Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins

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Role of tyrosine phosphorylation in the reassembly of occludin and other tight junction proteins. Am. J. Physiol. 276 (Renal Physiol. 45): F737–F750, 1999.—After the simulation of anoxia by ATP depletion of MDCK cell monolayers with metabolic inhibitors, the tight junction (TJ) is known to become structurally perturbed, leading to loss of the permeability barrier. Peripheral TJ proteins such as zonula occludens 1 (ZO-1), ZO-2, and cingulin become extremely insoluble and associate into large macromolecular complexes (T. Tsukamoto and S. K. Nigam. J. Biol. Chem. 272: 16133–16139, 1997). For up to 3 h, this process is reversible by ATP repletion. We now show that the reassembly process depends on tyrosine phosphorylation. Recovery of transepithelial electrical resistance in ATP-replete monolayers was markedly inhibited by the tyrosine kinase inhibitor, genistein. Indirect immunofluorescence revealed a decrease in staining of occludin, a membrane component of the TJ, in the region of the TJ after ATP depletion, which reversed after ATP repletion; this reversal process was inhibited by genistein. Examination of the Triton X-100 solubilities of occludin and several nonmembrane TJ proteins revealed a shift of occludin and nonmembrane TJ proteins into an insoluble pool following ATP depletion. These changes reversed after ATP repletion, and the movement of insoluble occludin, ZO-1, and ZO-2 back into the soluble pool was again via a genistein-sensitive mechanism. Rate-zonal centrifugation analyses of detergent-soluble TJ proteins showed a reversible increase in higher density fractions following ATP depletion-repletion, although this change was not affected by genistein. In 32P-labeled cells, dephosphorylation of all studied TJ proteins was observed during ATP depletion, followed by rephosphorylation during ATP repletion; rephosphorylation of occludin was inhibited by genistein. Furthermore, during the ATP repletion phase, tyrosine phosphorylation of Triton X-100-insoluble occludin, which is localized at the junction, as well as ZO-2, p130/ZO-3 (though not ZO-1), and other proteins was evident; this tyrosine phosphorylation was completely inhibited by genistein. This indicates that tyrosine kinase activity is necessary for TJ reassembly during ATP repletion and suggests an important role for the tyrosine phosphorylation of occludin, ZO-2, p130/ZO-3, and possibly other proteins in the processes involved in TJ reformation.

occludin; zonula occludens 1; cytoskeleton; tyrosine phosphorylation; ATP depletion

The integrity of the epithelial permeability barrier is largely maintained by the tight junction (TJ) (1, 12, 32, 37). The TJ is composed of many proteins, both membrane and peripheral, linked to the actin-based cytoskeleton (7). The transmembrane protein of the TJ, occludin, is thought to play an important role in sealing the junction, but a number of proteins on the cytoplasmic face of the TJ are likely to play important structural and functional roles as well (22). These include the highly homologous proteins zonula occludens 1 (ZO-1) and ZO-2 as well as p130/ZO-3 (2, 5, 25, 29). At least under certain conditions, ZO-1 seems to associate directly or indirectly with actin-binding proteins such as fodrin (29, 52). Additional proteins of the TJ with unclear function include symplekin, cingulin, 7H6 antigen, and the recently identified claudins (11, 21, 31, 56). Several yet-to-be-identified phosphoproteins of 330 and 65 kDa have also been found to coimmunoprecipitate with known TJ proteins (47). Thus the molecular structure of the TJ is likely to be very complex.

