Protein phosphatase 2A associates with and regulates atypical PKC and the epithelial tight junction complex

Viyada Nunbhakdi-Craig,1 Thomas Machleidt,2 Egon Ogris,3 Dennis Bellotto,1 Charles L. White III,1 and Estelle Sontag1

1Department of Pathology and 2Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX 75390 3Institute of Medical Biochemistry, Division of Molecular Biology, Vienna Biocenter, A-1030 Vienna, Austria

Tight junctions (TJs) play a crucial role in the establishment of cell polarity and regulation of paracellular permeability in epithelia. Here, we show that upon calcium-induced junction biogenesis in Madin-Darby canine kidney cells, ABaC, a major protein phosphatase (PP)2A holoenzyme, is recruited to the apical membrane where it interacts with the TJ complex. Enhanced PP2A activity induces dephosphorylation of the TJ proteins, ZO-1, occludin, and claudin-1, and is associated with increased paracellular permeability. Expression of PP2A catalytic subunit severely prevents TJ assembly. Conversely, inhibition of PP2A by okadaic acid promotes the phosphorylation and recruitment of ZO-1, occludin, and claudin-1 to the TJ during junctional biogenesis. PP2A negatively regulates TJ assembly without appreciably affecting the organization of F-actin and E-cadherin. Significantly, inhibition of atypical PKC (aPKC) blocks the calcium- and serum-independent membrane redistribution of TJ proteins induced by okadaic acid. Indeed, PP2A associates with and critically regulates the activity and distribution of aPKC during TJ formation. Thus, we provide the first evidence for calcium-dependent targeting of PP2A in epithelial cells, we identify PP2A as the first serine/threonine phosphatase associated with the multiprotein TJ complex, and we unveil a novel role for PP2A in the regulation of epithelial aPKC and TJ assembly and function.

Introduction

Tight junctions (TJs)* serve as occluding barriers, maintain polarity and homeostasis, and regulate the permeability characteristics of the paracellular space in epithelia (Mitic et al., 2000). ZO-1, a member of the MAGUK family of proteins, acts as a scaffold to organize transmembrane TJ proteins and recruit various signaling molecules and the actin cytoskeleton to TJs (Gonzalez-Mariscal et al., 2000). Occludin binds to ZO-1 and regulates TJ paracellular permeability (Furuse et al., 1993; Wong and Gumbiner, 1997). Several lines of evidence point to proteins of the claudin family as key components of TJ fibrils and the primary seal-forming proteins responsible for mediating TJ’s physiological barrier (Tsukita and Furuse, 2000). Although TJs serve as organizing centers for numerous other proteins involved in trafficking and signaling (Mitic et al., 2000), little is known about the molecular mechanisms involved in the dynamic regulation of these multiprotein complexes. Translocation of occludin and ZO-1 from the cytoplasm to the membrane during Ca2+-induced TJ biogenesis is accompanied by their phosphorylation (Stuart and Nigam, 1995; Wong, 1997; Sakakibara et al., 1997; Farshori and Kachar, 1999). However, so far, how dephosphorylation events are involved in the structural/functional regulation of TJs remains obscure.

Protein phosphatase (PP)2A enzymes are major Ser/Thr protein phosphatases. The core enzyme is a dimer containing a catalytic subunit (C) and a regulatory subunit (A), which can associate to a regulatory subunit (B). Several families of B subunits have been identified and modulate PP2A catalytic activity and substrate specificity (Sontag, 2001). Distinct B subunits contribute to targeting PP2A to defined intracellular domains and recruiting PP2A to signaling complexes, thereby ensuring its functional specificity (Sim and Scott, 1999; Sontag, 2001). Notably, the holoenzyme containing the Bα subunit is a major PP2A isoform involved in cell growth and cytoskeletal regulation in numerous cell
types (Sontag, 2001). Here, we chose to undertake a detailed analysis of ABαC behavior in epithelial cells, a cell type for which PP2A properties and functions are poorly documented. We show that ABαC is targeted to the TJ complex and identify a novel role for PP2A in TJ regulation.

Results

Ca2+-dependent recruitment of ABαC to regions of cell–cell contact

In Madin-Darby canine kidney (MDCK) cells, Ca2+ depletion from the culture medium results in disruption of intercellular junctions and cell rounding; conversely, the formation of functional junctional complexes can be triggered upon transferring cells cultured in low Ca2+ (LC) medium to normal Ca2+ (NC) medium (Gonzalez-Mariscal et al., 1990; Cereijido et al., 2000). Because the β subunit is always complexed to the AC core enzyme (Sontag, 2001), immunofluorescent microscopy and immunoblotting with anti-βα antibodies were used to assess the behavior of ABαC during Ca2+-switch experiments in confluent MDCK cells. Ca2+-deprivation of cells resulted in progressive cell retraction and disappearance of the peripheral membrane staining for ABαC (Fig. 1 A). When cells were Ca2+ starved overnight and then switched to NC medium to induce junction biogenesis, a pool of ABαC gradually reconcentrated at cell–cell contact sites (Fig. 1 B). Significant amounts of ABαC copurified with the membrane fraction from cells cultured in NC medium, whereas most of the holoenzyme was present in the cytosolic fraction from cells transferred to LC medium (Fig. 1 C). The proportion of membrane-bound ABαC increased considerably during junction biogenesis and remained unchanged 24 h after the Ca2+ switch, at which time TJs were completely reformed (Fig. 1 D).

Ca2+-dependent interaction of ABαC with the TJ complex

The dependency of the membrane localization of ABαC on the presence of both Ca2+ and cell–cell contacts suggests that ABαC associates with junctional complexes. Thus, we compared by confocal microscopy the distribution of ABαC with that of known junctional proteins in well-polarized MDCK cells. Immunolabeling with anti-βα antibodies indicated that endogenous ABαC was present at areas of intercellular contact (Fig. 2 A). A similar belt-like pattern of membrane immunostaining was observed with anti-HA antibody in MDCK cells stably expressing HA-tagged βα (MDCK-βα), confirming the specificity of the signal obtained with anti-βα antibodies. Images of x-z sections performed across the cell monolayers showed that endogenous or expressed βα subunits were concentrated at both the apical membrane and apex of lateral cell borders, but essentially absent from basolateral domains. By comparison, the distribution of ZO-1, occludin, and claudin-1 was characteristically restricted to the apex of the lateral cell borders, whereas E-cadherin, a marker of adherens junctions (AJs), was primarily enriched along the lateral membrane (Fig. 2 B). Double labeling of cells further confirmed that ABαC partially colocalized with ZO-1 at the apical membrane, but not with E-cadherin in the basolateral borders (Fig. 2 C). Immunofluorescent results indicate that a portion of membrane-associated ABαC is in proximity to TJs in MDCK cells. Because the apical junctional complex is better defined in intestinal cells, immunogold–electron microscopy with anti-βα antibodies was used to examine ABαC localization in human colon, and revealed a pool of TJ-associated PP2A (Fig. 2 D).

