1. INTRODUCTION

1.1. Tight junctions.

1.1.1 The morphology and functions of tight junctions.

The existence of separate fluid compartments with different chemical compositions is particularly important for the development and maintenance of multicellular organisms. These compartments are delineated by various cellular sheets, which function as barriers to maintain the distinct internal environment of each compartment. For example, renal tubules, blood vessels and the peritoneal cavity are lined with epithelial, endothelial and mesothelial cellular sheets, respectively. Within these sheets, individual cells are linked together by a variety intercellular junctions (Fig. 1.1) including desmosomes (*maculae adherentes*), intermediate junctions (*zonulae adherentes*) and tight junctions (*zonulae occludentes*) (Farquhar and PALADE, 1963).

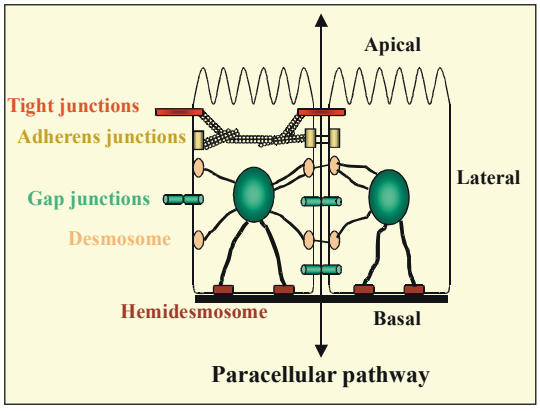

Fig. 1.1 Epithelial intercellular junctions. A schematic representation of a polarised absorptive epithelial cell. The different types of intercellular junctions, as well as hemidesmosomes, a type of cell-extracellular matrix adhesion, are shown. Tight junctions (red) and adherens junctions are linked to the actin cytoskeleton, whereas desmosomes and hemidesmosomes are linked to intermediate filaments.

Tight junctions are specialised plasma membrane microdomains highly enriched in cholesterol (Nusrat et al., 2000b) that encircle cells at the apical end of the lateral membrane. The morphology of tight junctions has been intensively analysed by freeze-fracture replica electron microscopy. The fracture plane often passes through the hydrophobic middle of lipid bilayers, thereby exposing the interior of cell membranes. On freeze-fracture replica electron micrographs, tight junctions appear as a set of continuous, anastomosing intramembranous particle strands or fibrils (tight-junction strands (Staehelin, 1973), see Fig. 1.2). In all tissues, the particles are ~10 nm across and spaced at a center-to center distance of 18 nm (Anderson, 2001). The number of tight junction strands as well as the frequency of their ramification varies notably depending on cell type, producing marked variation in the morphology of tight-junction strand networks.

On ultrathin section electron micrographs, tight junctions appear as a series of apparent fusions called kissing points, involving the outer leaflets of the plasma membranes of adjacent

cells (Farquhar and PALADE, 1963). At the kissing points, the intercellular space is completely obliterated, whereas in adherens junctions and desmosomes the apposing membranes are 15-20 nm apart (Tsukita et al., 2001). These observations led to a comprehensive understanding of the three-dimensional structure of tight junctions (Fig. 1.3). It is postulated that plasma membranes of adjacent cells are fused together by continuous tight junction strands of transmembrane particles.

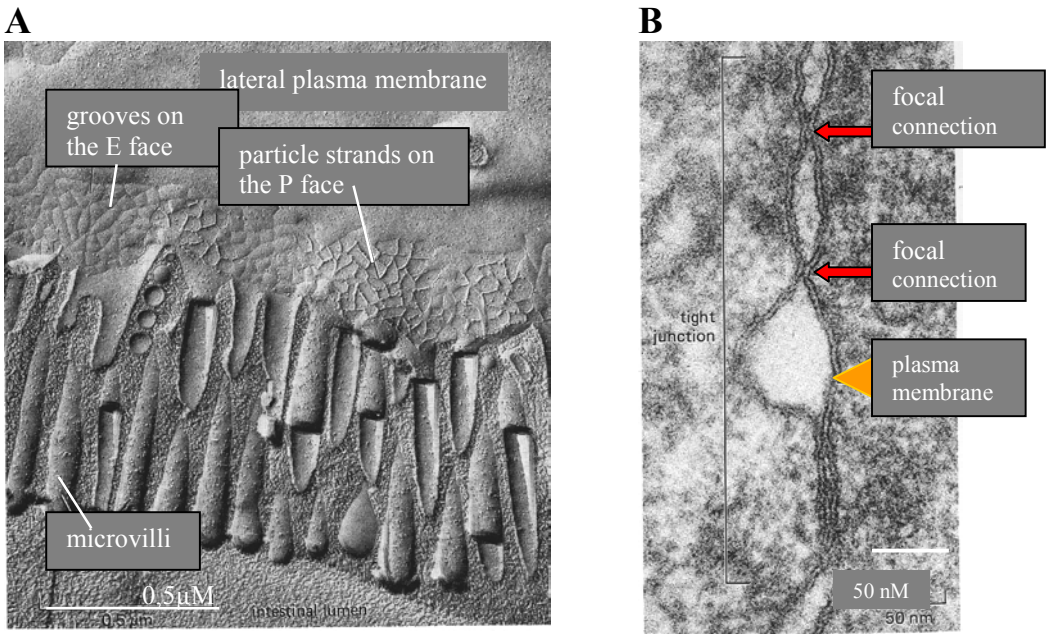

Fig. 1.2 Structure of tight junctions. A Freeze-fracture replica electron microscopic image of intestinal epithelial cells. When tight junctions are freeze-fractured, fracture planes run between the cytoplasmic and extracytoplasmic leaflets of plasma membranes, giving the P- or E-face images of TJs. The P (protoplasmic) face is the inner leaflet viewed from the outside, whereas the E (extracytoplasmic) face is the outer leaflet viewed from the inside.
B Ultrathin sectional view of tight junctions. TJs are detected as very close approximations of two neighbouring plasma membranes, which appear as focal hemifusions (kissing points).
(A and B, from Alberts et al., 1989)

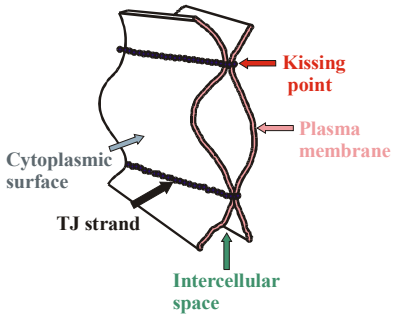

Fig. 1.3 Tight junction model. Schematic three-dimensional structure of tight junctions. Each tight-junction strand within a plasma membrane associated laterally with another tight-junction strand in the opposed membrane of an adjacent cell to form a paired tight-junction strand, obliterating the intercellular space (kissing point).

Such an architectural organisation confers two important properties to TJs. They act as (a) a selective intercellular barrier regulating diffusion of molecules and ions across the paracellular

route (gate function), and (b) a fence within a plasma membrane, impairing the intermixing of apical and basolateral membrane proteins and lipids (so called fence function) (Dragsten et al., 1981).

The life of higher organisms would be impossible without tight junctions which are crucial for the formation of blood-tissue barriers. For example, tight junctions in Sertoli cells form the basis of blood-testis barrier, which is essential for normal spermatogenesis. In hepatocytes, tight junctions seal the canalicular lumen from the sinusoidal space and serve as paracellular barrier between bile and blood. The TJs between brain capillary endothelial cells provides the basis for blood-brain barrier that maintains the homeostasis of the central nervous system and protects the brain from harmful materials circulating in the blood. Mice deficient in the major cell adhesion molecule of TJs in brain endothelia, claudin-5, died within 10 hours after birth (Nitta et al., 2003). In the brain of this claudin-5-deficient mice, no morphological abnormalities of blood vessels were found. However, the abnormal leakage of small molecule fluorescent tracer from the blood into the brain was revealed as compared to wild-type mice brain impermeable to this tracer.