The regulation of the assembly and disassembly of the TJ has attracted a great deal of interest in recent years. These issues are of obvious relevance in the context of epithelial tissue morphogenesis and development, where the permeability barrier must be formed de novo (23, 46). However, TJ assembly and disassembly is also a key feature of sublethal epithelial injury-repair processes, which disrupt the permeability barrier, as well as during the movement of inflammatory and other cells across tight epithelial structures (19). Some of these processes have been modeled in vitro. For example, ATP depletion using metabolic inhibitors in tight polarized epithelial monolayers has been used to model ischemic injury (8, 14, 33, 35, 52). Although there are important differences between this model system and in vivo ischemic injury, it has been shown that the model induces many changes characteristic of those seen in ischemic epithelial tissue such as the kidney (16, 30, 51). Among the alterations known to occur in these cultured cell models are perturbations in the actin-based cytoskeleton, misshaping of certain transporters, and disruption of the TJ [i.e., loss of transepithelial electrical resistance (TER) and alterations in the structure of the TJ] as shown by electron microscopy (3, 19, 52).

The biochemical basis of these alterations is only beginning to be unraveled. We have recently shown that, upon ATP depletion of Madin-Darby canine kidney (MDCK) cells, a cell line widely used in cell biological studies of polarized epithelia, several nontransmembrane TJ proteins, including ZO-1, ZO-2, and cingulin, aggregate into large macromolecular complexes and associate with the cytoskeleton (52). These proteins become remarkably insoluble to detergent-containing buffers, and the cytoskeletal protein, fodrin, can be coimmunoprecipitated in a complex containing ZO-1. For up to 3 h of ATP depletion, the process...
appears to be largely reversible. It is not known how this reassembly process is regulated, a matter which must be understood if agents that restore the permeability barrier after injury are to be rationally developed.

Ever since it was shown that the sorting and assembly of TJ proteins and the establishment of a permeability barrier is regulated by phosphorylation (40), considerable evidence has accumulated on the role of classic signaling mechanisms in the assembly of the TJ. Using the so-called “calcium switch” model, it has been shown that the regulation of the assembly of the TJ involves a heterotrimeric G protein (13, 15), thapsigargin-sensitive endoplasmic reticulum calcium stores (4, 48), and activation of protein kinase C (15, 47). Small GTP binding proteins also appear to play a role (41, 53, 55), and ZO-1 is known to be phosphorylated on tyrosine residues as well (28, 34, 44, 50), although the physiological significance of this latter phosphorylation remains to be worked out.

We now show that the functional and biochemical reassembly of the TJ during ATP depletion appears to be dependent upon a genistein-sensitive tyrosine kinase pathway, which is capable of differentially phosphorylating several TJ proteins and affects the association of TJ proteins with the detergent-insoluble (possibly “cytoskeletal”) fraction.

**MATERIALS AND METHODS**

Cell culture and chemicals. MDCK cells were maintained in DMEM supplemented with heat-inactivated 5% FCS and antibiotics, as previously described (48, 52). Genistein, tyrophostin-25, herbimycin A, and lavendustin A were purchased from Calbiochem (San Diego, CA). Anti-ZO-1 rat monoclonal antibody (R40.76) and anti-ZO-2 rabbit polyclonal antibody (p130/ZO-3) and possibly other TJ proteins are known to undergo phosphorylation on serine-threonine residues (2, 11, 43, 47, 56), and ZO-1 is known to be phosphorylated on tyrosine residues as well (28, 34, 44, 50), although the physiological significance of this latter phosphorylation remains to be worked out.

ATP depletion-repletion. ATP depletion-repletion was performed as previously described (52). In brief, after ATP depletion-repletion, monolayers were rinsed three times with ice-cold PBS. Extractions were performed by overlaying the cells with 300 µl of CSK-1 buffer (0.5% Triton X-100, 100 mM NaCl, 10 mM Tris·HCl, pH 7.4, and 300 mM sucrose) plus a protease inhibitor cocktail consisting of 1 mM phenylmethylsulfonyl fluoride (PMSF) + aprotinin, leupeptin, pepstatin A, and antipain (each 20 µg/ml) for 20 min at 4°C on a gently rocking platform. The extract (“E” fraction) was completely aspirated, and the residue (“R” fraction) was dissolved in 300 µl of 2× sample buffer. To get a linear density on the blot, two volumes of E fraction and one volume of R fraction of each condition were subjected to SDS-PAGE and Western immunoblotting.