These observations prompted us to use immunoprecipitation assays to investigate whether PP2A could interact with TJ proteins. Because most antisubunit antibodies do not quantitatively immunoprecipitate PP2A heterotrimers, cell extracts were prepared from MDCK-βα cells and MDCK cells stably expressing HA-tagged wild-type C (MDCK-Wt C), and immunoprecipitated using anti-HA antibody (Ogris et al., 1997; Goedert et al., 2000). Large amounts of ZO-1, occludin, and claudin-1 were detected in the immunoprecipitates prepared from MDCK-Wt C and MDCK-βα, but

Figure 1. Ca2+-dependent membrane localization of ABαC in MDCK cells. (A) Confluent MDCK cells grown on glass coverslips in NC medium were switched for 5 min, 30 min, or 2 h to LC medium containing 1 mM EGTA to induce rapid TJ disassembly (NC to LC), and then processed for indirect immunofluorescence with rabbit anti-βα Δ237 antibody. (B) MDCK cells were Ca2+ starved overnight then switched to NC medium to induce TJ biogenesis (LC to NC). Cells were fixed 15 min, 45 min, and 5 h after initiation of cell–cell contact and analyzed as described in A. Bars, 10 μm. (C) MDCK cells cultured in NC medium (NC) were switched overnight to LC medium (LC). Equivalent amounts of proteins from cytosolic (Cy) and membrane (M) fractions were analyzed by immunoblotting. (D) MDCK cells were switched from LC to NC medium for 0, 5, or 24 h. Equivalent amounts of proteins from cytosolic (Cy) and membrane (M) fractions were analyzed by immunoblotting for the presence of PP2A βα and Cα subunits.
not from control cells (Fig. 3 A). TJ proteins were enriched, whereas E-cadherin could not be detected in the HA-Box immunoprecipitates, strengthening the hypothesis that ABαC preferentially interacts with TJ proteins. Detergent insolubility is commonly used as an indicator of the cytoskeletal association and incorporation of proteins into large junctional complexes (Stuart and Nigam, 1995; Wong, 1997). Substantial amounts of ABαC were detected in ZO-1, occludin, claudin-1, but not in E-cadherin immunoprecipitates prepared from detergent-insoluble MDCK cell fractions (Fig. 3 B). Other Ser/Thr phosphatases, including PP1α or PP2A holoenzymes containing the B56 subunit, were not found in the immunoprecipitates, in support of a specific association of ABαC with the TJ complex. ABαC was also present in occludin immunoprecipitates prepared from rat colon or kidney tissue homogenates (Fig. 3 C). Comparative analysis of immunoprecipitates from cytosolic and membrane fractions prepared from MDCK cells cultured in NC medium or transferred to LC medium demonstrated that the interaction between PP2A and TJ proteins was Ca2+-dependent and primarily occurred in the membrane fraction (Fig. 3 D).

PP2A can dephosphorylate TJ proteins at the membrane, resulting in enhanced TJ permeability

Because PP2A is appropriately localized to dephosphorylate TJ-associated proteins, we next examined how changes in PP2A activity influence TJ protein regulation and TJ barrier function. Potent inhibition of endogenous PP2A activity was achieved by treating MDCK cells with the toxic okadaic acid (OA) (Haystead et al., 1989). Increased levels of endogenous PP2A activity were obtained after expression of Wt C subunit in MDCK cells. Analysis of stable polarized MDCK-Wτ C cells by confocal microscopy indicated that HA-tagged Wt C subunits were homogenously expressed in polarized MDCK cells (Fig. 4 A). First, to determine whether changes in PP2A activity affect the phosphorylation state of TJ proteins at mature TJs, ZO-1, occludin, and claudin-1 were immunoprecipitated from membrane fractions prepared from polarized MDCK, OA-treated MDCK or MDCK-Wτ C cells, and analyzed by immunoblotting with anti-phosphoserine and anti-TJ protein antibodies (Fig. 4 B). Cells were examined before and after incubation with sodium butyrate, which significantly enhances the expression of transfected proteins. After treatment with sodium butyrate, endogenous PP2A activity levels were increased by ~30% in MDCK-Wτ C relative to control cells (unpublished data). Cell incubation with OA resulted in ~70% PP2A inhibition. As expected from previous studies showing that TJ-associated ZO-1 is a Ser phosphoprotein (Anderson et al., 1988),

Figure 2. Analysis of the distribution of ABαC at junctional complexes in polarized MDCK cells and in human colon. (A–C) Analysis of polarized cells by confocal microscopy. Arrows in x-z images indicate the thickness of the monolayers. Bars, 10 μm. (A) Representative apical x-y sections and transversal x-z views obtained in MDCK cells labeled with either Δ237 (1), P9 (2), or 2G9 (3) anti-Box antibodies, and in MDCK-Box cells labeled with anti-HA antibody (anti-HA). (B) Representative x-z sections of ZO-1, occludin, claudin-1, or E-cadherin staining in MDCK cells. (C) MDCK cells were double labeled with rabbit anti-Box and either rat anti-ZO-1 or mouse anti-E-cadherin antibodies. Representative apical (for Box/ZO-1) and basolateral (for Box/E-cadherin) x-y sections, and corresponding x-z views are shown. (D) Immunogold labeling was performed with the anti-B Δ237 antibody (diluted 1:80) on frozen ultrathin sections obtained from a normal human adult colon tissue biopsy. Note the concentration of gold particles in the TJ region beneath the lumen of the microvilli. Similar results were obtained with the P9 antibody; no TJ labeling was obtained with corresponding preimmune serum (not depicted). Bar, 150 nm. D, desmosome.

Figure 3. Ca2+-dependent association of ABαC with membrane-associated TJ proteins. (A) Immunoblots of total cell lysates prepared from MDCK, MDCK-Wτ C, or MDCK-Box cells and immunoprecipitated with anti-HA-coupled affinity matrix. (B) Immunoblots of detergent-insoluble fractions prepared from MDCK cells and immunoprecipitated with either anti-ZO-1, –occludin, –claudin-1, or –E-cadherin antibodies (+), or no antibody (−). (C) Immunoblots of homogenates prepared from rat colon or kidney tissue and immunoprecipitated with (+) or without (−) anti-occludin antibody. (D) Immunoblots of cytosolic (Cy) and membrane (M) fractions prepared from MDCK, MDCK-Box, and MDCK-Wτ C cells cultured in NC medium (NC) then switched overnight to LC medium (LC), and immunoprecipitated with either anti-ZO-1 or anti-HA antibodies.
The cellular diffusion of [3H]-mannitol and [3H]-inulin was determined in four distinct stable MDCK-Wt C populations. (E) Parabutyrate, and are the mean tracer flux measured in untreated cells before addition of sodium butyrate. Subsets of cells were incubated with sodium butyrate–induced Wt C expression, and was sensitive to 100 nM OA (Fig. 4 D). 50 nM OA had no effect, whereas incubation of MDCK cells with 100–250 nM OA slightly dehydrated the TER decrease induced by expressed Wt C. The paracellular diffusion of radioactive tracers across MDCK-Wt C monolayers increased by up to fourfold after sodium butyrate–induced Wt C expression, and was sensitive to 100 nM OA (Fig. 4 D). 50 nM OA had no effect, whereas incubation of MDCK cells with 100–250 nM OA slightly decreased tracer fluxes (Fig. 4 E). At higher concentrations, OA induced prominent TJ leakiness, but this effect was associated with substantial cell rounding. Thus, changes in PP2A activity can influence the phosphorylation state of ZO-1, occludin, and claudin-1 at mature TJs, and modulate TJ barrier properties.