In intestinal, lung and skin epithelia TJs secure the integrity of these tissues and protect the organism from the damaging substances in the external environment. The importance of TJs in barrier function of skin epithelia was demonstrated by Furuse et al (Furuse et al., 2002). These authors generated claudin-1-deficient mice, which died within one day of birth with wrinkled skin. Although the layered organisation of keratinocytes appeared to be normal, the absence of TJ protein claudin-1 resulted in the disruption of the epidermal barrier leading to dehydration of organism.

Alterations in tight junction morphology has been found to occur in many diseases including hepatitis, Celiac Spruce, Crohn's disease, and gastritis, in which the tight junction fibrils of the respective epithelia become discontinuous and exhibit poor organization (Madara and Trier, 1980; Swift *et al.*, 1983; Hollander, 1988; Posalaky *et al.*, 1989). However, it is unclear how TJ disorders contributes to observed pathological symptoms.

Tight junction barrier breakdown is often associated with brain tumours and correlates with the induction of severe brain tumour edema. Tumour cells secrete vascular endothelial growth factor (VEGF), which primarily effects tight junctions and increases endothelial permeability (Mori *et al.*, 1999; Wang *et al.*, 2001; Kempski, 2001). The disruption of TJs permits tumour cells to pass forcibly through the vascular endothelium and metastasize (Mori et

al., 1999; Martin and Jiang, 2001). In addition, the TJ is the target for several bacterial toxins that produce diarrhea by altering the barrier (Fasano et al., 1991).

A rare human hereditary disease, nonsyndromic recessive deafness DFNB29, results from a recessive mutation in CLDN14, the human gene encoding tight junction protein claudin-14. Mutations of CLDN14 were found in two large Pakistani families in which many members were profoundly deaf (Wilcox et al., 2001). Tight junctions in the cochlear duct separate two compartments with different ionic composition, the K^+- rich endolymph from Na^+-rich perilymph. The absence of claudin-14 from TJs between cells in the organ of Corti alters the ionic permeability of the paracellular barrier of the reticular lamina, which results in degeneration of cochlear hair cells, most probably due to K^+ intoxication.

1.1.2 Model systems to study junction assembly and function.

The most widely used systems to study the tight junction function employ epithelial or endothelial cell monolayers cultured on a permeable support. Two parameters are used to analyse the gate function: the transepithelial electrical resistance (TER) and the paracellular permeability. TER, which is reciprocal to ionic conductivity across cellular sheets, characterises the integrity of TJs in terms of their relative permeability to ions. The TER varies by several orders of magnitude between so-called tight and leaky epithelia. Depending on cell type, TJs show a slight cation selectivity and have a pore size in the range of 8-20 Å (Powell, 1981; Reuss *et al.*, 1991). High TER values are observed only in cells in which mature tight junctions are developed. The paracellular permeability of uncharged hydrophilic traces of different sizes gives the information about size selectivity of a particular paracellular barrier.

Analysis of tight junctions generally starts with investigation of junction assembly with morphological methods. Localisation of TJ proteins is determined by indirect immunofluorescence and, generally, involves confocal microscopy. To study the process of TJ assembly a calcium switch model which is thought to mimic processes occurring during the morphogenesis of epithelial tissues (compaction) is applied (Rodriguez-Boulan and Nelson, 1989; Nigam and Brenner, 1992). This model has been established on MDCK cell line and based on the observation that extracellular Ca^{2+} is essential for maintenance of TJ structure. When cultured in low calcium media (LC media) MDCK cells lack TJs. Upon “switching” to normal calcium media (NC media), tight junctions assemble and seal within several hours, providing an opportunity to study this process in detail (Gonzalez-Mariscal et al., 1985). Alternatively, *de*

novo TJ formation can be studied during early embryonic development. In embryos, the outer cells of the compacted morula generate the trophectoderm, which represents a true epithelial cell layer, with complete junctional complex (Ohsugi et al., 1997). In this model the trophectoderm compaction and differentiation into polarised epithelia is paralleled to the adherence- and tight-junctions formation. In both, cell-culture and embryonic, models the cellular distribution of TJ proteins is controlled by indirect immunofluorescence.

1.1.3 Molecular components of the tight junction.

The complexity of TJ organisation and function is reflected by the growing number of molecular components and binding partners (Fig 1.4). TJ molecules can be divided for simplicity to several groups: transmembrane proteins, adaptors, regulatory proteins and transcription factors.

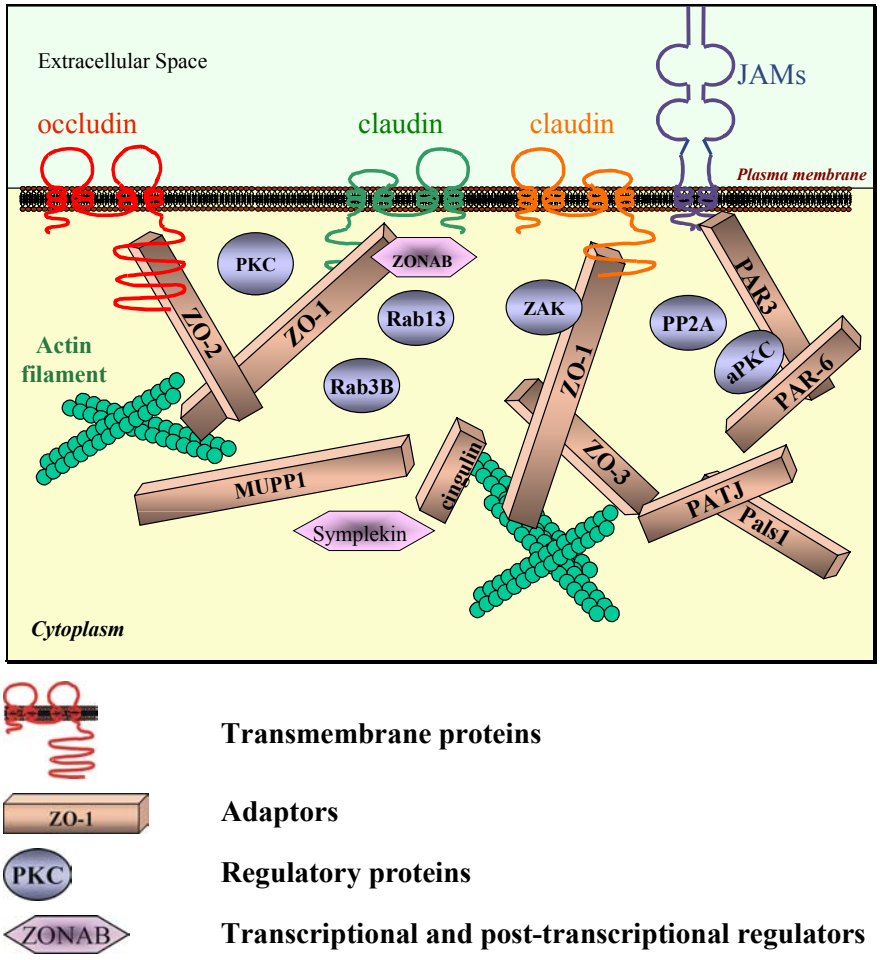

Fig. 1.4 The composition of the tight junction. The molecular composition of epithelial tight junctions is outlined. For simplicity, the junctional components have been grouped into transmembrane proteins, adaptors, regulatory proteins, and transcriptional regulators. Although examples are given for each group, the list provided is not complete and only proteins that are best studied are shown.
aPKC, atypical protein kinase C; JAMs, junctional adhesion molecules; MUPP1, multi-PDZ domain protein 1; Pals1, Protein associated with Lin-7 1 PAR, Partitioning defective; PATJ, Pals1-associated tight junction protein; PKC, protein kinase C; PP2A, protein phosphatase 2A; ZO, zonula occludens; ZONAB, ZO-1-associated nucleic-acid binding

Transmembrane proteins identified so far in tight junctions include occludin (Furuse et al., 1993), the claudin family (Furuse et al., 1998a; Morita et al., 1999) and junctional adhesion

molecule (JAM) (Martin-Padura et al., 1998). The integral membrane proteins occludin and the claudins constitute tight junction strands, which behave as regulated "channels" for the paracellular passage of ions and small molecules (described later). JAM is involved in the establishing of cell polarity in epithelial cells (Ebnet et al., 2001).

