludin. Furthermore, the expression of FcOCD did not have any detrimental effects on transepithelial electrical resistance independent of the expression level and/or the presence of cross-linking ligand.

All known basolateral targeting determinants mediate direct basolateral sorting of newly synthesized proteins. To test whether the C-terminal cytoplasmic domain of occludin would also do this, we metabolically labeled transfected cells grown on permeable supports and, after different chase periods, biotinylated either the apical or the basolateral cell surface. Biotinylated chimeras were then isolated and analyzed by SDS-PAGE. The fluorographs in Fig. 4 show that the chimera could be biotinylated predominantly at the basolateral cell surface, even when it was just starting to appear at the cell surface (Fig. 4B). Thus, the C-terminal cytoplasmic domain of occludin is capable of mediating basolateral expression of a membrane protein.
occludin is able to mediate direct basolateral transport of newly synthesized chimera.

**The C-terminal cytoplasmic domain of occludin mediates endocytosis**

Basolateral sorting signals are often co-linear with clathrin-coated pit domains; hence it was interesting to test whether the C-terminal cytoplasmic domain of occludin could also mediate endocytosis. Indeed, the confocal sections in Fig. 3B also showed some punctate staining, suggesting a possible endosomal or lysosomal localization. To test endocytosis, we incubated subconfluent MDCK cells, which had been grown on coverslips and expressed either FcOCD or control Fc receptors, with the anti-FcRII antibody on ice. The cells were then washed and subsequently incubated at 37°C for 10 minutes. The cells were then cooled again and bound antibody was visualized by binding a fluorescently labeled secondary antibody either to non-permeabilized cells, to reveal any antibody that remained at the cell surface, or to permeabilized cells in order to reveal both cell surface and internalized antibodies.

Fig. 5 shows that staining of permeabilized cells expressing FcLR5-22, a FcRII chimera containing the clathrin-coated pit domain of the human LDL receptor (left column), which were allowed to internalize the antibody for 10 minutes, resulted in cell surface labeling as well as some punctate staining inside the cells, visible in the lower optical section (Fig. 5A). If the same cells were labeled without prior permeabilization (Fig. 5B), or if the labeled permeabilized cells were not incubated at 37°C (Fig. 5C), this punctate staining could not be seen, confirming the intracellular location. If the same experiment was done with a cell line expressing FcRII-B1, the lymphocyte isoform that is unable to mediate endocytosis (Miettinen et al., 1989) (middle column), all three labeling conditions mentioned above resulted in essentially the same staining, with no intracellular punctate staining. Thus, this immunofluorescence assay works well as a test for endocytosis mediated by FcRII chimera.

If this experiment was done with cells expressing FcOCD (right column), intracellular punctate staining could also be observed in permeabilized cells after 10 minutes of uptake (Fig. 5A). As in the case of FcLR5-22, no punctate staining was obtained in cells that were not permeabilized or that were permeabilized but not incubated at 37°C (Fig. 5B,C). This indicates that FcOCD was able to internalize antibody. This conclusion is also supported by the punctate staining obtained in permeabilized cells labeled in the absence of ligand (Fig. 3B). The C-terminal cytoplasmic domain of occludin is thus also able to mediate endocytosis.

**Glycosylated occludin accumulates in the basolateral membrane**

To test whether the basolateral membrane is an obligatory intermediate of occludin on its way to tight junctions, we tried to generate mutants that would not become integrated into tight junctions and, hence, accumulate in the lateral membrane. Within tight junctions, neighboring plasma membranes are in very close contact or even partially fused (Kachar and Reese, 1982; Pinto da Silva and Kachar, 1982). Therefore, we introduced in each extracellular loop an N-linked glycosylation site in the hope that the oligosaccharides would be too bulky for integration into tight junctions. The glycosylation sites were introduced into the cDNA coding for HAoccludin, resulting in HAoccludinGL1+2 (Fig. 1). HAoccludin is an epitope-tagged full-length occludin, which becomes incorporated into tight junctions like wild-type occludin (Balda et al., 1996).

