4. DISCUSSION

4.1. PKC-mediated phosphorylation and redistribution of occludin under low calcium conditions.

The involvement of PKC in TJ assembly was suggested almost a decade ago, following the observation that the treatment of MDCK cells maintained in low calcium medium with PKC activators PMA and diC8 induced the redistribution of TJ proteins from the cytoplasm to the plasma membrane and the formation of TJ strands (Balda et al., 1993). Specifically, tight junction proteins ZO-1, ZO-2, ZO-3 and cingulin were shown to be recruited to the cell-cell contact sites after cell treatment with diC8, although their phosphorylation state remained unchanged, thus excluding the possibility that PKC may regulate TJ formation by the phosphorylation of these proteins (Balda et al., 1993; Citi and Denisenko, 1995). In fact, TJ proteins which undergo phosphorylation by PKC during TJ assembly could not be identified. Furthermore, the diC8-dependent TJ assembly was not paralleled by the adherens junction formation, suggesting a TJ-specific regulatory mechanism (Balda et al., 1993).

Numerous novel proteins localising at tight junction have been identified in recent years (Gonzalez-Mariscal et al., 2003). One of them, occludin, was shown to be heavily phosphorylated on serine and threonine residues, with the more phosphorylated occludin molecules being selectively concentrated at TJ (Sakakibara et al., 1997). Therefore, occludin was a promising candidate for a protein that is phosphorylated by PKC during TJ assembly. To evaluate this possibility we tested the effects of PKC activators on occludin phosphorylation and localisation in MDCK cells in which cell junctional complex has been disrupted by Ca^{2+} chelation.

Previous observations indicated that phospho-occludin can be specifically detected on Western-blots by its decreased electrophoretic mobility in SDS-PAGE gels (Sakakibara et al., 1997). Using commercially available polyclonal anti-occludin antibodies, we were able to distinguish between highly phosphorylated occludin from MDCK cells cultivated in NC conditions and less phosphorylated occludin from cells grown in LC conditions by observing their differing electrophoretic mobility (Fig. 3.1, A). Upon the addition of PMA or diC8 to MDCK cells cultivated in LC medium, a profound upward band shift of occludin was observed (Fig 3.1, A). These changes in electrophoretic mobility could be attributed entirely to the phosphorylation of occludin since the treatment with calf intestinal phosphatase resulted in the complete disappearance of the high molecular weight bands (Fig. 3.1, B). The above mentioned results clearly indicate that occludin is phosphorylated following stimulation of MDCK cells

maintained in LC medium with PKC activators PMA and diC8. Notably, occludin is the first tight junction protein in which the incorporation into tight junction following PKC activation was found to correlate with a marked increase in phosphorylation. Hence, the phosphorylation of occludin may represent an important step in PKC-mediated TJ assembly.

The PMA-induced increase in the phosphorylation state of occludin was found to be dose- and time-dependent (Fig. 3.2). When added for 60 min, PMA (2.5 nM) induced a small but easily distinguishable upward shift in the electrophoretic mobilityof occludin (Fig. 3.2, A). The maximum effect on occludin phosphorylation was observed at 25 nM of PMA (Fig. 3.2, A), resulting in a level of occludin phosphorylation which was identical (within the accuracy of immunoblot technique) to that from MDCK cells maintained in normal calcium conditions. Higher levels of occludin phosphorylation could not be achieved with further increase in PMA concentration, indicating that the level of occludin phosphorylation is tightly regulated by as yet unknown mechanisms.

The time course of occludin phosphorylation induced by PMA (25 nM) revealed that the first changes in the phosphorylation state of occludin occurred after 5-10 min and reached a maximum at 40 min (Fig. 3.2, B). Our data indicate that occludin phosphorylation in response to the treatment with PMA proceeds much faster than that found after tight junction assembly induced by calcium switch: occludin phosphorylation has been reported to reach a maximum 3-4 hours after calcium addition ((Sakakibara et al., 1997), see also Fig. 3.6). The faster kinetics of occludin phosphorylation induced by PMA may be due to higher levels of PKC activation by phorbol esters. Alternatively, there may be pathways which counteract occludin phosphorylation induced by a calcium switch more efficiently than during TJ assembly induced by PMA or diC8.

Occludin has been shown to interact with actin filaments in vitro (Wittchen et al., 1999). Furthermore, the overexpression of occludin in rat endothelial cell line induced the re-organisation of actin and the formation of circumferential fine actin bundles in close proximity to occludin-positive sites (Atsumi et al., 1999). Thus, one of occludins functions may be to link TJ complexes to the perijunctional actomyosin belt. Since resistance to TritonX-100 extraction is an indirect indicator of protein association with cytoskeleton, the detergent solubility of occludin was determined. Highly phosphorylated forms of occludin which appeared after the treatment of MDCK cells in LC by PMA or diC8 were found to be invariably un-extractable in buffers containing TX-100 (Fig. 3.1, A). Hence, the phosphorylation state of occludin may regulate its association with cytoskeleton. Importantly, the addition of diC8 into LC medium induces not

only the phosphorylation and redistribution of occludin (our data), but also triggers the reorganisation of actin filaments (Balda et al., 1993). In LC conditions actin is most concentrated around cell nuclei (Balda et al., 1993), while occludin is diffusely distributed in the cytoplasm. The treatment of MDCK cells with PMA or diC8 results in the redistribution of actin to the cell periphery (Balda et al., 1993). Thus, it is tempting to speculate that phosphorylated occludin may stimulate reorganisation of the actin cytoskeleton, although the present study does not provide sufficient evidence to prove this assumption. On the other hand, phospho-occludin may associate with detergent-insoluble membrane microdomains (also known as lipid rafts) which were recently shown to be a part of tight junctions (Nusrat et al., 2000b). Future investigations should clarify which detergent-insoluble cellular structures interact with occludin and how the phosphorylation of occludin regulates these interactions.

Immunofluorescent staining of occludin has been performed to examine how the changes in occludin phosphorylation correlate with its cellular distribution. Both PMA and diC8 cause the redistribution of occludin from cytoplasm to the regions of cell-cell contact in MDCK cells maintained in LC medium (Fig. 3.3, D, H). This relocation probably reflects the incorporation of occludin into tight junctions, since the formation of fragmentary TJs fibrils has been reported under identical experimental conditions (Balda et al., 1993). Staining of occludin at intercellular contacts was not as continuous or intensive in diC8 treated cells as in the cells grown under NC conditions. Occludin is known to concentrate only at those plasma membrane sites, which contact apposing plasma membranes of adjacent cells, and to be absent at plasma membrane of single isolated cells (data not shown). Since LC conditions cause cell rounding and detachment of some cells from the substrate, the observed discontinuity in occludin staining is probably due to the lack of a direct contact between neighbouring cells.