**Inhibition of PP2A promotes TJ protein phosphorylation and TJ assembly**

To explore the potential role of PP2A in TJ assembly, the distribution of ZO-1, occludin, and claudin-1 was compared by immunofluorescent microscopy and immunoblotting during Ca\(^{2+}\) switch experiments performed in untreated or OA-treated control MDCK cells, and in MDCK-Wt C cells (Figs. 5 and 6). Cells were Ca\(^{2+}\)-starved overnight to induce TJ downregulation, resulting in internalization/redis-tribution of TJ proteins from the cell periphery to the cytosol, and then transferred to NC medium to induce TJ biogenesis. The Ca\(^{2+}\) switch initiates a rapid sorting of TJ proteins from the cytosol to the membrane; however, complete TJ stabilization and rescaling, as measured by TER restoration, is only achieved >20 h after the calcium switch (Stuart and Nigam, 1995; Farshori and Kachar, 1999). 1 h after the Ca\(^{2+}\) switch, a portion of total TJ proteins had already migrated to the cell periphery in control MDCK cells, but this redistribution was largely inhibited after expression of Wt C (Fig. 5 A). The peripheral membrane staining for TJ proteins appeared more continuous after incubation of ZO-1 was immunoreactive with the anti-phosphoserine antibody in MDCK cell membrane fractions. OA-mediated inhibition of PP2A activity induced an increase in the amounts of phosphorylated ZO-1 that correlated with an upward shift in its electrophoretic mobility. Expression of Wt C resulted in marked dephosphorylation and increased electrophoretic mobility of ZO-1. TJ-associated occludin migrates as multiple bands encompassing slow-migrating, high molecular weight occludin forms that are primarily phosphorylated on Ser residues, and fast-migrating, low molecular weight, dephosphorylated occludin species (Wong, 1997). Accordingly, only slow-migrating occludin bands were immunoreactive with the anti-phosphoserine antibody in MDCK cells. OA induced a significant increase in the phosphorylation levels of occludin that was accompanied by changes in its banding pattern. Expressed Wt C dephosphorylated occludin, as judged by the complete disappearance of the phosphorylated, upper occludin band. The amounts of slow-migrating, phosphorylated claudin-1 were increased after incubation of MDCK cells with OA, whereas expressed Wt C caused the accumulation of a fast-migrating, dephosphorylated claudin-1 species.

Next, we examined how PP2A-mediated changes in the phosphorylation levels of TJ-associated proteins affected TJ barrier properties. TJs not only regulate paracellular ion flow, as measured by transepithelial resistance (TER), but also the diffusion of nonionic molecules through the paracellular space. Increasing levels of expressed Wt C caused a gradual and substantial decrease in the TER of MDCK monolayers (Fig. 4 C). Treatment with 100 nM OA marginally enhanced the TER of control cells, but markedly inhibited the TER decrease induced by expressed Wt C. The paracellular diffusion of radioactive tracers across MDCK-Wt C monolayers increased by up to fourfold after sodium butyrate–induced Wt C expression, and was sensitive to 100 nM OA (Fig. 4 D). 50 nM OA had no effect, whereas incubation of MDCK cells with 100–250 nM OA slightly decreased tracer fluxes (Fig. 4 E). At higher concentrations, OA induced prominent TJ leakiness, but this effect was associated with substantial cell rounding. Thus, changes in PP2A activity can influence the phosphorylation state of ZO-1, occludin, and claudin-1 at mature TJs, and modulate TJ barrier properties.
MDCK cells with OA. The critical role played by PP2A during early TJ biogenesis was even more clearly demonstrated during Ca$^{2+}$ switch experiments performed in the absence of serum (Fig. 5 B). OA promoted, whereas expression of WT C severely prevented the accumulation of TJ proteins at cell-cell contact sites. The inhibitory effect of expressed WT C could be partially reversed by OA, reinforcing the idea that PP2A negatively regulates the initial sorting of TJ proteins to the membrane. 4 h after the Ca$^{2+}$ switch, a complete junctional distribution of ZO-1 was achieved over the entire circumference of MDCK cells (Fig. 6 A). OA accelerated the formation of the TJ network. Expression of WT C noticeably delayed the accumulation of TJ proteins at junctional areas, as revealed by the predominant cytosolic concentration and fragmentary staining of ZO-1 at cell–cell boundaries. 24 h after the Ca$^{2+}$ switch and thereafter, all the cells displayed the typical chicken wire staining pattern of ZO-1. However, cytosolic pools of TJ proteins were still visible at that stage in MDCK-Wt C cells. Significantly, in contrast to TJ proteins, changes in PP2A activity did not affect the redistribution of E-cadherin to areas of cell–cell contact during AJ formation.

TJ assembly is associated with resistance of newly formed TJ protein complexes to nonionic detergent extraction (Stuart and Nigam, 1995; Wong, 1997; Farshori and Kachar, 2002).
To further determine how PP2A influences the phosphorylation state and association of ZO-1, occludin, and claudin-1 with TJs, we compared by immunoblotting their distribution in detergent-soluble/insoluble cell fractions during Ca²⁺ switch experiments (Fig. 6 B). The experimental conditions were optimized to allow for visualization of changes in the electrophoretic mobility/banding pattern of TJ proteins, which could be linked to changes in their phosphorylation levels (Fig. 4 B). When MDCK monolayers were exposed to prolonged Ca²⁺ starvation, ZO-1, occludin, and claudin-1 were predominantly observed in the detergent-soluble fraction, as reported previously (Stuart and Nigam, 1995; Wong, 1997; Farshori and Kachar, 1999). Expressed Wt C stimulated the accumulation of detergent-soluble, dephosphorylated TJ proteins. TJ reassembly was induced by switching MDCK cells from LC to NC medium and correlated with the progressive appearance of detergent-insoluble, slow migrating phosphorylated ZO-1 and occludin species (Wong, 1997). In accordance with literature data showing that claudin-1 becomes more resistant to detergent extraction during TJ assembly (Chen et al., 2000), the Ca²⁺ switch was marked by the buildup of a detergent-insoluble, slow-migrating claudin-1 band. 24 h after the Ca²⁺ switch, most of ZO-1 and occludin were phosphorylated and detergent-insoluble in MDCK cells, as expected from previous studies (Stuart and Nigam, 1995; Sakakibara et al., 1997; Farshori and Kachar, 1999) and from our immunofluorescent pictures showing complete membrane redistribution of ZO-1 (Fig. 6 A). Claudin-1 was more detergent extractable than ZO-1 and occludin (Nishiyama et al., 2001), and slow-migrating claudin-1 species were present in both soluble/insoluble fractions. Expression of Wt C dramatically inhibited the phosphorylation and recruitment of TJ proteins to detergent-insoluble fractions during TJ biogenesis. Even 24 h after the Ca²⁺ switch, higher levels of detergent-soluble, dephosphorylated TJ proteins were still present in MDCK-Wt C compared to control cells, in agreement with our immunofluorescent data showing residual ZO-1 cytosolic staining in MDCK-Wt C cells (Fig. 6 A). Reciprocally, the relative amounts of detergent-insoluble, phosphorylated TJ proteins were largely increased in OA-treated cells during TJ assembly, supporting the hypothesis that inhibition of PP2A activity promotes the phosphorylation and association of ZO-1, claudin-1, and occludin with TJs. In contrast, changes in PP2A activity did not affect the redistribution of E-cadherin during junctional biogenesis.