Analyses of cell lysates by sucrose density gradients. Cells were lysed in a buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 10 mM HEPES (pH 7.5), 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 4 mM EDTA, and 2 mM sodium orthovanadate (NaV), plus a protease inhibitor cocktail. Supernatants were separated by centrifugation (14,000 g, 4°C for 30 min) and the pellets were dissolved in the same volume of 2× sample buffer as the lysis buffer. Both fractions were separated by SDS-PAGE and subjected to Western blotting. Blots were quantified with NIH image software.

For sucrose gradient analysis, supernatants were fractionated over linear 5–20% sucrose gradients prepared in the lysis buffer. Each fraction was subjected to Western blotting. Blots were quantified with NIH image software.

Metabolic labeling and immunoprecipitation. For phosphorylation studies, confluent MDCK cell monolayers were labeled for 4 h in phosphate-free DMEM in the presence of inorganic 32P (50 µCi/ml, Dupont-NEN). At the end of the labeling period, cells were rinsed three times with PBS and treated with a buffer for ATP depletion. After 1 h of ATP depletion, cells were further incubated with phosphate-free DMEM containing 32P (50 µCi/ml) for 3 h. For immunoprecipitation of Triton X-100-insoluble occludin, cells were washed three times with ice-cold PBS, scraped in 600 µl of CSK-A buffer (0.5% Triton X-100, 50 mM Tris·HCl, pH 7.4, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM NaV, 300 mM sucrose, and a protease inhibitor cocktail), and extracted for 20 min on a rocking platform at 4°C. After centrifugation at 14,000 g for 10 min,
the insoluble pellet was further dissolved with 100 µl of CSK-B buffer (1% SDS, 50 mM Tris- HCl, pH 7.4, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM Na3V, 300 mM sucrose, and a protease inhibitor cocktail), homogenized with 25-gauge needle passing, and extracted by gentle vortexing for 10 min at 4°C. After dilution with 500 µl of CSK-A buffer, the lysate was passed through a 25-gauge needle again. Particulate matter was separated by centrifugation at 14,000 g for 10 min, and the supernatant (which now contained the Triton X-100-insoluble occludin) was used for immunoprecipitation. After preclearing, supernatants were incubated with anti-occludin antibody overnight at 4°C. Protein G Sepharose beads (Zymed) were added, followed by incubation for 1 h at 4°C. Beads were collected by centrifugation, washed five times with the immunoprecipitation buffer, and dissolved in 2× sample buffer.

For immunoprecipitation of the detergent (1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS)-soluble fraction (containing ≈95% of total ZO-1 and ZO-2), cells were solubilized with the lysis buffer, described above in the sucrose gradient analysis, and supernatant was separated by centrifugation at 14,000 g for 30 min. After preclearing, supernatants were incubated with anti-ZO-1 antibody overnight at 4°C. Immune complexes were collected with goat anti-rat IgG Sepharose beads (Cappel). Then beads were collected by centrifugation, washed five times with the immunoprecipitation buffer, boiled in 2× sample buffer, and subjected to SDS-PAGE, immunoblotting, and autoradiography (46, 52).

Alkaline phosphatase treatment. Confluent MDCK monolayers were lysed with a buffer for immunoprecipitation containing 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS, as described above, then processed for immunoprecipitation with an antibody against ZO-1 or occludin, as described above. Beads were washed three times with the same buffer and three times with a buffer for alkaline phosphatase treatment (50 mM Tris·HCl, pH 8.2, 50 mM NaCl, 1 mM MgCl2, 1 mM dithiothreitol, and 1 mM PMSF), then resuspended in 40 µl of the buffer containing 100 U of alkaline phosphatase (calf intestine, specific activity, 2,500 U/mg; Boehringer-Mannheim). After 30 min of incubation at 30°C, the reaction was stopped by adding 40 µl of 2× sample buffer. Proteins were analyzed by 6% SDS-PAGE (ZO-1 and ZO-2) or 7.5% SDS-PAGE (occludin) and then subjected to Western immunoblot (43).