The small amounts of occludin redistributed to the lateral cell membrane in response to the treatment with 2.5 nM PMA correlated well with the small upward band shift due to phosphorylation (cf Figs. 3.1, A and 3.3). The amount of occludin translocated to the lateral plasma membrane increased with the increase in PMA concentration to 25 nM, but the staining of occludin became more discontinuous (Fig. 3.3 E and F). This observation is consistent with studies reporting the disruption of TJs and an increase in occludin proteolysis in response to very high (0.1-1 μM) concentrations of PMA (Farshori and Kachar, 1999; Clarke et al., 2000b). Consistent with this assumption, we also noted the decrease in the cellular level of occludin in response to the treatment with 25 nM PMA (Fig. 3.1, A, lane 5 (S)).

Significantly, the level of occludin phosphorylation reached a maximum 40 min after treatment with 25 nM PMA, whereas the PMA-induced occludin redistribution from the cytoplasm to the lateral plasma membrane was observed after only 2 hours. Forty min after treatment with 25 nM PMA the amount of occludin translocated to the cell-cell contact sites was negligible (data not shown). In addition, other members of TJ complex, e.g. ZO-1 and F-actin, also were reported to accumulate at cell-cell contact sites 2 hours after addition of diC8 or PMA (Balda et al., 1993). These observations suggest that the translocation of occludin to the plasma membrane and the assembly of TJ complexes is a consequence of rather than a prerequisite for, PMA-induced occludin phosphorylation. Therefore, the phosphorylation of occludin may indeed be a signal which mediates its targeting to cell-cell contact sites and incorporation into TJ strands.

DAG and PMA bind to the C1 domains of many different proteins besides PKC (reviewed in Introduction). To verify that PMA or diC8 exert their effect on occludin phosphorylation and sub-cellular distribution primarily through PKC, we applied a specific PKC inhibitor GF-109203X to cultured MDCK cell). Unlike unspecific C1-targeted inhibitors, GF-109203X competes for the ATP-binding site of conventional and novel PKC isotypes, the only PKC isozymes which are known to be activated by DAG (the IC_{50} of the compound is in low nanomolar range against cPKC and 200-700 nM against different nPKC (Toullec *et al.*, 1991; Martiny-Baron *et al.*, 1993). The affinity of this inhibitor for DAG-insensitive atypical PKC is much lower (IC_{50}≈5.8 μM for PKCζ) (Martiny-Baron et al., 1993). The treatment of MDCK cells with GF-109203X completely abolished the PMA- and diC8-induced translocation of occludin to intercellular contacts, confirming the involvement of PKC. The inhibitory effect of GF-109203X was clearly seen at 5 μM (Fig. 3.4), but was not seen at lower concentrations (e.g. at 1 μM, data not shown). Although aPKC are known to be regulators of tight junction assembly (Suzuki et al., 2001; Suzuki et al., 2002), this subfamily is not activated by diacylglycerols. In addition, aPKC is unlikely to be significantly inhibited by 5 μM of GF-109203X because of the high IC_{50} value and restricted permeability of the compound through the plasma membrane. Complete intracellular inhibition of conventional PKC can be achieved at submicromolar concentrations of GF-109203X (Toullec et al., 1991; Martiny-Baron et al., 1993). Therefore, the inhibition of occludin translocation by 5μM GF-109203X implicates a DAG-sensitive **novel** PKC as a mediator of diC8 and DAG-induced TJ assembly. Occludin phosphorylation triggered by PMA could be also inhibited by 5 μM of GF-109203X (Fig. 3.4, B), but was not inhibited at

lower concentrations (not shown). These data further strengthen the link between the phosphorylation of occludin and its incorporation into TJ.

4.2. Tight junction assembly by calcium switch: the role of conventional PKC.

Our data indicating the involvement PKC in occludin phosphorylation under specific LC conditions tempted us to investigate whether PKC is responsible for the regulation of occludin phosphorylation under more physiological conditions. For this purpose we have chosen the "calcium switch" model of tight junction assembly. Epithelial cells placed in low-calcium medium lose intercellular contacts (Martinez-Palomo et al., 1980) and apical-basolateral polarity (Vega-Salas et al., 1987). Upon return to normal calcium medium, cell monolayers form functional tight junctions (Martinez-Palomo et al., 1980) and develop cell polarity within few hours. This calcium-switch procedure is the most useful method of studying tight junction assembly and has been validated in a large number of reports. Moreover, the resulting synchronized formation of junctional complex is thought to mimic many features of early morphogenesis of epithelial tissue (Stuart and Nigam, 1995a).

Consistent with previously published data (Sakakibara et al., 1997), we observed a restoration of the phosphorylation level and honey-comb staining pattern of occludin 4 hours after the addition of calcium to the extracellular medium (Fig. 3.5), indicating the re-establishment of tight junction structure. The application of GF-109203X during calcium switch profoundly inhibited the sorting of occludin to intercellular contacts (Fig. 3.5, A). In contrast, the phosphorylation of occludin induced by calcium switch was only slightly inhibited by GF-109203X (Fig. 3.5, B). Significantly, GF-109203X inhibited the appearance of the upper most intense band, but had almost no effect on the intermediate diffuse bands of occludin. It is therefore unlikely that PKC alone accounts for the phosphorylation of occludin under normal physiological conditions. However, since the inhibition of PKC abrogates occludin targeting to the regions of cell-cell contacts, we suggest that it is PKC-mediated phosphorylation of occludin that regulates the incorporation of occludin into tight junction.

Like the experiments designed to study TJ assembly by PMA and diC8, described above (part 3.1), GF-109203X exerted its effects on the phosphorylation and cellular distributuon of occludin at 5 μM and was ineffective at lower concentrations. As discussed above (page 71), the relatively high concentration of GF-109203X required to achieve inhibition indicates that conventional PKC is unlikely to regulate the phosphorylation and incorporation of occludin into

TJ. To further evaluate the role of conventional PKC in tight junction assembly, we used kinase inhibitor Gö6976 to selectively inhibit cPKC. When used in combination with GF-109203X, the application of Gö6976 allows dissection of the relative contribution of cPKC. This is because both compounds are equally potent inhibitors of cPKC, but Gö6976 (in contrast to GF-109203X) is absolutely inactive toward nPKC and aPKC (Martiny-Baron et al., 1993; Way et al., 2000).

The effects of Gö6976 indicated that the enzyme activity of cPKC is required neither for the incorporation of occludin into TJ nor for its phosphorylation (Fig.3.5, A and B). Moreover, close examination of obtained data revealed that the presence of Gö6976 during calcium switch resulted in more prominent and continuous staining of occludin at intercellular contacts (framed images on Fig. 3.5), indicating that the compound may *promote* the formation of TJ. Our data and the results of other authors (Sakakibara et al., 1997; Wong, 1997) demostrate that the phosporylation level of occludin is a very sensitive indicator of the maturation status of tight junctions. Thus, if Gö6976 is really capable of promoting TJ formation during calcium switch, the phosphorylation level of occludin is expected to be increased by the presence of Gö6976.