TJ assembly also correlates with the development of TER. When MDCK-Wt C cells were switched from LC to NC medium to induce TJ rescaling, they developed TER with much slower kinetics than control cells (Fig. 6 C). This delay in TER development was exacerbated when the Ca²⁺ switch was performed under serum-free conditions (LC to DC), validating our hypothesis that PP2A activity negatively regulates TJ assembly.

Effects of PP2A deregulation on F-actin

The complete reestablishment of the actin cytoskeleton architecture plays a crucial role during Ca²⁺-mediated junctional biogenesis, and the contraction of perijunctional F-actin critically regulates the TJ permeability barrier (Denker and Nigam, 1998). Thus, we addressed the hypothesis that PP2A regulates TJs via F-actin remodeling. As shown in Fig. 7, we could not observe any significant difference in the organization of F-actin in control MDCK or MDCK-Wt C cells switched for 2 h from LC to NC medium or cultured in NC medium. Subsets of control cells were incubated for 1 h with 100 nM OA before staining. (B) MDCK cells were transiently transfected with the pcDNA3.1 vector encoding HA-tagged Wt C, and seeded at confluency on coverslips 24 h posttransfection. After plating, cells were either incubated overnight in LC medium then switched to DC or NC medium for the indicated time, or cultured in NC medium. Cells were double stained for the HA epitope and F-actin. For A and B, representative apical and basal x-y sections are shown for cells cultured in NC medium. Bars, 10 μM.
Regulation of tight junction proteins by PP2A | Nunbhadk-Crae et al. 973

(see “apical” sections) and stress fibers (see “basal” sections) present in confluent cells cultured in NC medium. However, now and then, the pattern of stress fibers appeared interwoven or less dense in confluent MDCK-Wt C cells.

**aPKC-dependent regulation of TJ proteins by PP2A**

The aPKC isoforms, PKCζ, and PKCα, are major TJ-associated protein kinases that critically participate in the establishment of epithelial TJ structures (Suzuki et al., 2001). In light of the functional importance of aPKC in TJ biogenesis, we investigated its role in PP2A-dependent TJ protein regulation. First, we found that ABαC was able to dephosphorylate in vitro PKCζ-phosphorylated ZO-1, occludin, and claudin-1 in an OA-sensitive manner (Fig. 8 A). We then examined the effects of inhibiting aPKC on OA-induced redistribution of TJ proteins. aPKC activity was blocked by incubating MDCK cells with the cell-permeable, myristoylated PKCζ pseudosubstrate (ZI), which directly inhibits aPKC autophosphorylation and transactivation (Standaert et al., 1999a). Although OA alone was sufficient to promote the translocation of ZO-1, occludin, and claudin-1 from the cytosol to the membrane in MDCK cells incubated in LC medium, this effect was blocked by preincubating cells with ZI (Fig. 8 B). OA-stimulated accumulation of TJ proteins at junctional areas was also greatly affected by ZI in cells switched from LC to DC medium, as indicated by the disrupted junctional staining pattern (Fig. 8 B) and reduced amounts of detergent-insoluble TJ proteins (Fig. 8 C) in ZI-treated cells.

**PP2A interacts with and regulates aPKC**

We have previously reported that PP2A is a critical regulator of PKCζ signaling in fibroblasts (Sontag et al., 1997). Moreover, OA activates aPKCζ/α in adipocytes (Standaert et al., 1999b), raising the possibility that PKCζ is a target for PP2A in epithelial cells. Indeed, ABαC colocalized with PKCζ at the apical membrane of MDCK cells (Fig. 9 A) and associated with both cytosolic and membrane-associated PKCζ during immunoprecipitation assays (Fig. 9 B). OA clearly promoted the membrane translocation of PKCζ in LC medium and its accumulation at junctional areas during TJ assembly performed under serum-free conditions (Fig. 9 C). In contrast, severe defects in the recruitment of PKCζ into junctional complexes were observed in MDCK-Wt C cells during TJ biogenesis. Compared to control cells, the staining pattern of PKCζ at the membrane appeared irregular and discontinuous. Abundant cytoplasmic pools of the kinase remained present in MDCK-Wt C cells, even 24 h after the Ca²⁺ switch. In support of a direct role of PP2A in the regulation of aPKC during TJ assembly, OA induced a ~2.7-fold activation of membrane-associated aPKC activity in MDCK cells switched from LC to DC medium, whereas aPKC activity was inhibited by ~50% in MDCK-Wt C cell membrane fractions, relative to the control (Fig. 9 D). Phosphorylation of PKCζ on Thr410 is essential for intrinsic PKCζ activity and subsequent activation (Standaert et al., 1999a). Accordingly, OA promoted, whereas expressed Wt C prevented the phosphorylation of PKCζ on Thr410 during TJ assembly performed in the absence of serum (Fig. 9 E). As with PKCζ, PP2A also associated with and regulated PKCα activity and distribution (unpublished data). Thus, PP2A directly participates in the regulation of aPKC in MDCK cells.
**Discussion**

We show that a pool of ABoC is recruited to peripheral regions of intercellular contact in a Ca$^{2+}$-dependent manner. To our knowledge, this is the first example of Ca$^{2+}$-dependent translocation of ABoC. Although the importance of signal-regulated translocation of protein kinases is well recognized (Sim and Scott, 1999), the concept of signal-depen-
dent redistribution of specific PP2A enzymes is fairly groundbreaking. We show that ABoC is concentrated at the apical membrane of polarized MDCK cells, where it interacts with TJ proteins. Our experimental conditions were aimed at visualizing membrane-associated PP2A; PP2A is likely present in other subcellular compartments not described here. Because aPKC indirectly binds to ZO-1 via the junctional-adhesion molecule (JAM) (Ebnet et al., 2001; Itoh et al., 2001) and may interact with occludin (Nusrat et al., 2000), and ZO-1 binds to both occludin (Furuse et al., 1994) and claudin-1 (Itoh et al., 1999), the precise PP2A binding partner at the TJ has yet to be identified. Nevertheless, our conclusion that ABoC interacts with a multiprotein TJ complex is strengthened by our findings showing the Ca$^{2+}$ dependency of the association of PP2A with TJ proteins at the membrane, and the presence of PP2A at TJs in human colon. In contrast to other signaling molecules, a direct interaction of specific Ser/Thr phosphatases with TJs has never been described. Thus, it is significant that ABoC associates with the TJ complex; because of its localization, it is a strong candidate for regulating the phosphorylation state of TJ proteins.