All transmembrane TJ proteins directly interact with the peripheral core TJ proteins that function as adaptors. Adaptors contain several protein–binding domains. These structural features permit them to scaffold many TJ proteins and link them to signalling molecules and the cytoskeleton. Among the TJ adaptors, zonula occludens-1 (ZO-1) (M_r 220 kD) (Stevenson et al., 1986), ZO-2 (M_r 160 kD) (Gumbiner et al., 1991), and p130/ZO-3 (M_r 130 kD) (Balda *et al.*, 1991; Haskins *et al.*, 1998) attract particular attention. These three proteins belong to the membrane-associated guanylate kinase (MAGuK) family of proteins, since they contain the three characteristic PDZ domains, followed by one SH3 and one GuK domain. Other MAGuK family members have been shown to be involved in clustering receptors or channels in the plasma membrane (Kim et al., 1995). Thus, it is tempting to speculate that the ZO-proteins act as molecular scaffolds bringing together many different proteins of TJs. Indeed, ZO-1 binds claudins, occludin, ZO-2, ZO-3, cingulin, and actin (Furuse *et al.*, 1994; Fanning *et al.*, 1998; Wittchen *et al.*, 1999; Cordenonsi *et al.*, 1999a; Fanning *et al.*, 2002) and so far may organise the transmembrane proteins and couple them to cytoplasmic proteins and/or actin microfilaments.

Another TJ adaptor protein PAR3 is important for the establishment of epithelial polarity. PAR3 contains three PDZ domains and a region that binds to the catalytic domain of atypical PKCs (Izumi et al., 1998). Each PDZ domain interacts with a variety of binding partners including JAM, which recruits the complex to TJ (Itoh et al., 2001) and PAR6, a protein that binds the small GTPase Cdc42 or Rac (Lin et al., 2000). Thus, the PAR3-PAR6 interactions may transfer signals from Rho family GTPases and atypical PKCs to TJs.

Recently discovered TJ-associated protein PATJ (Pals1 associated tight junction protein) (Kamberov *et al.*, 2000; Roh *et al.*, 2003) and MUPP1 (multi-PDZ domain protein 1) (Hamazaki et al., 2002) could also serve as adaptors, but their interacting partners are not yet well studied.

There is increasing evidence that TJ-associated MAGUK proteins may regulate gene expression and cell proliferaion. The analysis of ZO-1 amino acid sequence revealed that it is homologous to the *Drosophila* Disc-large tumour suppressor, which negatively regulates cell growth (Willott et al., 1993; Tsukita et al., 1993). Therefore, ZO-1 is a potential cell growth regulator in mammals. Involvement of TJ components in nuclear processes has also been

proposed on the basis of reports indicating that ZO-1 and ZO-2 can localise to the nucleus. The nuclear localisation of the MAGUK proteins ZO-1 and ZO-2 might be dependent upon the proliferation state of the cells, as well as on other unknown parameters (Gottardi *et al.*, 1996; Balda and Matter, 2000; Reichert *et al.*, 2000; Glaunsinger *et al.*, 2001; Islas *et al.*, 2002; Traweger *et al.*, 2003). Furthermore, the screening of MDCK expression library with a GST fusion protein containing the third PDZ and SH3 domains of ZO-1 identified Y-box transcription factor, ZONAB (ZO-1 associated nucleic acid-binding protein). In MDCK cells ZONAB and ZO-1 functionally interact to modulate the expression of the erbB-2 proto-oncogene, indicating a direct linkage between TJs and the regulation of gene expression (Gottardi *et al.*, 1996; Balda and Matter, 2000). Another TJ protein that can localise to the nucleus is symplekin (Keon et al., 1996), a protein that has been linked to a messenger RNA processing (Takagaki and Manley, 2000; Hofmann et al., 2002).

1.1.4 Aqueous pores within TJ strands.

As discussed above, the TJ ionic selectivity could be characterised by measuring the TER across an epithelial monolayer. By comparing the TER and the morphology of tight-junction strands in various epithelia, Claude et al. found that as tight-junction strands increase in number, the TER value increases not linearly but logarithmically (Claude, 1978). To explain this relationship, the existence of aqueos pores within the branched tight junction strands, taking both open and closed states, was postulated (Claude, 1978; Tsukita and Furuse, 2000). Provocative but currently incomplete evidence supports speculation that members of claudin family of proteins create TJ pores and that individual charged residues in extracellular loops of claudins determine the ion selectivity of these aqueous pores (Stevenson *et al.*, 1988b; Simon *et al.*, 1999; Anderson, 2001; Furuse *et al.*, 2001).

The most direct evidence that claudins constitute ion-selective pores came from the studies of TER in two strains of Madin-Darby canine kidney (MDCK) epithelial cells – MDCK I and MDCK II. Stevenson et al. reported that MDCK I cells have a 30-60-fold higher TER than MDCK II cells, but the number of tight junction strands in these strains is very similar (Stevenson et al., 1988b). This difference in tightness could be explained by the heterogeneity of aqueous pores in terms of their probability of being open or closed. Furuse and colleagues found that each strain of MDCK cells has an individual expression pattern of claudins, where MDCK I express only claudin-1 and claudin-4 and MDCK II cells also express large amounts of claudin-2

in addition to claudins-1 and –4 (Furuse et al., 2001). When claudin-2 was introduced into MDCK I cells, the TER value of these MDCK I transfectants fell to the level of MDCK II cells without any changes in the number of tight junction strands. Therefore, it is likely that claudin-2 constitutes aqueous pores with high conductance within tight junction strands of MDCK II cells.

Recent studies on renal hypomagnesemia provided further evidence that claudins are responsible for creating aqueous pores and determine variable properties of paracellular transport. Simon and co-workers discovered that mutations in claudin-16/ paracellin-1 provide the basis for this hereditary disease (Simon et al., 1999). Normally, most Mg^{2+} is resorbed from the urine through the paracellular pathway in the thick ascending limb of Henle; coincidentally, this is the principal site where claudin-16 is expressed. In patients with heredity hypomagnesemia, the reabsorbtion of Mg^{2+} is reduced and it is lost in the urine. The disease phenotype can be rationalised by proposing that claudin-16 creates a cation-selective pore through the tight junction. This hypothesis is supported by the acidity of the claudin-11 extracellular loops and the correlation found by Collegio and co-authors between charge selectivity and changed amino acid composition (Colegio et al., 2003).

1.2. Occludin.

1.2.1 Structure and function of occludin.

Occludin (~60 kDa) is the first cloned transmembrane protein of tight junctions (Furuse et al., 1993). The name of this protein derives from the Latin word “occludere” which means to occlude. It is widely expressed by epithelial and endothelial tissues and its presence has also been reported in neurons and astrocytes (Furuse et al., 1993; Hirase et al., 1997; Bauer et al., 1999). Morphological studies prove that occludin is a constituent of tight junction strands. In immuno-replica electron microscopy, antibodies against occludin exclusively labelled tight-junction fibrils, indicating that occludin is directly incorporated into tight-junction strands (Saitou et al., 1997). In addition, when overexpressed in mouse L fibroblasts, which do not have TJs, occludin concentrates in a punctate manner at the cell-cell borders, resulting in the formation of very short TJ strand-like strands (Furuse et al., 1998b).

The analysis of amino acid sequence of occludin suggests that it contains four transmembrane domains, two extracellular loops and three cytoplasmic domains (one intracellular short turn, a short amino-terminal domain and a long carboxy-terminal domain) (Furuse et al., 1993). Consistent with this topological prediction, a monoclonal antibody against the C-terminal tail labelled only the cytoplasmic side of junctions in thin section electron

microscopic images, whereas an antibody raised against the first extracellular loop labeled living nonpermeabilised cultured epithelial cells (Furuse et al., 1993; Van Itallie and Anderson, 1997). No occludin-related genes have been identified yet, but two isoforms of occludin, with slightly different N-termini are generated by alternative splicing (Muresan et al., 2000).