Western immunoblot analysis. Electrophoresed proteins were transferred to nitrocellulose filters (MSI) by electroblotting. After blocking with a buffer containing 2% fat-free dry milk, 1% Triton X-100, 50 mM Tris·HCl, pH 7.4, and 10 mM EDTA, membranes were incubated with primary antibodies for 1 h at room temperature. In the case of anti-phosphotyrosine blots, membranes were blocked with PBS + 0.1% Tween 20 containing 0.5% BSA. After washing, immunoblots were developed using the SuperSignal CL-HRP substrate system (Pierce, IL) with horseradish peroxidase-conjugated secondary antibodies (Jackson Laboratories). Radioactive samples were then subjected to autoradiography after stripping antibodies with a stripping buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, and 0.7% β-mercaptoethanol) for 30 min at 60°C.

RESULTS

Genistein markedly inhibits TER recovery after ATP repletion but has little effect in confluent monolayers. As shown in Fig. 1A, transient metabolic inhibition with 2 mM 2-deoxy-D-glucose and 10 µM antimycin A induced a rapid drop of TER in MDCK cell monolayers grown on filters, as previously described (35, 52). After changing to a normal growth medium, TER levels increased gradually to the initial level within 5 h (52, 56). The peak level after ATP repletion varied from 80% to 125% of the initial level. In this assay, recovery of TJ function was reproducibly observed and TER levels remained stable for up to 24 h after ATP repletion. To examine the hypothesis that this recovery of TJ function might, as in the MDCK cell "calcium switch" model, be regulated by signaling mechanisms that affect the phosphorylation of cellular proteins, we employed a variety of broad spectrum kinase inhibitors. An impressive and highly reproducible effect was observed with the tyrosine kinase inhibitor, genistein, which had minimal if any effect on TER in steady-state...
MDCK monolayers (Fig. 1B), but markedly suppressed TER recovery after ATP repletion (Fig. 1A). Genistein did not significantly affect ATP levels after ATP depletion-repletion (data not shown). Although 100 µM tyrphostin-25 had a similar (although weaker) effect, two other tyrosine kinase inhibitors (2 µM herbimycin A as well as 12.5 µM lavendustin A) did not have an inhibitory effect on TJ functional recovery. This pronounced effect of genistein but not other tyrosine kinase inhibitors raises the possibility that one or a limited subset of tyrosine kinases is involved in functional TJ recovery after ATP repletion.

Occludin reversibly shifts to an intracellular site after ATP depletion-repletion while several other TJ proteins remain near the cell margin. We next examined the subcellular localization of an integral membrane TJ protein, ZO-1, in MDCK monolayers after ATP depletion-repletion. Since occludin has been demonstrated to localize at the lateral membrane as well as at the TJ (43), we sought to identify these two pools of occludin by a laser-scanning immunofluorescent microscope. Using a z-series reconstructed image from 10 x-y plane sections, we could separate two pools of occludin: 1) occludin at the TJ, which gave a "honeycomb-like" linear staining pattern; and 2) occludin at the lateral membrane, which gave a weak crescentic staining pattern along lateral border of intact MDCK monolayers (Fig. 2A). Moreover, in agreement with a previous report (43), this lateral staining of occludin mostly disappeared after extraction with CSK-1 buffer that contains 0.5% Triton X-100 (Fig. 3C).