So far, the evidence for a role for Ser/Thr phosphatases in TJ regulation is fragmentary, and derives entirely from studies utilizing OA and calyculin A, which inhibit PP2A/PP1 enzymes in vivo in a dose- and time-dependent manner. Interestingly, these inhibitors are naturally occurring toxins that modulate intestinal paracellular permeability and are responsible for diarrheic shellfish poisoning in humans (Tripuraneni et al., 1997; Okada et al., 2000). As reported previously in other epithelial cells (Singer et al., 1994; Tripuraneni et al., 1997; Okada et al., 2000), we observed that high concentrations of OA or prolonged incubation of MDCK cells with this inhibitor induced cell rounding and TJ leakiness. Under these conditions, not only PP2A but also PP1 enzymes became inhibited, and a direct role for PP2A in TJ regulation cannot be meaningfully evaluated. Instead, lower OA concentrations more selective for PP2A did not appreciably alter the organization of the TJ network and the resistance and paracellular permeability of MDCK cells grown in NC medium, in agreement with previous studies (Pasdar et al., 1995; Tripuraneni et al., 1997; Okada et al., 2000). At first sight, this may suggest that PP2A does not regulate TJs once formed. However, enhanced PP2A activity induced dephosphorylation of membrane-associated TJ proteins, leading to decreased TER and increased TJ permeability. Based on previous studies pointing to an important role for occludin dephosphorylation in promoting TJ opening (Farshori and Kachar, 1999; Clarke et al., 2000; Simonovic et al., 2000), reduced levels of TJ-associated, phosphorylated occludin may contribute in part to enhanced TJ leakiness in MDCK-Wt C cells. The fact that OA can inhibit the effects of expressed Wt C suggests that PP2A-dependent changes in the phosphorylation state of TJ-bound proteins modulate the dynamic opening/closing of mature TJs. Many signaling molecules mediate changes in TJ barrier properties via remodeling of the actin cytoskeleton (Denker and Nigam, 1998). Yet, we were unable to demonstrate that changes in PP2A activity induce clear-cut effects on F-actin organization under our experimental conditions.
Likewise, OA does not significantly affect F-actin distribution in carcinoma cells (Strnad et al., 2001). However, the effects of OA on F-actin are dose- and time-dependent (Leira et al., 2001); high OA concentrations consistent with PP1 inhibition disrupt F-actin (Fiorentini et al., 1996).

OA promoted the phosphorylation and recruitment of TJ proteins to TJs during junctional assembly. Reciprocally, expressed Wt C induced the accumulation of soluble, dephosphorylated forms of TJ proteins, which correlated with a severe delay in the sorting of TJ proteins from the cytosol to TJs, and retardation in TER development. The observation that OA induces the membrane translocation of TJ proteins in LC medium and reverses the effects of Wt C in the absence of serum is consistent with the hypothesis that inhibition of PP2A promotes TJ assembly. Thus, the phosphorylation state of TJ proteins is controlled by PP2A and is closely linked to their ability to redistribute to the membrane during junctional biogenesis. It has been proposed that the nucleation of AJ assembly by cadherin/catenin complexes controls TJ assembly (Gumbiner et al., 1994). Although PP2A Cα subunit associates with and stabilizes the β-catenin/E-cadherin complex in immature blastocysts (Gotz et al., 2000), we were unable to colocalize or coimmunoprecipitate ABεC and E-cadherin in polarized MDCK cells. The accumulation of E-cadherin at regions of cell–cell contact also proceeded normally despite PP2A deregulation, suggesting that PP2A-dependent defects in TJ assembly do not occur secondary to abnormalities in AJ formation. Together with the observations that AB56C, but not ABuC, regulates β-catenin in the Wnt signaling pathway (Li et al., 2001), and that ABεC, but not AB56C, associates with TJ proteins, our findings suggest that the regulation of TJs by PP2A likely involves an E-cadherin–independent pathway (Stuart and Nigam, 1995) controlled by ABεC. However, the hierarchical regulation of junctional complexes is not absolute but rather multifaceted, as inhibition of cadherin adhesion can exert both negative and positive effects on TJ biogenesis in MDCK cells (Troxell et al., 2000). It is thus possible that distinct PP2A holoenzymes differentially affect separate signaling pathways that converge on TJs.

The results from our inhibitor studies indicate that aPKC participates in the regulation of ZO-1, occludin, and claudin-1 by PP2A. OA induced a 2.7-fold activation of membrane-bound aPKC, in agreement with the finding that membrane-associated PKC activity more than doubles during TJ assembly (Stuart and Nigam, 1995). Our conclusions support those of previous reports showing the importance of aPKC-mediated TJ protein phosphorylation for Ca²⁺-induced TJ assembly (Stuart and Nigam, 1995; Suzuki et al., 2001). PP2A associated with both soluble and membrane-bound aPKC and modulated aPKC activity and distribution. Thus, PP2A may be part of and directly regulate the aPKC/Par-3 signaling complex that is involved in regulation of TJ formation and cell polarity. The aPKC/Par-3 complex is tethered to TJs via JAM. It is noteworthy that, as with Wt C, overexpression of a JAM mutant disrupts the localization of aPKC and ZO-1 without significantly affecting the localization of E-cadherin (Ebnet et al., 2001).

In conclusion, we demonstrate that ABεC is recruited to and is a novel component and regulator of the TJ signaling complex. The regulation of TJs by PP2A is dependent on functional aPKC. We establish PP2A as a novel regulator of aPKC and identify the TJ proteins, ZO-1, occludin, and claudin-1, as targets of PP2A/aPKC signaling in epithelial cells.