Amino acid sequences of the human, murine and canine occludin show high homology (90% identity), whereas they diverge considerably from those of chicken and rat-kangaroo (~50% identity) (Ando-Akatsuka et al., 1996). Structural and functional data suggest that occludin carboxy-terminal cytoplasmic tail (corresponding to amino acids 266-522 of human occludin) has at least two sub-domains. A distal sub-domain encompassing the C-terminal 150 amino acids is highly charged, relatively conserved across species, and binds directly to ZO-1, ZO-2, and ZO-3 (Furuse *et al.*, 1994; Haskins *et al.*, 1998; Itoh *et al.*, 1999). Two studies provided evidence that this sub-domain contains the information for occludin targeting to the TJ. Furuse *et al*. reported that occludin constructs lacking the distal COOH-terminal sub-domain do not localise to the tight junction when transfected into a bovine kidney cell line (Furuse et al., 1994). Furthermore, when COOH-terminal 150-amino acid portion of occludin was fused with the transmembrane NH_2 terminus of rat connexin 32 (cx32), the resulting chimeras were localised to the tight junction, even though the NH_2 terminal half of cx32 possesses gap junction targeting information (Mitic et al., 1999).

The second occludin sub-domain that localised to the carboxy-terminal half encompasses about 100 amino acids proximal to the cell plasma membrane. This sub-domain is not known to interact with other proteins, is less charged and less conserved across species as compared to the distal sub-domain (Ando-Akatsuka et al., 1996).

The extracellular loops of occludin attract particular attention because of their unusual composition. Specifically, the first loop lacks charged amino acid residues and has a high percentage of tyrosine and glycine (25% and 36% respectively in chicken occludin) while the second loop contains only a few charged side chains. This has led to the speculation that the extracellular loops of two occludin molecules on opposite plasma membranes may adhere to each other in the ion-rich paracellular environment simply through energetically favorable hydrophobic interactions (Ando-Akatsuka et al., 1996). Supporting this hypothesis, Van Itallie and Anderson found, that occludin expression induced cell-cell adhesion in NRK and Rat-1 fibroblasts, which originally do not have tight junction and show poor cell-cell adhesion properties(Van Itallie and Anderson, 1997). Occludin-induced adhesion was abolished by adding

peptides corresponding to the occludin's first extracellular loop. Lacaz-Vieira showed that resealing of opened tight junctions (TJs) in A6 *Xenopus* kidney epithelial cell monolayers is impaired when peptides homologous to segments of the first external loop of chick occludin where added to cells (Lacaz-Vieira et al., 1999). These results suggest that the first exracellular loop of occludin can mediate intercellular adhesion and sealing through homophilic interactions.

Interestingly, brain capillary endothelial cells that form the highly selective blood-brain barrier express high levels of occludin, whereas endothelial cells in non-neural tissues, with poor barrier properties, express lower levels of occludin (Hirase et al., 1997). The evidence that occludin is a functional component of the transepithelial barrier came from several studies analysing the effect of occludin overexpression on epithelial barrier properties. The occludin overexpression in MDCK epithelial cells induced a modest increase in TER and a 15% increase in the number of TJ strands (McCarthy et al., 1996). Other studies in MDCK cells using tailless occludin also showed an increased TER indicating that the C-terminal tail is not necessary to induce an electrically tighter seal (Balda et al., 1996). Thus, occludin contributes to the function of the paracellular barrier through the extracellular loops.

One report suggests that occludin may organise actin filaments linked to TJs. Thus, the transfection of occludin into rat endothelial cell line, which originally expresses only a trace amounts of occludin, resulted in actin rearrangements; specifically, circumferential fine actin bundles developed in close relation to the sites of occludin-positive cell-cell contacts (Atsumi et al., 1999). Additionally, in vitro studies showed that occludin may directly interact with F-actin (Wittchen et al., 1999). The attachment of the actin cytoskeleton to occludin-positive cell-cell contacts could be also mediated by ZO-1, which associates directly with actin filaments and occludin (Fanning et al., 1998).

Huber et al showed that the expression of occludin with modified N-terminal cytosolic domain up-regulated transepithelial migration of neutrophils, suggesting that occludin may regulate opening and closing of epithelial intercellular junctions (Huber et al., 2000).

Taken together, these studies provide an evidence that occludin is important TJ constituent. However, tight junction strands can also be formed without occludin, in some cell types like endothelial cells in non–neuronal tissue and in human/guinea pig Sertoli cells indicating that occludin is not a main structural component of TJ strands (Hirase et al., 1997; Moroi et al., 1998). This assumption has been proven directly, because occludin knockout

embryonic stem cells still make claudin-containing tight junction fibrils and form intercellular diffusion barriers (Saitou et al., 1998).

At present, the physiological function of occludin is still a mystery. Studies of occludin-deficient mice did not give the exact answer. These mice were born seemingly normal, but as they grew up, they began to show complex phenotypes, including marked growth retardation, chronic inflammation and hyperplasia of the gastric epithelium, and mineral depositions in the brain (Saitou et al., 2000). No histological abnormalities were found in the testis as well as the ovary, but their sexual behaviour was affected. Occludin -/- males had normal spermatogenesis but produced no litters when mated with wild-type females, and occludin -/- females produced litters normally when mated with wild-type males but did not suckle them. Tight junctions in most organs of occludin-deficient mice that have been studied so far, such as intestinal epithelial cells, seem normal in terms of their morphology and TER. Thus, the relatively normal phenotype of occludin-deficient mice and embryonic stem cells disputes the results, obtained through cell culture models that demonstrated the importance of occludin in creating the paracelluar barrier. It is possible that in the organism, occludin is important for other TJ functions, including the regulation of paracellular flux of non-charged solutes or even the regulation of transcription, since the forced expression of occludin was recently shown to suppress the v-raf-induced transformation of cultured epithelial cells (Li and Mrsny, 2000).

1.2.2 Heavily serine/threonine phosphorylated occludin is a component of the TJ.

On SDS-PAGE occludin migrates as a tight cluster of multiple bands ranging between 62 and 82 kDa (Furuse et al., 1993; Sakakibara et al., 1997). This multiple banding of occludin was found in all species examined so far and is a result of multiple phosphorylation (Sakakibara et al., 1997; Wong, 1997).

Since detergent insolubility is commonly used as an indicator of protein incorporation into large complexes such as the cytoskeleton and intercellular junctions, the solubility of the multiple forms of occludin in the nonionic detergents was determined. Detergent fractionation with the use of TX-100 (Wong, 1997) and NP-40 (Sakakibara et al., 1997) showed that in MDCK cells the low molecular forms of occludin were mostly detergent-soluble, whereas high molecular forms of occludin were detergent-insoluble. Phosphoamino acid analysis showed that in NP-40-soluble occludin, both serine and threonine residues were phosphorylated, whereas in higher Mr bands of NP-40-insoluble occludin, serine residues were predominantly

phosphorylated with slight threonine phosphorylation (Sakakibara et al., 1997). The exact phosphorylation sites were not identified. Phospho-tyrosine was undetectable in both occludin fractions. Considering that TJ strands are resistant to detergent extraction (Stevenson and Goodenough, 1984; Stevenson *et al.*, 1988a; Stevenson *et al.*, 1988b) these findings suggest that highly phosphorylated occludin is predominantly incorporated into tight junction. Indeed, a monoclonal antibody recognising only phosphorylated occludin stained only tight junction of intestinal epithelial cells, whereas other antibodies predominantly recognising low Mr bands stained also the basolateral membranes and the cytoplasm (Sakakibara et al., 1997).