The analysis of the kinetics of occludin phosphorylation during tight junction assembly induced by calcium in the presence and absence of Gö6976 revealed strong acceleration of occludin phosphorylation by Gö6976 (Fig. 3.6, A). The maximum level of occludin phosphorylation was reached about 3 hours after the switch to NC conditions; in the presence of Gö6976 the maximum phosphorylation was observed after only 1 hour. Consistent with a low IC_{50} of Gö6976 toward cPKC (23 nM for PKCα, (Martiny-Baron et al., 1993)), the acceleration of occludin phosphorylation by Gö6976 was observed at relatively low concentrations (80-150 nM, see Fig. 3.6, B).

In parallel experiments, Gö6976 in a dose-dependent manner accelerated the incorporation of occludin into newly forming tight junction (Fig. 3.7), again illustrating the link between the assembly of occludin into TJ and its phosphorylation. With the exception of diacylglycerols (Balda et al., 1991; Balda et al., 1993) which have broad range effects in cells, Gö6976 is the first compound reported to accelerate tight junction assembly. Taking into account the remarkable potency and specificity of Gö6976 toward cPKC, we conclude that the inhibition of cPKC during calcium switch promotes the assembly of tight junction. In other words, cPKC seems to antagonise the *de novo* assembly of tight junction during calcium switch. Although further work is required to confirm that the effects of Gö6976 are specifically due to the inhibition of cPKC, the literature supports this assumption. PKC, especially conventional PKCα

has been implicated in cell and tissue barrier dysfunction in a number of pathologies. These include the increased pulmonary endothelial permeability and respiratory distress syndrome caused by inflammatory cytokines, oxidative stress and phorbol esters {Siflinger-Birnboim, 2003 302 /id}. Thus, PKC–dependent pathway(s) in epithelial cells may antagonise the function of a barrier. The effects of Gö6976 can therefore be reasonably explained in terms of inhibiting the basal cPKC activity which antagonises the formation of tight junction.

Significantly, cPKC has not been previously implicated as an antagonist of tight junction assembly. Our data thus expand the possible therapeutic applications of cPKC inhibitors: the inhibition of cPKC seems to be promising for clinical applications in which rapid re-establishing of functional tissue barrier is desirable; for instance, under acute renal failure or intestinal ischemia.

4.3. Tight junction assembly by calcium switch: the role of novel PKC.

Since the involvement of cPKC in calcium-induced tight junction assembly could be completely excluded by the experiments with Gö6976, some other PKC isoforms must regulate TJ assembly in this system. As discussed above, aPKC is unlikely to be markedly inhibited by GF-109203X at the concentration used to block TJ assembly. In addition, aPKC isoforms are not sensitinve to diacylglycerols and hence could not mediate the assembly of tight junction by DAG. Beside cPKC and nPKC DAG may also activate PKCμ, but this isoform is not sensitive to GF-109203X (Zugaza et al., 1996; Chiu and Rozengurt, 2001). From these considerations, it logically follows that enzyme activity of novel PKC is likely to be essential for occludin phosphorylation and tight junction formation.

Previous studies using indirect immunofluorescence demonstrated that out of four known novel PKCs MDCK cells express δ, ε and η isozymes (Dodane and Kachar, 1996; Chen et al., 1997). PKCθ was not detected in confluent monolayers of MDCK cells by immunostaining(Dodane and Kachar, 1996). However, these results should be interpreted with caution since immunostaining may be not sensitive enough to detect low levels of PKCθ. In addition, maximum expression of PKCθ may precede the formation of confluent cell monolayer with completely assembled TJs. Until recently, there were no known kinase inhibitors specific for nPKC until rottlerin was identified. Rottlerin, extracted from *Mallotus philippinensis*has, has been recently shown to selectively inhibit θ and -δ nPKC isotypes (IC_{50} ≈3-6 μM for both PKCθ and -δ (Gschwendt et al., 1994; Villalba et al., 1999). Rottlerin inhibits conventional PKC with

significantly reduced potency (IC_{50} ≈30-42 μM), and has a negligible effect on other PKC isotypes ε, η and ξ (IC_{50} ≈80-100 μM).

To evaluate the role of PKCθ and -δ isotypes in occludin phosphorylation and incorporation into TJs, we performed Ca^{2+} switch assays in the presence of rottlerin. A moderate inhibition of occludin phosphorylation was observed at 10 μM (Fig. 3.8, A) – a concentration required for substantial intracellular inhibition of PKCθ and -δ (Gschwendt *et al.*, 1994; Villalba *et al.*, 1999; Minami *et al.*, 2003). The same concentration of rottlerin was sufficient to significantly block the assembly of occludin into tight junctions upon the switch to NC medium (Fig. 3.8, B). The effect of rottlerin reached a maximum at 20 μM: even 4 hours after the induction of TJ formation, the phosphorylation state and the distribution pattern of occludin resemble that of cells cultivated in LC medium (*cf*. Figs. 3.5, A and 3.8, B). Thus, PKCθ and -δ may participate in the regulation occludin phosphorylation and cell localisation.

To test whether PKCθ and/or -δ regulate the formation of the entire TJ complex we studied the effects of rottlerin on the sorting of TJ scaffold protein ZO-1. ZO-1 is believed to bind and organise many TJ proteins including claudins, occludin, ZO-2, ZO-3, cingulin, and actin (Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Cordenonsi *et al.*, 1999a). Hence, miss-localisation of ZO-1 would result in the disruption of the TJ structure. Immunofluorescent staining of ZO-1 revealed that rottlerin, in a dose-dependent fashion, abolished the redistribution of ZO-1 to cell-cell contact sites (Fig. 3.9), suggesting that the signalling by novel PKC isotypes θ and δ is essential for the TJ biogenesis. Interestingly, inhibitory effects of rottlerin at 10 μM were different for ZO-1 and occludin; the intensity of occludin staining at all cell-cell contacts was equally decreased (Fig. 3.8A, b), whereas ZO-1 staining remained intense at most cell borders with occasional thickenings and even more strong staining at some multicellular corners, but was completely absent at certain intercellular contacts (Fig. 3.9, B).

These differential effects of rottlerin on the sorting of occludin and ZO-1 imply that the localisation of these proteins at cell junctional complexes is differentially regulated by kinases. To our knowledge, rottlerin is the first compound which causes differential sorting of tight junction proteins to cell junctions and may, therefore, be a valuable tool to investigate TJ assembly. The investigation of the molecular mechanisms underlying the effects of rottlerin will likely allow the dissection of pathways regulating the incorporation of ZO-1 and occludin into TJs.

To determine precisely which of two PKC isotypes, θ or-δ regulates occludin and ZO-1 incorporation into newly forming TJs, we applied an isotype-specific PKCθ inhibitor, MPPI. MPPI is a 19 amino acid cell-permeable myristoylated peptide derived from a PKCθ pseudosubstrate sequence (Osada et al., 1992). This sequence occupies the substrate-binding cavity of PKCθ and thus inhibits its activity in intact cells. Since this inhibitor of PKCθ did not block the incorporation of occludin and ZO-1 into assembling TJs, we conclude that occludin phosphorylation and the formation of tight junction in MDCK cells requires the signalling by PKCδ.