Materials and methods
Plasmids and antibodies
The pcDNA3.1 vector encoding HA-tagged Wt C has been described previously (Ogris et al., 1997; Goelet et al., 2000). The pcDNA3.1 vector (Invitrogen) encoding HA-tagged Wt C was prepared from pcDNA1/Amp-HA-Bu (Yu et al., 2001) via NdeI/XhoI restriction sites. Antibodies included: anti-HA (Covance); anti-ZO-1 (Chemicon International, Inc.); anti-claudin-1, -occludin, and -phosphoα-Ser (Zymed Laboratories, Inc.); anti-Cα, anti-E-cadherin, and anti-ΔPKCα (Transduction Laboratories); anti-ΔPKCβ (Alexis Corporation); anti-ΔPKCζ (C-20), anti-p-αPKCζ (Thr410) (Santa Cruz Biotechnology, Inc.); anti-ΔPKCε (Calbiochem); anti-B56ε subunit (Tebrani et al., 1996); and affinity-purified FITC- and Texas red-coupled secondary antibodies (Jackson Immunoresearch Laboratories). The rabbit anti-Bα23β antibody was raised against a fusion protein containing a 6× His tag fused to amino acids 238–447 of the rat βs subunit. The rabbit anti-B P9 and monoclonal 2G9 antibodies were raised against a synthetic peptide corresponding to amino acids 398–411 of the rat βs subsequence. Antibodies raised against Bu may crossreact with the β, 8, or γ isomers of the Bu subunit family, but expression of Bβ and Bγ is restricted to testis and/or brain, and B8 is a cytosolic subunit almost undetectable in kidney (for review see Sontag, 2001).

Cell culture and characterization of stable MDCK cell lines
All experiments were performed in the highly polarized MDCK strain II D5 clonal cell line (Brewer and Roth, 1991). Cells were maintained on plastic dishes in DME (Gibco BRL) containing 10% FBS (HyClone). For most experiments, cells were plated at high density on Transwell filters (Costar) and cultured until complete polarization, as verified by measuring TER. Cells were transfected using Lipofectamine Plus reagent (Gibco BRL). Stable clones were obtained after selection with 800 μg/ml geneticin (Gibco BRL), analyzed for expression of the transfecant, and pooled together to minimize the effects of clonal variations. Seven separate pooled populations of stable transfectedants were utilized throughout our studies. The expression level of transfected proteins was constantly monitored by immunofluorescence and immunoblotting. Immunoblotting indicated that HA-tagged Wt C subunit was expressed at ~30–50% of the levels of endogenous C in stable MDCK cell lines, as previously reported in NIH 3T3 cells (Ogris et al., 1997). HA-tagged Bu subunits were expressed at ~20% of the levels of endogenous Bu. Stable MDCK cells transfected with the pcDNA3.1 vector were used as controls (control MDCK cells) and behaved like the nontransfected cells in our experiments. Sodium butyrate (2.5–5 mM; Sigma-Aldrich) was added in the culture medium for ~16 h before each experiment to enhance expression of the transfected proteins, and had no detrimental effects on any of the parameters measured in our studies, as reported previously (Brewer and Roth, 1991).

Calcium switch experiments
To induce rapid TJ disassembly, monolayers of MDCK cells grown in normal Ca²⁺ (NC) medium (DMEM + 10% FBS; 1.8 mM CaCl₂) were incubated in LC medium (CaCl₂-free S-MEM; Gibco BRL) containing 1 mM EGTA. For prolonged CaCl₂ removal, cells were incubated overnight in LC medium containing 1% diazoyed FBS. For the CaCl₂ switch, CaCl₂-starved cells were transferred to either NC or DC (DMEM + 1% diazoyed FBS) medium. If indicated, OA (Alexis Biochemicals) or the vehicle (100% DMSO) was added to the medium during the CaCl₂ switch.

Transepithelial resistance measurements
Cells were plated at confluency and grown on sixwell Transwell filters in NC medium. TER values were measured in duplicate wells using an Endohm™ voltohmmeter (World Precision Instruments). TER values (Ωcm²) were normalized to the area of the monolayer (filter), and calculated by subtracting the blank values (~18 Ωcm²) from the filter and the bathing medium. All cell culture media were supplemented with 25 mM Hepes, pH 7.4, and the integrity and cell density of the monolayers were carefully monitored during TER measurements.
Paracellular diffusion measurement

Cells were grown on 6.5-mm Transwell filters until complete polarization. TJ leakiness was assessed by measuring the diffusion of [3H]-inulin (Amer- sham Biosciences) and [3H]-mannitol (NEN™ Life Science Products, Inc.) across the membrane in monolayers of equivalent cell density (Wong and Gumbiner, 1997). The tracer diffusion was examined by replacing the apical compartment medium by fresh NC medium containing the tracer (2.5 μCi/ml), and the basal compartment medium with the isosmotic, or cytosolic/ media. When indicated, OA or the vehicle (DMSO) was added in both basal and apical bathing media. The compartment media were collected 60 min afterwards, and counted by liquid scintillation.

Confocal microscopy

Cells grown on Transwell filters or on glass coverslips were fixed with methanol for 5 min at −20°C, and labeled sequentially for 1 h with primary and secondary antibodies at a 1:100 dilution (Sontag et al., 1995). For the visualization of F-actin, cells were fixed for 20 min with 4% paraformaldehyde, permeabilized for 5 min with 0.1% Triton X-100, and labeled for 30 min with FITC- labeled phalloidin (Sigma-Aldrich). The samples were mounted using Fluoromount-G (Fisher) and examined on a Leica TCS SP confocal microscope using a 63× objective. Images (16 x-y or x-z sections across cells) were directly captured, saved and transferred to Adobe Photoshop® 5.5 for printing. The specificity of the labeling was verified by omitting first or second antibodies during the staining procedures, and by using pre-immune sera and antibodies that had been preadsorbed with the corresponding antigen.

Electron microscopy

Cryosections (80 nm) from normal human adult colon fixed in 1% glutaraldehyde/4% paraformaldehyde were picked up using a 1.8-mm loop with a droplet of frozen 2.3 M sucrose, thawed, moved onto Formvar-covered and carbon-coated glow-discharged 100 mesh copper grids and stored on buffer. Immunogold labeling of the cryosections was performed by the Tokuyasu method (Tokuyasu, 1986) by which the grids are moved through series of droplets containing blocking agents, antibodies and are ultimately coated with a layer of 0.2% uranyl acetate in 2% methylcellulose. The cryosections were incubated for 30 min each with serial dilutions of the primary antibody in PBS containing 5% milk, and then with protein A gold (10 nm diameter; 1:70 dilution; a gift from G. Posthuma, Utrecht University School of Medicine, The Netherlands). The cryosections were examined using a JEOL 1200EX Transmission Electron Microscope operating at 120KV. Electron micrographs were taken using Eastman Kodak SO-163 electron image film.

Cell fractionation and Western blotting

To prepare detergent-soluble and -insoluble fractions, confluent cells were washed in PBS, harvested in 400 μl buffer S containing 50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.5 mM EDTA, 1 mM sodium fluoride, 100 μM sodium orthovanadate, and incubated for 5 min at 30°C. The extracts were then centrifuged at 4°C to pellet nuclear material. In other experiments, the colon and kidney were minced, homogenized in buffer S containing 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1 mM sodium fluoride, 100 μM sodium orthovanadate and centrifuged at 13,000 g for 15 min to remove insoluble material. Aliquots of the supernatants were immunoprecipitated with mouse anti-occludin antibody and analysed as described above.