The immunoblots in Fig. 2 show that the anti-HA antibody recognized only one band in MDCK cells transfected with either the cDNA for HAoccludin (lanes 9 and 10) or with cDNA for HAoccludinGL1+2 (lanes 11 and 12). HAoccludinGL1+2 migrated with a higher molecular weight, confirming the glycosylation. If HAoccludinGL1+2 was immunoprecipitated from metabolically labeled cells, two different bands could be observed (Fig. 6, total). Without chase, HAoccludinGL1+2 appeared as a sharp band that became weaker with increasing times of chase; in addition a higher molecular weight, more diffuse band appeared as chase times increased, suggesting...
conversion to a complex glycosylated protein. If glycosylation was inhibited with tunicamycin, a band appeared that had a lower apparent molecular weight than either band observed in untreated cells, confirming the presence of N-linked carbohydrates (Fig. 6). If occludin mutants with only one or the other glycosylation site were expressed, the resulting proteins exhibited an intermediate molecular weight, suggesting that both sites became glycosylated (not shown). The efficient glycosylation of these sites supports the predicted membrane topology of occludin.

We next asked whether HAoccludinGL1+2 would still become incorporated into tight junctions. To do this, filter-grown MDCK cells were fixed and permeabilized with the Triton X-100/ethanol procedure – this method was found previously to result in a clear co-localization of HAoccludin with the tight junction protein ZO-1 (Balda et al., 1996) – and processed for immunofluorescence using a polyclonal antibody against the HA-epitope and a monoclonal antibody specific for ZO-1.

Fig. 7A shows that HAoccludinGL1+2 could be detected primarily in the lateral membrane and very little staining was observed in sections derived from the apical part of the monolayer. Additionally, some intracellular punctate staining was observed. In contrast, the ZO-1 labeling still appeared as a sharp junctional staining, indicating that neither the distribution of this protein nor the gross junctional morphology was affected in MDCK cells expressing HAoccludinGL1+2. Unlike HAoccludin, HAoccludinGL1+2 did not significantly co-localize with ZO-1 (Fig. 7B). Also confocal Z-sections confirmed the preferential basolateral expression of HAoccludinGL1+2 (Fig. 7C).

To test whether newly synthesized glycosylated occludin was directly sorted to the basolateral membrane, we again combined metabolic labeling with cell surface biotinylation. Since occludin does not have any lysines in its extracellular loops, we used biotin-hydrazide to biotinylate the carbohydrates. Fig. 6 (surface) shows that HAoccludinGL1+2 appeared predominantly in the basolateral membrane. Since glycosylated occludin lacking the C-terminal domain is sorted apically, it can be excluded that the basolateral transport of HAoccludinGL1+2 was induced by glycosylation (A. Gut, F. Kappeler, N. Hyka, M.S. Balda, H.-P. Hauri and K. Matter; unpublished). Thus, full-length glycosylated occludin is sorted directly to the basolateral membrane during biosynthesis.

As mentioned above, the clear ZO-1 staining shown in Fig. 7 indicates that tight junctions were not affected by expression of HAoccludinGL1+2. Furthermore, measurements of
Transepithelial electrical resistance of clones expressing glycosylated occludin did also not suggest any negative effect on tight junctions.

**Transfected truncated occludin oligomerizes with endogenous occludin**

We have previously shown that a deletion mutant of occludin that lacks the C-terminal domain (Fig. 1, HAoccludinCT3) is transported to tight junctions (Balda et al., 1996). This is not what one would expect if the C-terminal domain is important for basolateral targeting. However, it could be that the transfected mutant occludin oligomerized with endogenous occludin and was thereby dragged to tight junctions. This possibility is supported by the co-clustering of transfected mutant and endogenous occludin within tight junctions and the inefficient transport of the truncated protein to tight junctions (Balda et al., 1996). To test such an oligomerization more directly, non-transfected or HAoccludinCT3-expressing cells were solubilized and endogenous occludin was immunoprecipitated with the antibody that was generated against the C-terminal domain of chicken occludin, which had been deleted in the transfected protein, and that cross-reacts with endogenous dog occludin. Then, the immunoprecipitates were analyzed by immunoblots using the anti-HA antibody to detect co-precipitated HAoccludinCT3.