This conclusion is inconsistent with data obtained by Mullin et al. These authors reported that overexpression of wild-type PKCδ isoform in LLC-PK1 epithelial cells induces both the structural and functional damage of TJs (Mullin et al., 1998). This contradiction may be explained by different signalling pathways regulating TJs in MDCK and LLC-PK1 epithelia. On the other hand, overexpression often results in high levels of PKC production exceeding the endogenous levels by an order of magnitude or more. As a result, overexpressed wild-type PKC isozymes may participate in signalling processes in which they are usually not involved (Brose and Rosenmund, 2002). Thus, basal PKC-δ activity may be essential for TJ formation while elevated PKC-δ activity may cause TJ disfunction.

The role of nPKC in tight junction assembly has not been addresed. However recent reports suggest that nPKC does have a positive influence on the barrier function. For example, the results of Harrington and colleagues (Harrington et al., 2003) demonstrated that overexpression of PKC-δ promotes basal endothelial barrier function by enhancing focal adhesion contact. Another report suggests that stimulation of confluent epithelial cells with PKC agonist bryostatin-1 enhances barrier properties of the monolayer and promotes the association of tight junction proteins with cytoskeleton, presumably via activation of PKCε (Yoo et al., 2003). Thus, accumulating evidence suggests a supporting role of novel PKCs in the barrier properties of tight junctions. Further studies will be required to elucidate the role of particular nPKC isoforms in the tight junction biogenesis. This important task is complicated by the fact that the physiological functions of all PKC isozymes seem to be very redundant: knockout mice have been produced for most PKC isoforms (reviewed in the Introduction, 1.4.1), but none of the mice have obvious alterations in tissue permeability or any other related drastic abnormalities. Cellular models of epithelial barriers seem to be more sensitive to the manipulations affecting PKC activity. In this regard, experiments with kinase-inactive point mutants of nPKC, which

often demonstrate dominant-negative effects, may be very promising in the further elucidation of the molecular mechanisms underlying the assembly of tight junction.

4.4. Gö6976 triggers the formation of tight junction in low calcium conditions and blocks the disassembly of tight junction.

The comparison of the effects exerted by GF-109203X and Gö6976 allowed us to make important conclusions about the role of conventional PKC in tight junction assembly induced by switch to normal calcium conditions. As a next step we characterised the involvement of cPKC in tight junction assembly induced by activators of PKC in LC conditions, by comparing the effects of GF-109203X and Gö6976. Two other questions were addressed in these experiments: (i) whether GF-109203X inhibits the formation of the entire TJ complex in LC conditions (e.g. if the inhibition of redistribution of TJ proteins other than occludin can be achieved with GF-109203X) and (ii) whether the observed effects of GF-109203X (Fig. 3.4) can be attributed entirely to the inhibition of PKC.

GF-109203X inhibited the diC8-induced redistribution of ZO-1 to intercellular contacts (Fig. 3.10) similarly to the previously observed effect on occludin (Fig. 3.4). We conclude from this data that GF-109203X does not specifically block the sorting of occludin into TJ, but instead inhibits the formation of the entire TJ complex. The application of myristoylated protein kinase C inhibitor (MPKCI) also resulted in a profound inhibition of ZO-1 re-distribution (Fig. 3.10). MPKCI, which is a short (only 8 amino acids) peptide mimicking the pseudosubstrate region of PKCα/β (Eichholtz et al., 1993), is expected to inhibit most PKC isoforms due to the remarkable similarities in the preferential phosphorylation substrates (Nishikawa et al., 1997). Since the treatment of MDCK cells with structurally unrelated pan-PKC inhibitors GF-109203X or MPKCI abolished the diC8-induced redistribution of ZO-1 (Fig. 3.10), it is obvious that the enzyme activity of some PKC isoforms is absolutely required for the assembly of tight junctions induced by diacylglycerols in LC conditions.

In contrast to MPKCI and GF-109203X, Gö6976 increased the amount of ZO-1 translocated to the cell-cell contacts (Fig. 3.10). The staining of ZO-1 at intercellular contacts was more continuous and intensive as compared to samples treated with diC8 alone, and completely disappeared from the cytoplasm. In parallel experiments, Gö6976 potentiated the redistribution of occludin and claudin-1 induced by the treatment of MDCK cells with diC8

(results not shown), indicating that the inhibition of cPKC not only did not block the formation of TJ complex, but also promoted the formation of tight junction.

What mechanisms could underlie the synergism of Gö6976 and diC8? Since Gö6976 is a kinase inhibitor, we proposed that it may block a kinase-regulated pathway which promotes the disassembly of tight junction. Alternatively, it may block a pathway which delays the process of tight junction assembly induced by treatment with diacylglycerols. These possibilities can be evaluated at least in part by studying of the effects of Gö6976 (without diC8) on the cellular distribution of tight junction proteins in LC conditions.

Significantly, in the present study Gö6976 was found to induce the formation of tight junction in LC conditions, similar to diacylglycerols. We draw this conclusion from the following results: (i) the treatment of MDCK cells with Gö6976 triggered the translocation of all four tested TJ proteins (ZO-1, ZO-2, claudin-1 and occludin) to intercellular contacts (Figs. 3.12, A and 3.13, A), (ii) occludin and ZO-1 showed complete co-localisation at intercellular contacts, indicating the formation of tight junction complexes (Fig. 3.13, B) and (iii) treatment with Gö6976 resulted in the phosphorylation and decreased solubility of occludin in TX-100-containing buffer (Fig. 3.12, B) which is characteristic of maturing tight junctions (Sakakibara et al., 1997).

None of the above mentioned effects of Gö6976 indicating the assembly of tight junction in LC conditions could be mimicked by other kinase inhibitors e.g. PD98059 (inhibitor of p42 MAP kinase) or by other PKC inhibitors (GF-109203X, rottlerin). Gö6976 and GF-109203X are equally potent inhibitors of cPKC but differ strongly in their ability to inhibit other PKC subfamilies (see page 72). The inability of GF-109203X to mimic the effects of Gö6976 provides additional evidence that the stability of tight junction under LC conditions is regulated not only by cPKC but also by members of other PKC subfamilies.

Interestingly, assembly of the TJ complex triggered by Gö6976 was not paralleled by the formation of adherens junctions, since we did not observe the accumulation of E-cadherin at cell-cell contact sites after the Gö6976 treatment of cells cultivated in LC medium (Fig. 3.12 A). Previously, intercellular E-cadherin-mediated adhesion was shown to be a prerequisite for the assembly of tight junctions (see introduction). In the present study, Gö6976 concentrations as low as 5 µM failed to induce cadherin translocation to cell-cell contacts, although 150 nM was sufficient to trigger tight junction formation (Fig. 3.12 and 3.13). Hence, the Gö6976-triggered mechanism of TJ assembly is independent from cadherin-mediated cell-cell adhesion. We noted

the strong similarity between the effects of PKC inhibitor Gö6976 and PKC activators diC8 and PMA on tight junction assembly in LC conditions: all these substances result in the formation of discontinuous TJ and profound occludin phosphorylation without having significant effect on cellular distributuon of cadherins. Hence PKC agonists diC8 and PMA and cPKC inhibitor Gö6976 seem to modulate the same pathway affecting the stability of tight junction under low calcium conditions.