Analysis of TJ protein phosphorylation

MDCK cell detergent-insoluble fractions were immunoprecipitated as described above with anti-ZO-1, -occludin, or -claudin-1 antibodies. The immunoprecipitates were washed and resuspended in P buffer (50 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 0.5 mM EDTA, 1 mM sodium fluoride, 100 μM sodium orthovanadate). Phosphorylation of TJ proteins was performed for 1 h at 30°C by adding 10 μg phosphatidylinserine, a gift from P. Sternweis (UT Southwestern, Dallas TX), ~1 μg recombinant human pPKCz (Calbiochem), and 100 μM [γ-32P]ATP (5 μCi) per reaction. The tubes containing phosphorylated TJ proteins were transferred on ice and carefully divided into 15-μl aliquots. Either 100 nM purified ABcAb (Goert et al., 2000), 100 nM ABcAb preincubated on ice for 20 min with 1 μM OA, or buffer alone, were added into the reaction mixtures. The samples (25 μl) were incubated for another 30 min at 30°C, after which the reactions were terminated by addition of 3× sample buffer for SDS-PAGE. The samples were boiled for 5 min then simultaneously analyzed by SDS-PAGE on 4-20% gradient gels (Bio-Rad Laboratories), followed by autoradiography. In other experiments, ZO-1, occludin, and claudin-1 were immunoprecipitated from membrane fractions, resolved by SDS-PAGE and analysed by immunoblotting using rabbit anti-phosphoserine antibody. The blots were reprobed with anti-ZO-1, occludin, and claudin-1 antibodies. Phosphorylation of PKCζ was examined by immunoblotting using p-nPKCζ (Thr410) antibody (Standart et al., 1999a). In parallel, aliquots of cell fractions were resuspended in AP buffer (50 mM Tris, pH. 7.4, 50 mM NaCl, 1 mM MgCl2, 1 mM DTT, 0.1% NP-40, and 50 μM aPKC substrate). The reaction mixtures were spotted onto P-81 phosphocellulose paper and washed with 75 mM phosphoric acid. Incorporation of 32P was determined by liquid scintillation counting.

aPKC activity assays

aPKC activity was measured in immunoprecipitates as described previously (Standart et al., 1999a). After washing in P buffer, aPKC immunoprecipitates were incubated for 30 min at 30°C with 50 μl of P buffer containing [γ-32P]ATP (1 μCi/2 μl), and a specific substrate analogue of PCKζ-pseudosubstrate (BioSource International), a selective aPKC substrate. The reactions were performed in the presence or absence of 10 μM PCKζ pseudosubstrate (BioSource International), as described previously (Sontag et al., 1997). The reaction mixtures were spotted onto P-81 phosphocellulose paper and washed with 75 mM phosphoric acid. Incorporation of 32P was determined by liquid scintillation counting.

PP2A activity assays

Aliquots of cell extracts were assayed for PP2A activity for 6 min at 30°C using the RRREEEeTIEE phosphatepeptidase (Biosynthesis, Inc.) as a substrate. Dephosphorylation of the peptide was determined by measuring the release of P, using a quantitative colorimetric assay (Sontag et al., 1999).
References

Anderson, J.M., B.R. Stevenson, L.A. Jesaitis, D.A. Goodenough, and M.S. Moosiker. 1988. Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. J. Cell Biol. 106:1141–1149.

Brewer, C.B., and M.G. Roth. 1991. A single amino acid change in the cytoplasmic domain alters the polarized delivery of influenza virus hemagglutinin. J. Cell Biol. 114:413–421.

Cereijido, M., L. Shoshani, and R.G. Contreras. 2000. Molecular physiology and pathophysiology of tight junctions. I. Biogenesis of tight junctions and epithelial polarity. Am. J. Physiol. Gastrointest. Liver Physiol. 279:G477–G482.

Chen, Y., Q. Lu, E.E. Schroenberger, and D.A. Goodenough. 2000. Restoration of tight junction structure and barrier function by down-regulation of the mitogen-activated protein kinase pathway in ras-transformed Madin-Darby canine kidney cells. Mol. Biol. Cell. 11:849–862.

Clarke, H., A.P. Soler, and J.M. Mullin. 2000. Protein kinase C activation leads to dephosphorylation of occludin and tight junction permeability increase in LLC-PK1 epithelial cell sheets. J. Cell Sci. 113:3187–3196.

Denker, B.M., and S.K. Nigam. 1998. Molecular structure and assembly of the tight junction. Am. J. Physiol. 274:F1–F9.

Denker, B.M., A. Suzuki, Y. Honoki, T. Hirose, M.K. Meyer Zu Brickwedde, S. Ohno, and D. Verreet. 2001. The cell polarity protein ASIP/PAR-3 directly associates with junctional adhesion molecule (JAM). EMBO J. 20: 3738–3748.

Farshori, P., and B. Kachar. 1999. Redistribution and phosphorylation of occludin during opening and rescaling of tight junctions in cultured epithelial cells. J. Membr. Biol. 170:147–156.

Fiorentini, C., P. Matarrese, A. Fattorossi, and G. Donelli. 1996. Okadaic acid induces changes in the organization of F-actin in intestinal cells. Toxicol. 34: 937–945.

Furuse, M., T. Hirase, M. Itoh, A. Nagafuchi, S. Yonemura, and S. Tsukita. 1993. Occludin: a novel integral membrane protein localizing at tight junctions. J. Cell Biol. 123:1777–1788.

Furuse, M., M. Itoh, T. Hirase, A. Nagafuchi, S. Yonemura, S. Tsukita, and S. Tsukita. 1994. Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. J. Cell Biol. 127: 1617–1626.

Goedert, M., S. Sarutmir, R. Jakse, M.J. Smith, C. Kamibayashi, C.L. White, and E. Sonntag. 2000. Reduced binding of protein phosphatase 2A to tau protein with frontotemporal dementia and parkinsonism linked to chromosome 17 mutations. J. Neurosci. 20:2155–2162.

Gonzalez-Mariacl, L., A. Betanzos, and A. Avila-Floreo. 2000. MAGUK proteins: structure and role in the tight junction. Semin. Cell Dev. Biol. 11:315–324.

Gonzalez-Mariacl, L., R.G. Contreras, J.J. Bolivar, A. Ponc, D.R. Chavez, and M. Cereijido. 1990. Role of calcium in tight junction formation between epithelial cells. Am. J. Physiol. 259:C978–C986.

Gorz, J., A. Probst, C. Misul, R.M. Nitsch, and E. Ehler. 2000. Distinct role of protein phosphatase 2A subunit Calpha in the regulation of E-cadherin and its B56 regulatory subunit inhibit Wnt signaling in Xenopus. EMBO J.