As previously described, the nontransmembrane TJ protein ZO-1 (like ZO-2 and cingulin) remained localized mainly to the lateral surface of the apical part of cells even after prolonged ATP depletion (Fig. 3E) (52). In contrast, the membrane component of the TJ, occludin (which was not examined in earlier studies), showed a significant redistribution and/or internalization after ATP deple-tion as analyzed by staining with an antiocludin polyclonal antibody (Fig. 2B). The same result was also observed with an anti-occludin monoclonal antibody (MOC 37) (data not shown). There was both a loss of the sharp honeycomb-like pattern and increased "fuzziness" of lateral cell surface staining as well as an increase in intracellular staining, which often appeared punctate in character (Fig. 2B). When MDCK cells were extracted with a buffer containing 0.5% Triton X-100 (CSK-1 buffer) and analyzed by confocal microscope, thinning of occludin staining was observed in ATP-depleted cells at the TJ level (colocalized with ZO-1) (Fig. 3D and E), suggesting that the intensity of occludin staining at the TJ may decrease to an intensity similar to that of the lateral border. Moreover, impressive punctate staining of occludin was observed intracellularly in basal confocal sections (Fig. 3F).

When the ATP depletion buffer was replaced with normal growth medium (ATP repletion), the (inextractable) punctate intracellular staining largely disappeared, and occludin staining was observed at the level of ZO-1 similar to that in steady-state monolayers (Figs. 2C and 3G). Treatment with 100 µM genistein after ATP repletion caused much of occludin to remain intracellularly localized in a pattern somewhat similar but not identical to that seen during ATP depletion (Figs. 2D and 3J and 3L). Treatment of genistein alone did not show any significant effect on distribution of occludin and ZO-1 (Fig. 3J and 3L). Together with the TER data, these findings indicate a correlation between the recruitment of occludin to the TJ and the reestablishment of a tight epithelial barrier after ATP depletion-repletion and suggest that a genistein-sensitive tyrosine kinase is important for the reassembly of the TJ during the ATP repletion phase.

A substantial fraction of occludin becomes detergent insoluble after ATP depletion, along with ZO-1 and ZO-2, although like these latter proteins, changing ionic strength does not affect the extractability of occludin in control and ATP-depleted monolayers. To clarify whether the changes in occludin in the ATP depletion-repletion model we observed by immunocytochemistry were accompanied by alterations in its biochemical
properties, we sought to examine its Triton X-100 solubility in comparison with the nonmembrane TJ proteins ZO-1 and ZO-2. We had previously shown that ZO-1, ZO-2, and cingulin all become highly inextractable to a Triton X-100-containing buffer after ATP depletion, and this is reversible with ATP repletion (52) (Fig. 4A). Initial analysis of occludin suggested a similar behavior. By Western blot (two volumes of E fraction and one volume of R fraction were loaded), most of the occludin in steady-state monolayers was found in the Triton X-100-extractable pool, although a small amount of occludin was also found in the residue at a variety of higher molecular weights corresponding to its less and more serine-phosphorylated forms, as described previously (Fig. 4) (43). After ATP depletion, both the extractable and residue fractions revealed a