Fig. 8 shows that if non-transfected cells were analyzed, neither the anti-HA antibody nor the anti-occludin antibody precipitated a protein that would have been recognized by the anti-HA antibody in immunoblots. In transfected cells, both antibodies precipitated a band that was recognized by the anti-HA antibody and had the right molecular weight for being HAoccludinCT3. Since the anti-occludin antibody was generated against the domain that was deleted in the mutant and, consequently, does not recognize the truncated protein (Balda et al., 1996), the HAoccludinCT3 that was found in the anti-occludin immunoprecipitate must have been associated with endogenous occludin. The specificity of the antibodies is also illustrated by the finding that the majority of the complex was dissociated in the presence of Empigen BB, resulting in very little co-precipitation of HAoccludinCT3. Thus, transfected HAoccludinCT3 associates with endogenous occludin. When this manuscript was in preparation, a paper appeared that described the same result for C-terminally truncated chicken occludin expressed in Xenopus embryos (Chen et al., 1997). While these data do not exclude the possible existence of additional but inefficient cell surface targeting determinants in the N-terminal half of occludin, they suggest that HAoccludinCT3 is dragged to tight junctions by endogenous occludin and can therefore be transported to tight junctions without containing a basolateral targeting domain.

This raises the question of whether the glycosylated mutant of occludin was also oligomerizing with endogenous occludin. If this had occurred, one would expect that either HAoccludinGL1+2 is also in tight junctions or that endogenous occludin is retained in the basolateral membrane. Since the anti-occludin antibody recognizes the endogenous and the chicken protein, we could not get conclusive results in the co-precipitation assay with HAoccludinGL1+2. Nevertheless, we could not detect significant amounts of HAoccludinGL1+2 in tight junctions, suggesting that transfected occludin was not dragged into tight junctions by the endogenous protein. Furthermore, since we do not possess an antibody that recognizes only dog and not chicken occludin, we could not directly test whether endogenous occludin is retained in the lateral membrane together with HAoccludinGL1+2.

**DISCUSSION**

In this study, we analyzed the biosynthetic transport of occludin to tight junctions. We found that the C-terminal cytoplasmic domain of occludin was capable of mediating basolateral targeting of a reporter protein, indicating that it contains a basolateral targeting determinant. Moreover, mutant occludin molecules that could not efficiently integrate into tight junctions accumulated in the basolateral membrane, suggesting that the biogenesis of tight junctions occurs from the basolateral membrane.

A model in which tight junctions are assembled from the basolateral membrane is also supported by the observation of lateral intramembrane strands in freeze-fracture replicas derived from epithelia treated, for instance, with proteases or calcium ionophores (for reviews, see Bentzel et al., 1991; Polak-Charcon, 1991). Furthermore, small amounts of occludin could recently be detected in the lateral membrane in chicken intestinal epithelial cells by immunocytochemistry.
indicating that endogenous occludin is also transported to the basolateral domain (Sakakibara et al., 1997).

The importance of the C-terminal cytoplasmic domain of occludin for cell surface transport and integration into tight junctions was already suggested by experiments analyzing the expression of occludin mutants with various deletions of different sizes in the C-terminal domain (Furuse et al., 1994; Balda et al., 1996; Chen et al., 1997). Interestingly, these mutants either became efficiently integrated into tight junctions or accumulated intracellularly and were only inefficiently, if at all, integrated into tight junctions. None of these mutants was expressed basolaterally, indicating that basolateral targeting is not just caused by a determinant that is overpowered by another transport determinant in the wild-type protein. This is also supported by the basolateral accumulation of glycosylated full-length occludin reported here. Since we found recently that N-linked glycosylation mediates apical transport of three different membrane proteins including HAoccludinCT3 (A. Gut, F. Kappeler, N. Hyka, M.S. Balda, H.-P. Hauri and K. Matter; unpublished), we can exclude the possibility that this result was due to a basolateral sorting activity of carbohydrates.

We have previously shown that a fraction of a truncated mutant of occludin that lacks the C-terminal domain, which contains the basolateral targeting determinant, became integrated into tight junctions (Balda et al., 1996). This indicates that the N-terminal half of occludin either contains additional determinants that mediate cell surface transport with a low efficiency, or is able to associate with endogenous components of tight junctions and is passively transported to tight junctions. We find now that this transfected mutant protein can be co-immunoprecipitated with the endogenous protein. This suggests that the mutant protein was transported to the junction since it was oligomerized with endogenous occludin, but does not exclude the existence of additional transport determinants. Nevertheless, that C-terminally truncated occludin is dragged to tight junctions by the endogenous protein is also supported by our previous finding that significant amounts of the transfected mutant protein accumulated in intracellular structures (Balda et al., 1996), as well as by similar experiments in Xenopus embryos that were reported recently (Chen et al., 1997).