Since Gö6976 is capable of inducing the formation of tight junction in low calcium medium independent of diacylglycerol, its effect seems to be due to the inhibition of a pathway promoting the disassembly of tight junction. To collect more evidence on the involvement of cPKC in the disassembly of tight junction we performed the switch from NC to LC conditions in the presence and in the absence of Gö6976. Our data clearly indicate that Gö6976 at 5 µM almost completely blocks the disassembly of TJ (Fig. 3.14), implying that the enzyme activity of cPKC is absolutely required for disassembly of tight junctions. As discussed below, this finding is of major importance for the understanding of the biogenesis of tight junction.

The mechanisms regulating the assembly of the tight junction are poorly understood. Even less is known about the molecular mechanisms responsible for the disassembly of TJs. The existence of mechanisms which effectively down-regulate tight junction permeability is generally assumed since TJ is thought to be a highly dynamic structure. For example, during a normal immune response, TJs between endothelial or epithelial cells open to permit the transmigration of leukocytes from the vasculature into tissues and then rapidly reseals (Huang et al., 1988). During wound healing, changes in tissue architecture require the rapid and efficient re-modelling of intercellualr contacts. However, the mechanisms by which cells control the transient opening of intercellular junctions remain uncharacterised. The phosphorylation of some unidentified proteins has been implicated in the disassembly of tight junction, since the disassembly could be blocked by unspecific kinase inhibitor H-7 (Citi, 1992). Mitogen-activated protein kinase (p42 MAPK) disrupts TJs in Ras-transformed MDCK cells, but no evidence of the involvement of MAPK in TJ biogenesis in non-transformed cells has been found (Chen et al., 2000). More recently, GF-109203X has been shown to block the disassembly of tight junctions, indicating the involvement of PKC (Avila-Flores et al., 2001). However, as the same substance also blocks TJ assembly (Fig. 3.5) these results did not contribute significantly to our understanding of the biogenesis of tight junction.

Although cPKC has been implicated in the down-regulation of TJ and, more widely, in the breakdown of tissue barrier function in a number of reports, the vast majority of data indicating the involvement of cPKC were collected under pathological conditions like carcinogenesis (Mullin *et al.*, 2000; Martin and Jiang, 2001) and/or under the action of various biologically active agents like phorbol esters (Rosson et al., 1997), toxins (Chen et al., 2002), cytokines (Ferro et al., 2000; Siflinger-Birnboim and Johnson, 2003) and so on. Our data indicate that the basal activity of cPKC acutely antagonises the stability of the tight junction both in resting cells cultivated in LC conditions (Figs. 3.12 and 3.13) and in cells in which cell-junctional complex is in remodelling process caused by switch from NC to LC conditions (Fig. 3.14). Together these data suggest that cPKC–regulated pathways may be a key mechanism controlling the stability of tight junction complex under physiological conditions.

Our suggestion that signalling by conventional PKCs opens intercellular junctions is supported by the observation that the binding of activated leukocytes to endothelial cells results in a rise in intracellular Ca^{2+}, one of the activators of conventional PKCs (Huang et al., 1993). Since occludin is a target for cPKC-mediated pathway, it could be involved in the coordinated opening and closing of paracellular space. Previously, it was shown that transfected occludin mutants up-regulate the transmigration of neutrophils across epithelial sheets, e.g. demonstrate dominant-negative effects. Thus, it is tempting to speculate that occludin regulates paracellular permeability in response to PKC signalling.

Our results suggest also that the involvement of cPKC in barrier dysfunction under various pathologies should be re-evaluated. This is because the protection of barrier integrity by cPKC inhibitors may be due to the inhibition of basal cPKC activity (which, as demonstrated in the present work, enhances the stability of tight junction at least under LC condition) rather than due to a blockade of a cPKC pathway which might have been activated under specific pathological conditions.

4.5. Conclusion: the assembly of TJs is regulated by the antagonism of conventional and novel protein kinase C isoforms.

The present and previous studies demonstrate that the treatment of MDCK cells in LC medium with PKC activators diC8 and PMA induce the assembly of TJs (Balda et al., 1993). However, these PKC activators did not induce the formation of continuous junctional structures which encircle the epithelial cell. Furthermore, in our experiments the increase in PMA

concentration resulted in more discontinuous staining of occludin at cell-cell contact sites. There are two explanations for these phenomena. First, PKC activities alone are not sufficient to promote the complete TJ assembly i.e. other signalling pathways must contribute to guarantee the formation of continuous tight junction. Second, given that diC8 and PMA activate both conventional and novel PKC, and that conventional PKC negatively regulates TJs, a plausible explanation is that activation of novel PKC isoforms in LC medium stimulates TJ assembly and that conventional PKCs antagonise this effect. To test the last hypothesis, we studied the distribution of ZO-1 and occludin in MDCK cells treated with PKC activator diC8 in the presence or absence of the conventional PKC inhibitor Gö6976 (Fig. 3.10). We found that inhibition of cPKC with Gö6976 increased the amount of ZO-1 and occludin translocated to the plasma membrane at regions of cell-cell contact, thus supporting the second hypothesis. The staining of ZO-1 and occludin was more continuous and intense as compared to samples treated with diC8 alone. On the other hand, inhibition of total cellular PKC activity with pan-PKC inhibitors GF109203X or MPKCI abolished TJ formation induced by diC8. As discussed above, GF109203X inhibits conventional and novel PKC isotypes. MPKCI is a cell-permeable oligopeptide homologous to pseudosubstrate motif of PKCα (Eichholtz et al., 1993). All PKC isotypes, except PKCμ, contain an autoinhibitory domain with substrate like properties, the so-called pseudosubstrate domain which supposedly keeps the enzyme in the inactive state, by interacting with the substrate binding site in the catalytic domain. Its sequence resembles a PKC phosphorylation site, but the phosphate acceptor Ser/Thr is replaced by Ala. Diacylglycerol or phorbol ester binding to the regulatory domain of PKC results in a conformational change that liberates the substrate binding domain from the bound pseudosubstrate, thereby activating the enzyme (Newton, 1995). Hence, MPKCI is a rather specific PKC inhibitor, but it does not distinguish between different PKC isotypes because of only negligible differences in substrate specificity (Nishikawa et al., 1997). The inhibitory effect of MPKCI and GF109203X on TJ assembly induced by diC8 (Fig. 3.10) proves that suppressing conventional PKC is not sufficient to trigger TJ assembly: the enzyme activity of novel PKCs is absolutely required for TJ assembly in a given experimental model. On the whole, all data of the present study are intrinsically consistent with the idea that the formation of TJs is regulated by antagonistic action of novel and conventional/PKCμ isoforms.