The relationship between occludin phosphorylation and tight junction formation has been further investigated using the Ca^{2+}-switch model of disassembly and reassembly of tight junctions (Gonzalez-Mariscal et al., 1990). In the LC medium, the highly phosphorylated occludin forms were barely detectable in MDCK cells. Almost all occludin was distributed in cytoplasm and could be solubilised with a non-ionic detergent. Tight junction assembly induced by the addition of calcium was paralleled by occludin phosphorylation and accumulation at the cell borders (Sakakibara et al., 1997). The correlation between the extent of occludin phosphorylation and the maturation status of tight junction suggests that occludin phosphorylation may be a molecular mechanism accounting for incorporation of occludin into tight junctions and regulation of tight junction biogenesis.

These conclusions are contrary to results obtained on *Xenopus laevis* embryos. Non-ionic detergents, regardless of the presence of SDS, solubilise essentially all occludin from Xenopus embryos, and TJ maturation is associated with a downshift in the electrophoretic mobility of occludin reflecting the occludin dephosphorylation (Cordenonsi *et al.*, 1997). Thus, the contribution of occludin phosphorylation in TJ maturation of developing *Xenopus laevis* is likely to be minor. Consistently, the occludin assembly at the nascent TJs within mouse trophectoderm is correlated with the downshift in the migration of occludin from approximately 72-75 to 65-67 kDa, which can be mimicked by treatment of occludin-containing extracts with phosphatase (Sheth et al., 2000b). However, the assembled occludin in mouse trophectoderm is rapidly converted from a detergent-soluble to an insoluble form.

Despite the discrepancy between the data obtained on embryos and those obtained on MDCK cells TJ assembly in all cases correlates with changes in the occludin phosphorylation pattern. These observations and the fact that occludin is the only TJ protein for which the link between phosphorylation and sorting to the TJ is existing, drive the investigation of molecular

mechanisms of occludin phosphorylation. To fully understand the role of phosphorylation in occludin function, occludin phosphorylation sites and kinases must be identified.

1.3. Tight junction assembly.

1.3.1 Assembly of the TJ: the role of cadherin mediated cell-cell adhesion.

In simple epithelia, TJ and adherence junctions (AJ) are located close to each other on the lateral membrane and are associated with the actin cytoskeleton. In TJs, ZO-1 acts as the linker between TJ transmembrane proteins and the cytoskeleton (Fanning et al., 1998). In AJs, the main adhesion receptor E-cadherin is associated at its cytoplasmic domain with catenins: β-catenin, and α-catenin (Gumbiner and McCrea, 1993; Rimm et al., 1995), which provide attachment to actin (Rimm et al., 1995; Aberle et al., 1996). These structural arrangements provide the molecular interaction between AJ and TJ through the underlying actin fibers or other components. Moreover, some studies suggested that intercellular Ca^{2+}-dependent binding of the E-cadherin molecules is absolutely required for TJ assembly. The assembly of TJs in mouse embryo can be inhibited by blocking the formation of cell-cell adhesion with antibodies to the extracellular domain of E-cadherin (Fleming et al., 1989). Similarly, incubation of MDCK cells with anti-cadherin- antibodies prevents the TJ assembly induced by Ca^{2+}-switch (Gumbiner and Simons, 1986; Gumbiner et al., 1988). The hypothesis that the cadherin-mediated adhesion is a prerequisite for TJ formation is consistent with the observation that during the initial stage of the formation of the intercellular junctional complex TJ- and AJ- proteins are co-localised. For example, in MTD-1A epithelial cells grown in culture, primordial spot-like junctions are formed at the tips of thin cellular protrusions radiating from adjacent cells, where E-cadherin and ZO-1 are precisely colocalised (Yonemura et al., 1995). At this early stage, occludin is not detected at the cell-cell contact sites. But, as cellular polarisation proceeds, occludin gradually accumulates at the ZO-1-positive punctate junctions to form the mature TJ, and in a complementary manner E-cadherin is sorted out from the ZO-1-positive spot-like junctions to form the mature AJ (Ando-Akatsuka et al., 1999).

These processes are very similar to the dynamics of tight junction formation during mouse embryo trophectoderm differentiation. Its first phase is characterised by the assembly ZO-1α$^-$ splicing isoform at the apicolateral region of membrane contact between blastomers. The assembly process first occurs at punctate foci where ZO-1α$^-$ co-localises with adherence junction proteins α- and β-catenins (Fleming et al., 1989; Sheth et al., 2000a). 12 hours after ZO-1α$^-$ delivery plaque TJ protein cingulin assembles and co-localises with ZO-1 (Fleming et al., 1993).

During the final phase ZO-1α^+ splicing isoform is transcribed *de novo* (Sheth et al., 1997). ZO-1α^+ isoform associates with occludin during the period of Golgi localisation; then the two proteins assemble together at the tight junctions (Sheth et al., 1997). Following occludin assembly, all TJ proteins are precisely co-localised and placed apically from proteins of the E-cadherin/catenin complex. Moreover, thin-section electron microscopy analysis prior to occludin assembly shows a single apicolateral junctional complex; but after occludin assembly, this structure splits into apical tight junctions and subjacent zonula adherens (Sheth et al., 2000a).

Several studies were aimed to elucidate the molecular mechanism of the regulation of TJ assembly by AJs. These studies suggest at least two distinct, but not exclusive, mechanisms. Both are based on the observation that cells in which the α-catenin gene is deleted cannot form TJs (Watabe et al., 1994; Watabe-Uchida et al., 1998). First, cadherin-catenin complex may provide an anchor for TJ adaptor proteins, like ZO-1. ZO-1 then recruits other TJ proteins to the sites of primordial junctions. Alternately, the cadherin-catenin complex may control actin cytoskeleton rearrangement, an essential part of TJ organisation and stability.

In support of the first model, it was shown that TJ scaffold protein ZO-1 interacts directly with α-catenin (Itoh et al., 1997; Imamura et al., 1999). It is therefore possible that the recruitment of ZO-1 to cadherin-positive primordial junctions is mediated through α-catenin. This suggestion is supported by the observation that in non-epithelial cells lacking TJs, ZO-1 localises to cadherin–based AJs and directly binds α-catenin (Itoh et al., 1993; Itoh et al., 1997) 1997). Thus, the cadherin-catenin complex may recruit ZO-1 to the sites of primordial cell-cell contacts. The question of whether ZO-1 possesses an instructive signal to recruit and tether other TJ proteins remains unanswered. One study reported that the delivery of occludin to the TJ membrane site is independent from both ZO-1 assembly and cadherin-cadherin adhesion. In mouse embryos, lacking the E-cadherin, ZO-1 failed to localise at cell-cell contact sites but occludin membrane localisation was unaltered (Ohsugi et al., 1997). It seems therefore that although the recruitment of occludin into the TJ occurs in association with the ZO-1α^+ isoform, the mechanism for occludin targeting to cell-cell contact sites differs from that of ZO-1.

In support of the second model, other data suggest that cadherin-cadherin adhesion controls actin cytoskeleton rearrangement. In mouse E-cadherin-null embryos actin filaments are abnormally prominent in regions of cell-cell contacts, suggesting the disturbance in microfilament arrangement (Ohsugi et al., 1997). In MDCK cells, Ca^{2+}-dependent cadherin adhesion results in the changes of Rac1 and RhoA activity which are known to regulate actin

dynamics (Noren et al., 2001), and the E-cadherin cytoplasmic domain is directly involved in these signalling effects, because its deletion abolishes the changes in activities of both GTPases. Besides the induction of signalling pathways regulating actin cytoskeleton organisation, cadherin-catenins complex may directly influence cytoskeletal organisation since adherence junctions are tightly bound to the circumferential filament band. As mentioned above, α-catenin links cadherins to the F-actin (Rimm et al., 1995). α-catenin also binds two other F-actin-binding proteins, vinculin and α-actinin (Nieset et al., 1997; Weiss et al., 1998) and the deletion of vinculin binding domain of α-catenin is sufficient to impair the targeting of occludin and ZO-1 to cell-cell contact sites (Watabe-Uchida et al., 1998). Furthermore, in α-catenin null cells, the expression of a chimeric protein consisting of NH2-terminal part of α-catenin fused with vinculin tail was sufficient to induce ZO-1 assembly at the cell-cell contact sites while amino-terminus of α-catenin alone was unable to cause ZO-1 redistribution (Watabe-Uchida et al., 1998). These results provide evidence that an α-catenin-vinculin interaction may regulate TJ assembly, presumably through actin-cytoskeletal rearrangement.