The next question is how occludin is integrated into tight junctions once inserted into the basolateral membrane. It had been suggested that integration of occludin into the junction is mediated by the C-terminal cytoplasmic domain involving an interaction with the submembrane cytoskeleton (e.g. with ZO-1). That other domains of occludin are at least as important as the C-terminal domain for integration into tight junctions was first suggested by experiments with the C-terminally truncated and N-terminally epitope-tagged mutant HAoccludinCT3 (Balda et al., 1996). This protein was expressed in tight junctions but, as revealed by immunofluorescence, formed patches along the junction instead of a continuous ring. Furthermore, the distribution of endogenous occludin was changed, forming the same discontinuous pattern as the transfected occludin, which may reflect the association of endogenous and transfected occludin. If these transfected cells were co-cultured with wild-type MDCK cells, however, the discontinuous distribution was corrected to a continuous one in junctions between a wild-type cell and a transfected cell (Balda et al., 1996). Thus, occludin is still able to oligomerize and to participate in intercellular complexes without the C-terminal domain.

The first direct evidence for the importance of the extracellular loops for the stabilization of occludin in tight junctions came from experiments in which a peptide corresponding to the second extracellular loop of occludin was added to epithelial monolayers (Wong and Gumbiner, 1997). This resulted in the disappearance of occludin from the junctions and subsequent degradation. Other proteins of tight junctions (e.g. ZO-1) and the gross morphology of the cells were not affected. Thus, the second extracellular loop of occludin is important for concentrating and/or stabilizing the protein within tight junctions. The effect of the peptide appeared to be very slow (i.e. 2 to 3 days in mature monolayers), suggesting that the peptide-induced loss of occludin was due to an effect on the integration of newly synthesized occludin into tight junctions, rather than on the stability of already integrated protein. Interestingly, in the presence of the peptide, occludin became degraded, suggesting that it had become internalized. Since the C-terminal domain of occludin is able to mediate endocytosis, this implies that without extracellular interactions, the C-terminal domain of occludin mediates internalization rather than stabilization at the junction.

A role for the extracellular loops in the accumulation of occludin in tight junctions is also suggested by the glycosylated occludin that accumulates in the lateral membrane, described here. There are two possible ways that oligosaccharides could have inhibited integration of occludin into tight junctions: (1) the carbohydrates are simply too bulky and inhibit integration sterically; (2) the carbohydrates block an interaction that is mediated by the extracellular loops and required for junctional accumulation of occludin. Interestingly, accumulation of glycosylated occludin in the lateral membrane could also be observed if only the first loop was glycosylated, suggesting that it might also be of importance for localization (not shown). This is also supported by experiments in transfected fibroblasts, which suggest that the first loop of occludin has adhesive properties (Van Itallie and Anderson, 1997).

The evidence discussed here suggests the following working model. Once newly synthesized protein arrives in the Golgi apparatus, it is sorted to the basolateral membrane by a targeting signal in the C-terminal cytosolic domain. Since occludin becomes phosphorylated if the formation of tight junctions is induced in calcium-switch experiments, it could be that this step is regulated by phosphorylation of occludin (Sakakibara et al., 1997). At the time of exit from the Golgi, occludin has probably already formed oligomers. Since intracellular HAoccludinCT3 accumulates in a perinuclear, Golgi-like compartment (Balda et al., 1996; A. Gut, F. Kappeler, N. Hyka, M. S. Balda, H.-P. Hauri and K. Matter, unpublished), occludin might also oligomerize in the Golgi apparatus, similar to connexins, proteins of gap junctions with the same membrane topology (Musil and Goodenough, 1993). On arrival at the lateral membrane, interactions occurring in the extracellular loops and possibly also in the transmembrane domains are then responsible for the accumulation of occludin in tight junctions. Once in tight junctions, cytosolic interactions appear to become important for a continuous junctional distribution of occludin, as suggested by the discontinuous distribution of transfected and endogenous occludin in cells expressing HAoccludinCT3 (Balda et al., 1996). Elegant in vitro studies with recombinant proteins containing parts of ZO-1 and occludin, respectively, demonstrated that the C-terminal
cytosolic domain of the latter protein directly interacts with ZO-1 (Furuse et al., 1994). Therefore and because of the results with HAoccludinCT3, it appears likely that this interaction between ZO-1 and occludin is responsible for the arrangement of occludin within the junction.

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