Nakazato, E., J.A. Chen, C.S. Foley, T.W. Liang, J. Tom, M. Cromwell, C. Quan, and R.J. Moriarty. 2000. The coiled-coil domain of occludin can act to organize structural and functional elements of the epithelial tight junction. J. Biol. Chem. 275:29816–29822.

Ogris, E., D.M. Gibson, and D.C. Pallas. 1997. Protein phosphatase 2A subunit assembly: the catalytic subunit carboxy terminus is important for binding cellular B subunit but not poliovirus middle tumor antigen. Oncogene. 15:911–917.

Okada, T., A. Nuzai, S. Matsunaga, N. Fusetani, and M. Shimizu. 2000. Assessment of the marine toxins by monitoring the integrity of human intestinal Caco-2 cell monolayers. Toxicol. In Vitro. 14:219–226.

Pardar, M., Z. Li, and H. Chan. 1995. Desmosome assembly and disassembly are regulated by reversible protein phosphorylation in cultured epithelial cells. Cell Mol. Gynecol. 30:108–121.

Sakakibara, A., M. Furuse, M. Saitou, Y. Ando-Akatsuka, and S. Tsukita. 1997. Possible involvement of phosphorylation of occludin in tight junction formation. J. Cell Biol. 137:1393–1401.

Sim, A.T., and J.D. Scott. 1999. Targeting of PKA, PKC and protein phosphatases to cellular microdomains. Cell Calcium. 26:209–217.

Simonovic, I., J. Rosenberg, A. Koutsouris, and G. Hecht. 2000. Enteropathic Escherichia coli dephosphorylates and dissociates occludin from intestinal epithelial tight junctions. Cell Microbiol. 2:305–315.

Singer, K.L., B.R. Stevenson, P.L. Woo, and G.L. Firestone. 1994. Relationship of serine/threonine phosphorylation/dephosphorylation signaling to glucocorticoid regulation of tight junction permeability and ZO-1 distribution in nontransformed mammary epithelial cells. J. Biol. Chem. 269:16108–16115.

Sonntag, E. 2001. Protein phosphatase 2A: the Trojan Horse of cellular signaling. Cell Signal. 13:7–16.

Sonntag, E., V. Nunbhakdi-Craig, G.S. Bloom, and M.C. Mumbry. 1995. A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. J. Cell Biol. 128:1131–1144.

Sonntag, E., J.M. Sonntag, and A. Garcia. 1997. Protein phosphatase 2A is a critical regulator of protein kinase C zeta signaling targeted by SV40 small t to promote cell growth and NF-kappaB activation. EMBO J. 16:5662–5671.

Sonntag, E., V. Nunbhakdi-Craig, G. Lee, R. Brandt, C. Kamibayashi, J. Kurct, C.L. White, III, M.C. Mumbry, and G.S. Bloom. 1999. Molecular interactions among protein phosphatase 2A, tau, and microtubules. Implications for the regulation of tau phosphorylation and the development of tauopathies. J. Biol. Chem. 274:25100–25498.

Standaert, M.L., G. Bandyopadhyay, L. Perez, D. Price, L. Galloway, A. Pekle-povic, M.P. Sajan, V. Cenni, A. Sirri, J. Moscat, et al. 1999a. Insulin activates protein kinases C zeta and C lambda by an autophosphorylation-dependent mechanism and stimulates their translocation to GLUT4 vesicles and other membrane fractions in rat adipocytes. J. Biol. Chem. 274:25088–25116.

Standaert, M.L., G. Bandyopadhyay, M.P. Sajan, L. Cong, M.J. Quon, and R.V. Farese. 1999b. Okadaic acid activates atypical protein kinase C (etalambda) in rat and 3T3-L1 adipocytes. An apparent requirement for activation of GLUT4 translocation and glucose transport. J. Biol. Chem. 274:14074–14078.

Strnad, P., R. Windoffer, and R.E. Leube. 2001. In vivo detection of cytokerin filament network breakdown in cells treated with the phosphate promoter inhibitor okadaic acid. Cell Tissue Res. 306:277–293.

Stuart, R.O., and S.K. Nigam. 1995. Regulated assembly of tight junctions by protein kinase C. Proc. Natl. Acad. Sci. USA. 92:6072–6076.

Suzuki, A., T. Yamamaka, T. Hirose, N. Manabe, K. Mizuno, M. Shimizu, K. Akimoto, Y. Izumi, T. Ohnishi, and S. Ohno. 2001. Atypical protein kinase C is involved in the evolutionarily conserved protein complex and plays a critical role in establishing epithelia-specific junctional structures. J. Cell Biol. 152:1183–1196.

Tehrani, M.A., M.C. Mumbry, and C. Kamibayashi. 1996. Identification of a novel protein phosphatase 2A regulatory subunit highly expressed in muscle. J. Biol. Chem. 271:5164–5170.

Regulation of tight junction proteins by PP2A | Nunbhakdi-Craig et al. 977

Mitc, L.L., C.M. Van Itallie, and J.M. Anderson. 2000. Molecular physiology and pathophysiology of tight junctions I. Tight junction structure and function: lessons from mutant animals and proteins. Am. J. Physiol. Gastrointest. Liver Physiol. 279:G250–G254.
Tokuyasu, K.T. 1986. Application of cryoultramicrotomy to immunocytochemistry. J. Microsc. 143(Pt 2):139–149.

Tripuraneni, J., A. Koutsouris, L. Pestic, P. De Lanerolle, and G. Hecht. 1997. The toxin of diarrheic shellfish poisoning, okadaic acid, increases intestinal epithelial paracellular permeability. Gastroenterology. 112:100–108.

Troxell, M.L., S. Gopalakrishnan, J. McCormack, B.A. Poteat, J. Pennington, S.M. Garringer, E.E. Schneeberger, W.J. Nelson, and J.A. Marrs. 2000. Inhibiting cadherin function by dominant mutant E-cadherin expression increases the extent of tight junction assembly. J. Cell Sci. 113:985–996.

Tsukita, S., and M. Furuse. 2000. Pores in the wall: claudins constitute tight junction strands containing aqueous pores. J. Cell Biol. 149:13–16.

Wong, V. 1997. Phosphorylation of occludin correlates with occludin localization and function at the tight junction. Am. J. Physiol. 273:C1859–C1867.

Wong, V., and B.M. Gumbiner. 1997. A synthetic peptide corresponding to the extracellular domain of occludin perturbs the tight junction permeability barrier. J. Cell Biol. 136:399–409.

Yu, X.X., X. Du, C.S. Moreno, R.E. Green, E. Ogris, Q. Feng, L. Chou, M.J. McQuoid, and D.C. Pallas. 2001. Methylation of the protein phosphatase 2A catalytic subunit is essential for association of Balpha regulatory subunit but not SG2NA, striatin, or polyomavirus middle tumor antigen. Mol. Biol. Cell. 12:185–199.