1.3.2 Assembly of the TJ: the role of protein kinase C.

Protein kinase C (PKC) is an early recognised potential regulator of TJ assembly. It was noted that TJ formation induced by addition of Ca^{2+} can be potentiated by the PKC agonist 1,2-dioctanoylglycerol (Balda et al., 1991). Furthermore, stimulating MDCK cells in low Ca^{2+} medium with diC8 mimicked, to a certain extent, the switch to NC medium, as evidenced by translocation of the ZO-1 to the plasma membrane, formation of junctional fibrils, decreased permeability of the intercellular space to mannitol, and reorganisation of actin filaments to the cell periphery (Balda et al., 1993). The diC8 target, presumably PKC, seems to be down-stream of E-cadherin in the signalling pathway for the TJ assembly, since diC8 treatment in LC does not induce E-cadherin redistribution and counteracts the effect of anti-E-cadherin antibodies (Balda et al., 1993). These data indicate that protein kinase C is required for the assembly of tight junctions; however, a positive correlation has not been obtained between the kinetics of tight junction assembly and the phosphorylation of tight junction proteins such as ZO-1, ZO–2, ZO-3 and cingulin (Balda et al., 1993; Citi and Denisenko, 1995).

The importance of PKC-dependent protein phosphorylation in TJ assembly was postulated because protein kinase inhibitors H7 and calphostin C inhibiting (among other kinases) PKCs, block junction assembly induced by Ca^{2+} switch (Citi and Denisenko, 1995;

Stuart and Nigam, 1995b). H7 and calphostin C, profoundly inhibited the increase in transepithelial electrical resistance (TER), a functional measure of tight junction development. This effect was paralleled by a delay in the sorting of the tight-junction protein ZO-1 to the tight junctions, while the assembly of desmosomes and adherens junction were not detectably affected (Stuart and Nigam, 1995b).

On the other hand, PKC has been implicated in TJ dysfunction in a number of studies. For example, the exposure of MDCK (Ojakian, 1981) and LLC-PK1 cells (Clarke et al., 2000a) at normal physiological conditions to phorbol esters, that mimic the action of DAG via PKC caused a decreased TER accross established monolayers. TPA treatment at supra-maximal concentrations (10^{-6} – 10^{-7} M) reduced the phosphorylation levels of occludin (Farshori and Kachar, 1999; Clarke *et al.*, 2000a). Furthermore, the disruption of cell-cell contacts caused by calcium removal can be prevented by the PKC inhibitor H-7 (Citi, 1992).

Apart from insights into PKC regulation of tight junction assembly and disassembly that have been obtained in pharmacological studies, the role of individual PKC isozymes was investigated through the overexpression of wild-type and dominant interfering PKC mutants in LLC-PK1 epithelia. The overexpression of the novel δ-isoform of protein kinase C was by itself sufficient to reduce transepithelial electrical resistance and to increase paracellular permeability of cell monolayers, resembling the effects of TPA on LLC-PK1 cells (Mullin et al., 1998), while the overexpression of classical PKCα isoform affected transepithelial permeability only in the presence of phorbol esters: the overexpression of wild-type PKCα rendered the LLC-PK1 cells more sensitive to the effects of 12-O-tetradecanoylphorbol–13 acetate (TPA) on barrier function of these cells. Conversely, expression of an inactive PKC-α rendered the cells more resistant to the effects of TPA (Rosson et al., 1997). These results indicate that α and δ PKC isoforms may regulate the disruption of tight junctions in LLC-PK1 cells caused by TPA, but molecular Scale bar, 20 μm mechanisms of this regulation remain unclear.

The participation of PKC both in the establishment of TJ structures and in their decomposition indicates the existence of complex regulatory pathways which are not yet understood. Some of this complexity arises from different signalling pathways operating in various cell types, but other factors like the existence of non-PKC DAG receptors also may be involved. To date, all pharmacological investigations of the PKC regulation of TJ function involved nonspecific PKC activators (DAG, phorbol esters) combined with the use of unspecific PKC inhibitors (calphostin C, H7) (Brose and Rosenmund, 2002). Because mammalian cells

have several types DAG receptors besides PKCs (see 1.4.3.), the results of these studies have to be interpreted with caution. With the emergence of more sensitive isozyme-specific inhibitors, the role of PKC in tight junction assembly needs to be re-evaluated.

On the other hand, the apparent paradox of opposing effects of the same agents might be explained by the fact that the PKC family is composed of related but structurally distinct isoenzymes (each a product of separate genes) with discrete cofactor requirements, substrate specificity, and tissue distribution. Since phorbol esters activate multiple isoenzymes of PKC (Newton, 1995; Brose and Rosenmund, 2002), the possibility is raised that each PKC isoenzyme may selectively mediate distinct, and often opposing, effects within one stimulated epithelial cell.

To further understand the relationship between PKC activity, tight junction assembly, and disassembly, it is important to address the following questions: 1) which junctional proteins are critical targets for PKC phosphorylation to bring about alteration of tight junction morphology? 2) which members of the PKC family may be responsible for this alteration?

Although Madin–Darby canine kidney (MDCK) cells express classical (α, β and γ), new (δ and ε) and atypical (ζ and ι) PKC isoforms (Dodane and Kachar, 1996) only atypical PKCs have been reported to concentrate at the TJ and their role has been intensively studied. The calcium- and DAG-independent aPKCs are critically involved in the development of the epithelial junctional structures and are thought to control the cell polarity of mammalian epithelial cells, by forming a ternary complex with PAR3 and PAR6 (Izumi et al., 1998; Suzuki et al., 2001; Suzuki et al., 2002). It was demonstrated that PAR6 binds directly to GTP-bound cell-division control protein 42 (Cdc 42), a Rho-family GTPase that is essential for epithelial-cell polarity (Kroschewski et al., 1999). This binding increases the intrinsic potential of PAR6 to activate aPKC and results in the assembly of the aPKC-PAR3-PAR6 complex (Lin *et al.*, 2000; Joberty *et al.*, 2000; Ohno, 2001). In wound-healing experiments, aPKC activity was not essential for the initial assembly of junctional components at sites of new cell-cell adhesion to form a primordial junction, but was required for the segregation of the junctional complex into distinct TJs and AJs (Suzuki et al., 2002). This indicates that aPKC mediates late stages of junctional maturation. Work by Nunbhakdi-Craig and co-authors suggests that aPKC activity is negatively regulated by PP2A (protein phosphatase 2A), which directly interacts with and dephosphorylates aPKC (Nunbhakdi-Craig et al., 2002). *In vitro*, PP2A can dephosphorylate TJ proteins that have been phosphorylated by aPKC, and the overexpression of its catalytic subunit

in MDCK cells has been shown to dephosphorylate TJ proteins and to increase paracellular permeability. These data indicate that the aPKC-PAR3-PAR6 complex and PP2A might represent opposing signalling pathways that regulate TJ assembly and disassembly.

Despite insights into relationship between aPKC and TJ dynamics, the roles of classical and novel PKC on tight junction assembly remain to be poorly understood.

1.4 Protein kinase C.

1.4.1 Protein kinase C family.

Members of the protein kinase C family are single polypeptides, with two major domains, an N-terminal regulatory domain and a C-terminal catalytic domain (Fig.1.4). To date, several isozymes of PKCs have been identified in mammalian tissues. Differences in their regulatory domains have permitted division of the isoforms into three groups: (i) conventional PKC (cPKC, includes $\alpha, \beta I, \beta II$ and γ isoforms) which are Ca^{2+} dependent and activated by both phosphatidylserine and diacylglycerol, (i) novel PKC (nPKC, includes $\delta, \varepsilon, \eta$ and θ isoforms) which are activated by both phosphatidylserine and diacylglycerol but are not dependent on calcium and (iii) atypical PKC (aPKC, includes ζ, λ and PRK; for PKC-related kinases) which are insensitive to DAG and Ca^{2+} (Toker, 1998).