Both diC8 and Gö6976 failed to restore the adherens junction under low calcium conditions (Fig. 3.11). Hence, if a balance between conventional and novel PKCc is shifted

toward activation of nPKC, TJs assemble even in the absence of cadherin-mediated cell-cell adhesion. At first glance, this conclusion is in contradiction with the previously reported observation, that prevention of E-cadherin adhesion by antibodies specific to the extracellular domain of cadherin blocks TJ formation (Behrens et al., 1985; Gumbiner et al., 1988; Balda et al., 1993). However, taking into account that the inhibitory effect of anti-E-cadherin antibodies on TJ formation could be counteracted with diC8 (Balda et al., 1993), it logically follows that PKC acts downstream from cadherin signalling. Hence, the next mechanism of tight junction assembly could be proposed: at the initial stages of junctional complex formation cadherin-mediated adhesion results in the activation of novel PKC which in turn triggers occludin phosphorylation and TJ assembly. The basal activity of conventional PKC attenuates this process.

4.6. Occludin as a substrate for PKC.

The simplest explanation for the observed occludin phosphorylation induced by PMA or diC8 is that stimulated PKC directly phosphorylates occludin, although the possibility that PKC activates an intermediate kinase(s) cannot be ruled out. Therefore the ability of recombinant PKC to phosphorylate C-terminal cytoplasmic tail of occludin was investigated. The importance of this C-terminal domain of occludin for integration into the TJs (Furuse et al., 1994; Mitic et al., 1999) and for its interaction with structural TJ proteins was reported previously. Specifically, C-terminal part of occludin was shown to bind TJ scaffolding proteins ZO-1, ZO-2 and F-actin in vitro and in vivo (Furuse et al., 1994; Wittchen et al., 1999). Pull-down assay with a small peptide derived from the occludin C-terminus identified ZO-1, PKCζ, the regulatory subunit of phosphatidylinositol 3-kinase and gap junction transmembrane protein connexin 26 in precipitates (Nusrat et al., 2000a). Thus, the occludin C-terminus has the potential to co-ordinate the binding of proteins which have been suggested to modulate TJ structure and function.

Based on our results indicating that novel PKCs, presumably PKCδ, regulate TJ assembly and occludin phosphorylation in MDCK cells, we selected PKCδ as a candidate for occludin kinase. The present study confirms that the COOH-terminal domain of occludin is phosphorylated *in vitro* by PKCδ (Fig. 3.16), which is known to be a serine/threonine kinase. Consistent with this finding, the functionally active form of occludin was shown to be heavily phosphorylated on serine and threonine residues but not on tyrosine residues (Sakakibara et al.,

1997). Taken together, these results suggest that PKCδ may directly phosphorylate occludin to exert its effects on TJs.

Previous attempts to phosphorylate the C-terminal domain of occludin *in vitro* by cdc2 kinase, casein kinase1, PKA, MAPK or syk tyrosine kinase failed (Cordenonsi et al., 1999b). Casein kinase 2 (CK2) has been reported to phosphorylate the COOH-terminal domain of occludin *in vitro (Cordenonsi et al., 1999b).* However, to date there are no data to indicate the involvement of casein kinase 2 in the regulation of TJ biogenesis. Nevertheless CK2 may have regulatory role, since it was shown that the phosphorylation of gap junctional membrane protein connexin (Cx45.6) (which has transmembrane topology similar to occludin) by CK2 leads to the destabilisation and degradation of Cx45.6 (Yin et al., 2000). Taking into account that gap junction and tight junction are closely co-ordinated (Kojima et al., 1999), it could be suggested by analogy that phosphorylation of occludin by casein kinase 2 may lead to its destabilisation.

Recently, c-Src was also shown to bind occludin *in vitro* and phosphorylate it on tyrosine residues (Kale et al., 2003). However, this phosphorylation would probably lead to the disruption of TJs, rather than to their assembly, since c-Src is involved in TJ disorder induced by oxidative stress (Basuroy et al., 2003). Moreover, tyrosine phosphorylation of recombinant occludin by c-Src reduces its binding to ZO-1, ZO-2, and ZO-3 scaffold proteins, which is thought to be important for incorporation into TJs (Furuse *et al.*, 1994; Van Itallie and Anderson, 1997; Kale *et al.*, 2003). Thus, to date PKCδ is the only kinase of those known to phosphorylate occludin in vitro for which the experimental data support a role in the incorporation of occludin into the tight junctions.

4.7. Purification of occludin from rat liver.

Phosphorylation is most common and perhaps most important post-translational modification of proteins and seems to be a major mechanism by which the cell regulates the activity of proteins. Previous studies indicate that the assembly/disassembly of tight junctions is always paralleled by the changes in occludin phosphorylation level, suggesting that occludin phosphorylation may play a regulatory role in TJ biogenesis (Sakakibara et al., 1997; Cordenonsi et al., 1997; Sheth et al., 2000b). For example the maturation of TJs in mammalian epithelial cell lines correlates with the appearance of highly phosphorylated occludin forms, which are predominantly phosphorylated on Ser residues (Sakakibara et al., 1997). In contrast, in mouse trophectoderm highly phosphorylated occludin forms disappear upon junctional complex

assembly (Sheth et al., 2000b). In primary bovine retinal endothelial cells the VEGF-induced disruption of TJs is paralleled by the increase in occludin phosphorylation level (Antonetti et al., 1999). In all these studies occludin phosphorylation sites and regulatory molecular pathways were not identified. However, opposite processes associated with occludin phosphorylation imply that occludin may be phosphorylated on different residues, and, depending on its phosphorylation pattern, may regulate both the assembly and disassembly of TJs.

Next series of experiments were aimed to identify the occludin residues which undergo phosphorylation by PKCδ. Phosphorylated occludin has been analyzed by several mass-spectrometric approaches to reveal the phosphorylation sites, however no phosphorylated residues could be identified. Taking into account that the information about *in vivo* occludin phosphorylation sites may provide key insights into the mechanisms of tight junction assembly we decided to isolate occludin from natural source (e.g. rat liver) for subsequent identification of phosphoryaltion sites. We decided to isolate occludin from a liver since occludin is highly expressed in this tissue (Furuse et al., 1993). Importantly, the junctional complex between hepatocytes constitutes a very tight barrier preventing bile leakage to the blood flow (blood-biliary barrier) (De Vos and Desmet, 1978).

First, we analysed the solubility of occludin in buffers containing non-ionic detergents. Similar to what we observed in confluent MDCK cell monolaers, highly phosphorylated occludin forms from rat liver were TritonX-100 insoluble (Fig. 3.17), indicating that occludin is associated with cytoskeleton. Interestingly, in mouse trophoectoderm highly phosphorylated occludin forms (72-75 kDa) could be easily extracted with TritonX-100, and these forms disappear upon TJ assembly (Sheth et al., 2000b). The discrepancy between our data and those obtained by Sheth may imply that modulation of occludin function by phosphorylation in adult tissues differs from that in embryonic development.