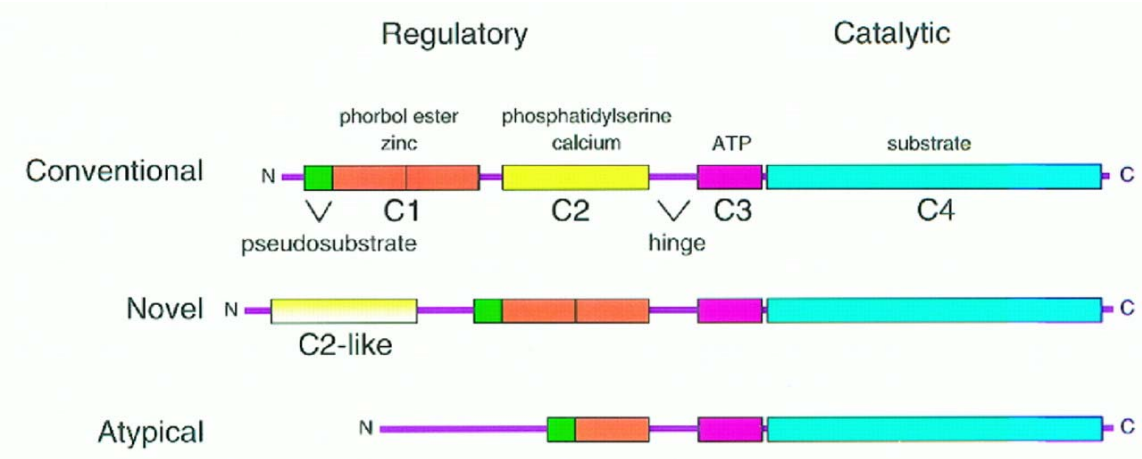

Fig. 1.4 Schematic representation of the primary structure of conventional, novel, and atypical protein kinase Cs. Indicated are the pseudosubstrate domain (green), C1 domain comprising one or two Cys-rich motifs (orange), C2 domain (yellow) in the regulatory half, and the ATP-binding lobe (C3, pink) and substrate-binding lobe (C4, teal blue) of the catalytic region. The C2 domain of novel protein kinase Cs lacks amino acids involved in binding calcium but has key conserved residues involved in maintaining the C2 fold (hence its description as ``C2-like''). Atypical protein kinase Cs have only one Cys-rich motif, and phorbol ester binding has not been detected. Reproduced from (Newton, 1995).

A recently described PKC isoenzyme, PKCμ (Johannes et al., 1994) or PKD1 (Valverde et al., 1994), does not fit into any of the major PKC subfamilies. It is phospholipid-dependent, calcium-insensitive, and activated by phorbol esters (Valverde et al., 1994; Rykx et al., 2003). Despite the similarity between the C1 domains of the PKCμ/PKD and the classical and novel PKCs, PKCμ/PKD is structurally different from any other PKC isotypes. PKCμ has a pleckstrin-homology domain, absent in all other PKC isozymes. Second, the amino acid sequence of the PKCμ catalytic domain is most similar to myosin light chain kinase of Dictyostelium (41% identity) and only 30-35 % to individual PKCs. And finally, PKCμ does not have an autoinhibitory pseudo-substrate sequence that can be found in most members of the PKC family (Valverde et al., 1994; Rykx et al., 2003).

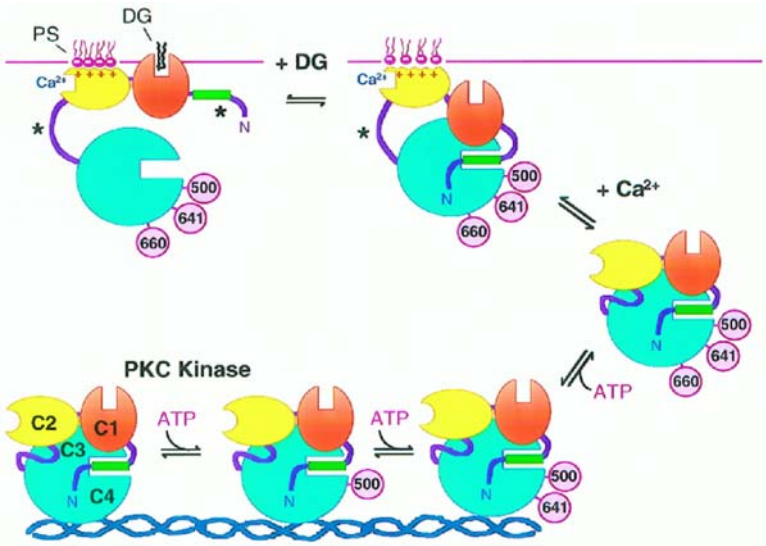

Fig. 1.5 Regulation of protein kinase C by 1) phosphorylation and 2) membrane binding and pseudosubstrate release. Unless phosphorylated, PKC associates with the detergent-insoluble fraction of cells (bottom left). cPKCs go through three phosphorylations (Thr-500, Thr-641 -PAGE and Western blot. SEITENREF _Toc74390513 \h 36 2.3.7 Silver staining of proteins n is mediated by diacylglycerol (DG) binding to the C1 domain and phosphatidylserine (PS) binding to the C2 domain. Protein kinase C can bind to membranes with low affinity with either C1 domain ligands (not shown) or with C2 domain ligands (top right). However, it is the high affinity binding (top left) mediated by both domains that results in pseudosubstrate release and maximal activation. Asterisks indicate the exposed hinge, which becomes proteolytically labile upon membrane binding (independently of pseudosubstrate release), and the exposed pseudosubstrate, which becomes proteolytically labile upon activation (independently of membrane binding) (Newton 1995).

The control of PKC activity has been studied in detail for conventional PKC isozymes. Newly synthesized PKC associates with the detergent-insoluble fraction of cells (Borner et al., 1989). It is processed to the mature, cytosolic form by three functionally distinct phosphorylations. First, transphosphorylation of threonine-500 (in PKCβII) at the activation loop renders the kinase competent to conduct autophosphorylation (Fig. 1.5). Second, autophosphorylation at the C terminus (Thr-641 in PKCβII) stabilizes the catalytically competent conformation; and a final autophosphorylation at the C terminus (Ser-660 in PKCβII) releases protein kinase C into the cytosol {Keranen, 1995 1 /id}. This triple phosphorylated mature form is inactive because the pseudosubstrate of its regulatory region occupies the substrate-binding

cavity. Membrane translocation and association leads to the release of the pseudo-substrate and causes maximal activation of the enzyme (Newton A.C. 1995). Membrane association may be mediated by DAG binding to the C1 domain or phosphatidylserine (*PS*) binding to the C2 domain. The affinity for acidic lipids is increased by Ca^{2+} bindind to C2 domain for conventional protein kinase Cs, likely by structuring the lipid-binding surface. Ca^{2+}-dependent phospholipid binding acts synergistically with DAG binding. This synergistic activation leads to the tight membrane association of the enzyme, which then causes a conformational change that reverts autoinhibition (Newton, 1995).