Highly phosphorylated occludin forms were split from less- or non -phosphorylated on the basis of detergent extractability. Simultaneously, TritonX-100 extraction eliminated most cytosolic and some membrane proteins. The phosphorylated occludin was solubilised from TX-100-insoluble fraction with 1% SDS. This step eliminated the protease activity in the sample, resulting in higher yields of purified occludin. Phospho-occludin was further enriched by sequential precipitation of proteins with ammonium sulphate. Western blot analysis of precipitates and supernatants showed that salting out with 7% or 11% of ammonium sulphate precipitates only negligible amounts of occludin, while precipitating the majority of genomic

DNA and many proteins, as could be judged from significantly reduced viscosity of 1% SDS extract and observation of bulky pellets. Subsequent precipitation with 14% of ammonium sulphate showed that occludin is quantitatively precipitated from obtained solution (Fig. 3.19). Thus, sequential precipitation permitted us not only to concentrate occludin fraction but also to separate occludin from genomic DNA, some proteins and, importantly, to reduce the SDS content which impedes the immunoprecipitation by destroying tertiary structure of antibodies. The biochemical fractionation described above seems to be very efficient, since it allowed to concentrate phospho-occludin several dozens times without apparent loss of protein (after the solubilisation with SDS).

Occludin was purified by immunoprecipitation with polyclonal anti-occludin antibodies covalently bound to Protein A gel. Classical method of immunoprecipitation omits the antibody cross-linking. In this case, antibody co-elute with antigen during the elution step contaminating the sample and complicating the subsequent mass spectrometry analysis. Covalent linkage not only permitted us to use the same antibody matrix for several repeated immunoprecipitations strongly reducing costs, but also prevented antibody contamination of the eluted occludin. Western blot analysis confirmed that phosphorylated occludin forms were successfully bound to antibody gel and eluted under acidic conditions (Fig. 3.20). To concentrate occludin obtained from many immnunoprecipitations occludin was bound to diatomaceous earth and eluted under specific conditions (see Materials and Methods). This procedure allowed also the purification of occludin from contaminating substances interfering with subsequent electrophoretic separation. SDS-PAGE followed by protein staining with Coomassie blue revealed only one smeared band the molecular weight of which corresponded to occludin. Subsequent MALDI MS mass finger printing analysis confirmed that the isolated protein is occludin (see below).

In conclusion, we developed simple, efficient and cost-effective procedure for the isolation of occludin from rat liver. Importantly, at least claudin-1 was found to co-fractionate with occludin (data not shown), indicating that the developed procedure may be used for the isolation of other tight junction proteins for subsequent characterisation by mass-spectroscopy. The identification of post-translational modifications in tight junction proteins will undoubtedly contribute to the better understanding of processes underlying TJ assembly and maintenance.

4.8. The identification of *in vivo* occludin phosphorylation sites.

MALDI MS mass finger printing analysis confirmed the occludin identity of isolated protein (Fig. 3.21 and Table 3.1). Furthermore, MS-Fit Database search identified occludin as the only protein present in the sample, in spite of the fact that many mass peaks did not match predicted occludin peptides. These additional masses may be a result of occludin degradation, may derive from the enzyme used for proteolytic digestion of occludin or from contaminating proteins from rat liver.

Amino acid sequence of occludin covered by peptides determined by MS analysis of peptide mixtures after enzymatic digestion with Asp-N and trypsin is presented in Fig.4.1.

Fig. 4.1 Amino acid sequence of occludin from Rattus norvegicus (Rat). The occludin peptides determined by MS analysis of peptide mixtures after enzymatic digestion with Asp-N and trypsin are marked with red and blue, respectively (see also Table 3.1 and Table 3.2). The sequence covered by both Asp-N and tryptic peptides is marked in violet. Matched peptides after trypsin cleavage which were determined only by LC-ESI MS are shown in green.

```
  1 MSVRPFESPP PYRPDEFKPN HYAPSNDMYG GEMHVRPMLS QPAYSFYPED EILHFYKWTS
 61 PPGVIRILSM LVIVMCIAVF ACVASTLAWD RAYGTGIFGG SMNYPYGSGF GSYGGGFGGY
121 GYGYGYGYGG YTDPRAAKGF LLAMAAFCFI ASLVIFVTSV IRSGMSRTRR YYLIVIIVSA
181 ILGIMVFIAT IVYIMGVNPT AQASGSMYGS QIYTICSQFY TPGGTGLYVD QYLYHYCVVD
241 PQEAIAIVLG FMIIVAFALI IVFAVKTRRK MDRYDRSNIL WDKEHIYDEQ PPNVEEWVKN
301 VSAGTQDMPP PPSDYAERVD SPMAYSSNGK VNGKRSYPDS LYKSPPLVPE VAQEIPLTLS
361 VDDFRQPRYS SNDNLETPSK RTPTKGKAGK AKRTDPDHYE TDYTTGGESC DELEEDWLRE
421 YPPITSDQQR QLYKRNFDAG LQEYKSLLAE LDEVNKELSR LDRELDDYRE ESEEYMAAAD
481 EYNRLKQVKG SADYKSKKNY CKQLKSKLSH IKRMVGDYDR RKT
```

Peptides identified by MS cover only a small portion of occludin sequence. There are several explanations for this fact. First, some peptides may become insoluble following digestion, especially peptides corresponding to hydrophobic transmembrane domains. In agreement with this assumption, we never registered peaks the mass of which would correspond to any transmembrane part of occludin. All experimentally determined peptides matched the soluble cytoplasmic N- and C-terminal parts of occludin, except one peptide corresponding to second extracellular loop of occludin. Second, peptides have different ionising properties and detection limits depending on their sequence and size. Probably, the amount of the analysed protein was not sufficient to detect the peptides with poor ionising properties. In addition, strongly ionising components can suppress the ionisation of more weakly ionising peptides. For example, matrix substances used in MALDI experiment can strongly influence the detectability of sample components. Last, some of digestion fragments are too short or too long to be specifically detected by MALDI-MS.

MALDI-MS peptide fingerprinting analysis not only ascertained occludin identity of the isolated protein, but also assigned potential putative phosphorylated peptides, allowing the selection of candidate sequences for the analysis by MS/MS. The phosphorylated peptides were identified by the detection of the 80 Da increments for each phosphorylated amino acid present as compared to the calculated masses of the corresponding non-phosphorylated peptides (Yip and Hutchens, 1992; Wang et al., 1993). Three peaks from the mass-spectum obtained after Asp-N digestion corresponded to peptides carrying a potential single modification with phosphate moiety. An inspection of the sequences of each putatively phosphorylated peptide indicates that D**Y**AERV and D**Y**RE peptides contained only one residue which may be phosphorylated, Tyr315 and Tyr468 correspondingly. Although in this case there is no ambiguity concerning the site of phosphorylation, it is still necessary to verify the sequence of the peptides corresponding to these ions, since the mass corresponding to phosphorylated peptide may arise from the unrelated impurity. The peptide DFRQPR**YSS**N contains three amino acids that might be phosphorylated, hence to precisely localise the site of phosphorylation this peptide must be sequenced by MS/MS approach.