PKC has been implicated in a multitude of physiological functions in the cell. Genetic studies of PKC function in mammals are difficult to interpret due to the presence of multiple genes and possible functional redundancy. In fact, all known PKC-deletion mutants show rather mild phenotypic changes. Nevertheless, they have yielded important insights into the function of individual PKC isozymes. PKCγ, one of the most prominent PKC isozymes in brain, has been shown to be important for brain functions involved in learning and memory (Abeliovich et al., 1993). Lack of PKCβ leads to an immunodeficiency by impairing humoral response and cellular B cell response (Leitges et al., 1996) and PKCß appears to be critically involved in B-cell-receptor-mediated survival signalling to NF-κB (Su et al., 2002). PKCε has been shown to be involved in the regulation of $GABA_A$ receptor function (Hodge et al., 1999). PKCθ appears to be involved in a unique signalling pathway linking T cell antigen receptor signalling to NF- κB activation in mature T lymphocytes (Sun et al., 2000). PKCδ-deficient smooth muscle cells exhibit increased apoptotic resistance (Leitges et al., 2001a). In addition, loss of PKCδ leads to increased antigen-induced mast cell degranulation (Leitges et al., 2002) and to the prevention of B cell tolerance due to maturation and differentiation of self-reactive B cells (Mecklenbrauker et al., 2002). PKCζ is important for the regulation of NF-κB transcriptional activity. Consequently, lack of PKCζ leads to impaired B cell receptor signalling, inhibition of cell proliferation and survival and defects in the activation of ERK and the transcription of NF-κB-dependent genes (Leitges et al., 2001b; Martin et al., 2002).

1.4.2 The 1,2-diacylglycerol production.

1,2-Diacylglycerol (DAG) can be produced by one of two different pathways for intracellular activation of PKC. In one mechanism, phosphatidylinositol is phosphorylated twice to form phosphatidylinositol–4,5 biphosphate (PIP2). A specific form of phospholipase C (PLC),

upon activation in a signal transduction pathway, hydrolyses PIP2 to form DAG and inosotol 1,4,5–triphosphate, which causes intracellular Ca^{2+} mobilisation (Liu and Heckman, 1998). PLC comprises a family of enzymes, of which at least PLCβ, PLCγ and PLC δ have been characterised. PLCβ isoforms are activated to a variable extent by the α subunits of heterotrimeric G_q and G_{11} proteins (Rhee and Choi, 1992) and by the G_i and $G_0\beta\gamma$ subunit complex (Clapham and Neer, 1993; Exton, 1997). PLCγ1 and γ2 are activated by receptor tyrosine kinases or by non-receptor tyrosine kinases in their cytosolic form (Rhee and Choi, 1992). The exact mechanism of activation of the PLCδ isoforms is still unclear (Nishizuka, 1995).

A second wave of DAG is produced by hydrolysis of phosphatidylcholine (Liu and Heckman, 1998). Phospholipase D (PLD), which produces phosphatidic acid and choline, is responsible for the first step in phosphatidylcholine degradation. Phosphatidic acid is then converted into DAG by the action of a phosphomonoesterase. Alternatively, phosphatidylcholine can be converted directly to DAG by a phosphatidylcholine-specific form of PLC (Zeisel, 1993). DAGs generated from either precursor, PIP2 or phosphatidylcholine, by either pathway are capable of activating PKC (Zeisel, 1993)

1.4.3 C1-domain proteins: PKC and alternative cellular effectors of DAG and phorbol esters.

The protein sequence responsible for high-affinity binding of DAG and phorbol esters was initially discovered in PKC isozymes and specifically designated the C_1 domain (Kaibuchi et al., 1989; Ohno, 2001). Depending upon the PKC type, the C_1 domain consists of one (aPKCs) or two (cPKCs and nPKCs) zinc-finger-like repeats that have a conserved pattern of cysteine and histidine residues and form a coordination site for two Zn^{2+} ions. Each zinc finger motif can form a single ligand-binding site for DAG. Only the single C_1 domain motifs of aPKCs do not bind DAG, and its function remains elusive (Mellor and Parker, 1998; Brose and Rosenmund, 2002).

In all cPKCs and nPKCs, kinase activation is closely coupled to DAG binding by the C_1 domain and the resulting membrane translocation (Newton, 1997; Newton, 2001). Hence, DAG and functionally analogous phorbol esters (natural diterpene secondary metabolites of *Euphorbiaceae* and *Thymelaceae*) have been used as tools to examine PKC function. Modulation of cellular processes by DAG and phorbol esters has been attributed exclusively to activation of PKCs, which is surprising because most eukaryotic cells contain at least five alternative types of

C1 domain containing proteins (Brose and Rosenmund, 2002). A number of observations indicate that the effects of DAG and phorbol esters are not always mediated by PKCs and may involve three alternative DAG targets in at least three key cellular processes. DAG- and phorbol-ester-mediated subcellular translocation of PKD1 is essential for protein transport from the trans-Golgi network to the cell surface (Matthews et al., 1999; Maeda et al., 2001; Rey et al., 2001; Baron and Malhotra, 2002; Van Lint et al., 2002). Activation of the Ras/Raf/MEK/ERK pathway in T lymphocytes is triggered by G-protein-coupled receptors and tyrosine-kinase-coupled receptors and is dependent on DAG-induced activation of RasGRP rather than PKCs (Dower et al., 2000; Jones et al., 2002). And finally, the stimulatory effects of DAG on neurotransmitter secretion from nerve cells are mediated by DAG/phorbol-ester receptors of the Unc-13/Munc13 family and not, as previously believed, by PKC isozymes (Betz et al., 1998; Lackner et al., 1999; Miller et al., 1999; Nurrish et al., 1999; Rhee et al., 2002).

These data reveal that the most frequently used pharmacological tools for PKC activation (i.e. phorbol esters, DAG) are not sufficiently specific to define PKC-mediated physiological effects and separate them from those caused by other C_1 domain proteins. Analogously, the C_1-domain-directed protein kinase inhibitors, such as widely used calphostin C, do not exclude the alternative to PKC pathways (Brose and Rosenmund, 2002). To date, for the functional separation of PKC-specific effects from those mediated by alternative DAG/phorbol-ester receptors the most promising agents developed are ATP-binding site inhibitors. Although many of these (e.g. H7, the indolocarbazole staurosporin, some balanol analogs, phenylaminopyrimidines, and rottlerin) inhibit protein kinases non-specifically, certain indolocarbazoles (e.g. Midostaurin/CGP41251, Gö6976, Gö7612, Gö7874, UCN-01) and bisindolylmaleimides (GF109203X, Gö6983, LY-333531, LY-379196, LY-317615) are rather PKC specific, some of them do show a preference for certain PKC isozymes (Way *et al.*, 2000; Barry and Kazanietz, 2001; Goekjian and Jirousek, 2001; Brose and Rosenmund, 2002; Swannie and Kaye, 2002).

1.5 Aims.

The proper assembly and functioning of tight junctions (TJ) is vital for the survival of many multicellular organisms. At the present time we lack a basic understanding of how an organism or even an individual cell regulates TJ assembly and the opening/closing of this intercellular seal. There are multiple indications in the literature that activators of PKC, diacylglycerols and the analogous phorbol esters are strong modulators of tight junction function. However, application of unspecific PKC inhibitors and activators has created an enormous amount of ambiguous and contradictory data concerning the role of PKC in TJ assembly and function. One rationale for the inconsistent data obtained using PKC inhibitors and activators may be that several diacylglycerol-sensitive PKCs isoforms, which differ in structure, substrate requirement and cellular localisation are involved in the regulation of many different cell functions. With the appearance of new-age isotype or subfamily-specific PKC-targeting pharmacological tools, the role of PKC in TJ assembly needs to be re-evaluated.

Further, the in-depth investigation of PKC-mediated signalling pathways regulating TJ assembly requires the identification of PKC targets. Previously, the investigation of PKC targets was problematic because the list of known TJ proteins was far from complete. In a last decade, a large number of new molecular constituents of TJ have been discovered. Among them the highly-phosphorylated transmembrane protein occludin is an attractive candidate for PKC-mediated signalling, since TJ maturation is always paralleled by changes in occludin phosphorylation state. It is obvious that the evaluation of occludin function is impossible without the identification of occludin phosphorylation sites and occludin kinases. Therefore, this work has focused on three major aims:

- to evaluate the involvement of protein kinase C pathway in the regulation of the occludin phosphorylation and sub-cellular distribution.
- to investigate how novel and conventional PKC, the two diacylglycerol-sensitive PKC subfamilies, are involved in tight junction assembly and disassembly.
- to identify *in vivo* sites of occludin phosphorylation.