Unfortunately all three potentially phosphorylated peptides of occludin which were successfully ionised by MALDI failed to become ionised in LC–ESI-MS, significantly complicating the mapping of phosphorylation sites. This problem could be partially resolved by a parallel digestion of occludin with trypsin and subsequent MS/MS analysis of tryptic peptides carrying presumably phosphorylated residues. However, we still were not able to detect the peaks with m/z corresponding to the phosphorylated tryptic peptides NVSAGTQDMPPPPSD**Y**AER and ELDD**Y**R in parent ion scan. Thus the Find-Mod - suggested phosphorylation of Tyr315 and Tyr468 remains to be proved by alternative approaches.

In spite of a fact, that we were not able to verify the modification of Tyr315 and Tyr468, the probability that at least one of suspected residues is phosphorylated is rather high, since occludin phosphorylation on Tyr residues was reported to occur in two different TJ re-assembly models (Tsukamoto and Nigam, 1999; Chen et al., 2000). First model simulated TJ-destruction during anoxia by ATP depletion of MDCK cell monolayers. In second model, TJs were down-regulated by transformation of MDCK cells with Ras oncogene. In both models TJ recovery either by ATP-repletion or inhibition of Ras-MEK-MAPK pathway was associated with significant increase in occludin phosphorylation on Tyr residues. These studies suggest important

role for the occludin tyrosine phosphorylation in the TJ re-assembly during injury-repair processes.

Table 4.1 Comparison of amino acid sequence surrounding putative phosphorylated ^{468}Tyr (rat sequence) residue of occludins of rat, mouse, human and dog. The phosphorylated Tyr residue is in bold red.

Rattus norvegicus (Rat)	RLDRELDD**Y**REESEE
Mus musculus (Mouse)	RLDKELDD**Y**REESEE
Homo sapiens (Human)	RLDKELDD**Y**REESEE
Canis familiaris (Dog)	RLDKELDD**Y**REESEE

The inspection of occludin amino acid sequences from different species shows that proposed phosphorylated Tyr315 and Tyr468 are highly conserved among mammalian species (tables 4.1 and 4.2), suggesting a vital importance of these residues. Furthermore, amino acids surrounding putatively phosphorylated ^{468}Tyr are also highly conserved among mammalian species (Table 4.2) and the ^{468}Tyr is situated within ZO-1 binding region. Thus, it is tempting to speculate that the phosphorylation of Tyr468 may regulate occludin-ZO-1 interaction. Future examination of point mutants that mimic Tyr468 phosphorylation for their binding affinity to ZO-1 is necessary to verify this assumption.

Table 4.2 Comparison of amino acid sequence surrounding putative phosphorylated ^{315}Tyr (rat sequence) residue of occludins of rat, mouse, human and dog. The phosphorylated Tyr residue is in bold red.

Rattus norvegicus (Rat)	QDMPPPPSD**Y**AERVD
Mus musculus (Mouse)	QDMPPPPSD**Y**AERVD
Homo sapiens (Human)	QDVPSPPSD**Y**VERVD
Canis familiaris (Dog)	QDMPPPPSD**Y**VERVD

Finally, one signal corresponding to the mass of tryptic phosphorylated peptide ^{369}YSSNDNLETPSK^{380} was visible in parent ion scan. The sequence of this peptide and the site of modification were determined by nano-ESI-MS/MS. The phosphorylation site was unambiguosly assigned to Ser^{371}. The amino acid sequence surrounding the phosphorylated Ser is

```
   -5  -3  -1 0+1  +3  +5
DDFRQ P R Y S S N D N L ETP
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Remarkably, at –3 position Arg is present, which is characteristic for substrate motives of many protein kinases belonging to AGC superfamily comprised of all PKC isotypes, PKA, PKG, CaM kinase II and PKB (Songyang *et al.*, 1996; Nishikawa *et al.*, 1997; Songyang and Cantley, 1998; Obata *et al.*, 2000). However, the entire sequence does not fit precisely to the substrate

consensus motif of any of these kinases, complicating the prediction of occludin kinase with high probability.

Importantly, analysis of amino acid sequence of occludin molecules from different mammalian species revealed that nine amino acids that precede phosphorylation site and phosphorylated Ser residue itself are highly conserved among all species (Table 4.3). Furthermore, phosphorylated Ser and Arg at –3 position are conserved even in such considerably diverged sequence as chicken occludin, suggesting a functional importance of the phosphorylation of this residue.

Table 4.3 Comparison of amino acid sequence surrounding identified phosphorylated residue of occludins from rat, mouse, human, dog and chicken. The phosphorylated Ser residue (red) and Arg at –3 position (underlined) are conserved in all species.

Rattus norvegicus (Rat)	**DDFRQPRYSS**NDNLETP
Mus musculus (Mouse)	**DDFRQPRYSS**NGNLETP
Homo sapiens (Human)	**DDFRQPRYSS**GGNFETP
Canis familiaris (Dog)	**DDFRQPRYSS**SGHLEPP
Gallus gallus (chicken)	RGDQPD**R**AL**S**ASPVHGE

The functional role of the Ser371 phosphorylation remains to be determined. Previously published works reported that antibodies recognising only highly phosphorylated chicken occludin forms stained exclusively tight junction strands, while antibodies directed against both phosphorylated and non-phosphorylated occludin forms stained also basolateral membrane and cytoplasm (Sakakibara et al., 1997). However, the phosphorylation sites of chicken occludin were not identified. It is tempting to speculate that the modification of Ser371 may regulate occludin targeting to the assembling tight junctions. Future investigations with antibodies specific to identified phosphorylated Ser371 should determine whether phosphorylation of Ser371 regulates incorporation of occludin into the tight junction.

Occludin analysed by MS was isolated from Triton-insoluble fraction, which is an indication for association with actin-based cytoskeleton. Thus, phosphorylation of Ser371 may regulate occludin attachment to the acto-myosin ring, which is known to be linked to tight junctions and regulate paracellular permeability (Turner, 2000). On the other hand, phosphorylation may regulate the association of occludin with its binding partners, e. g. ZO-1 or ZO-2 or some unknown proteins. Although Ser371 is located prior to ZO-1 binding domain of occludin (Furuse et al., 1994), phosphorylation may change occludin conformation and thus affect ZO-1 binding. Future investigations using point mutants and/or antibodies specific against phospho/Ser371 should clarify the functional significance of occludin phosphjorylation on Ser371.

Ser371 is the first *in vivo* phosphorylated residue identified in the proteins of tight junction. A past decade after the discovery of occludin in 1993 was very fruitful in terms of identification of new tight junction components (Gonzalez-Mariscal et al., 2003) providing a solid basement for the investigation of this intercellular structure. Now, we are starting to investigate the molecular mechanisms regulating the assembly and functioning of tight junction. Our long-range goals are aimed to understand the molecular basis for how tight junctions create selective and regulated barriers for the paracellular pathway and to apply this information to understand physiology of many organs (e.g. liver and kidney), alterations in disease and the potential for therapeutic interventions.