Fig. 3. Immunolocalization of occludin after ATP depletion-repletion in CSK-1 buffer-extracted MDCK monolayers. After ATP depletion-repletion, cells grown on collagen-coated coverslips were extracted with CSK-1 buffer for 20 min at 4°C, fixed with 1% paraformaldehyde for 15 min, and processed for immunofluorescence (occludin, A, C, D, F, G, J, L, M, and O; and ZO-1, B, E, H, K, and N). Then samples were examined through a confocal fluorescence microscope (Bio-Rad, model MRC-1024). Images were taken at the TJ level (ZO-1 as a indicator) and the basolateral level (1 µm above the base of the cell). Shown are control monolayers (A–C) and monolayers after 1 h of ATP depletion (D–F), 3 h of ATP repletion in presence (J–L), or absence (G–I) of 100 µM genistein, or treatment with 100 µM genistein alone in steady-state MDCK monolayers (M–O). Note that most intracellular occludin in control as well as ATP-replete monolayers is extracted with CSK-1 buffer at the basolateral level (C and I) (see Fig. 2A and 2C), whereas small punctate staining of occludin is still seen intracellularly and along cell-cell border in ATP-depleted monolayers (F). In genistein-treated cells after ATP repletion, granular staining of occludin is also seen at the basolateral level (L). ZO, zonula occludens. Bar = 25 µm.
Fig. 4. Analysis of Triton X-100 extractabilities of TJ proteins after ATP depletion in MDCK cells. A: time course of detergent-physiological salt extractabilities of TJ proteins after ATP depletion-repletion. MDCK cell monolayers were extracted at various times after ATP depletion-repletion with CSK-1 buffer. Two volumes of extractable fraction ("E") and one volume of residue fraction ("R") were separated by 7.5% SDS-PAGE and transferred to nitrocellulose. Western blots were probed with anti-ZO-1, ZO-2, and occludin antibodies (A-a). Blots were quantified with NIH Image software, and measurements were expressed as percentage of total density of both fractions (E/E+R) (A-b).

B: effect of salt concentration on extractability of occludin after ATP depletion. Cells were extracted after 1 h of ATP depletion with CSK buffer containing different concentrations of NaCl (0–500 mM) and subjected to Western blotting with anti-ZO-1 (B-a) or anti-occludin antibody (B-c). Blots were quantified and measurements were expressed as described above (B-b and B-d). Note that extractability of occludin in control and ATP-depleted monolayers was not affected by increasing concentration of NaCl, whereas extractability of ZO-1 increased with higher concentrations of NaCl in the cells. Solid circle, ZO-1 or occludin in control; solid square, ZO-1 or occludin after 1 h of ATP depletion. Data are representative of 6 separate experiments.
gradual collapse of occludin to the dephosphorylated 65-kDa form(s) when analyzed by SDS-PAGE (Fig. 4A-a) (discussed in more detail below). Densitometric analysis revealed a significant decrease in the amount of Triton X-100-soluble occludin after ATP depletion (Fig. 4A-b). This decrease in extractability of occludin was completely reversible upon ATP repletion, as was also the case for ZO-1 and ZO-2 (Fig. 4A). Moreover, the higher-molecular-mass smear of inextractable occludin (corresponding to its phosphorylated forms) was increased after ATP repletion (Fig. 4A-a). Genistein treatment of monolayers not subjected to ATP depletion did not alter the extractability of occludin, ZO-1, or ZO-2 (data not shown).

Since, after ATP depletion, the nontransmembrane TJ proteins ZO-1, ZO-2, and cingulin become extractable in a detergent buffer containing high concentrations of NaCl (52), we examined the effect of ionic strength on the extractability of occludin after ATP depletion. When the NaCl concentration in the extraction buffer (see MATERIALS AND METHODS) was varied from 0 to 500 mM, the extractability of occludin was not affected (Fig. 4, B-c and B-d), although the extractability of ZO-1 was clearly sensitive to ionic strength (Fig. 4, B-a and B-b). Thus, although a substantial fraction of occludin becomes inextractable after ATP depletion, as is the case for other TJ proteins, the lack of occludin sensitivity to high ionic strength buffers containing Triton X-100 suggests the biochemical basis of occludin inextractability may be due to a somewhat different mechanism from that for ZO-1 and ZO-2. It should be noted here that occludin was the only integral membrane TJ protein analyzed in this study (others were cytosolic).

Movement of occludin, ZO-1, and ZO-2 back into the soluble pool after ATP repletion is retarded by tyrosine kinase inhibition. Since genistein inhibited the movement of the inextractable intracellular occludin which accumulates after ATP depletion back to the TJ (Figs. 2 and 3), we sought to analyze the effect of genistein on the reversibility of occludin movement from the inextractable to the extractable fraction after ATP repletion; it seemed plausible that the genistein-sensitive punctate intracellular staining might represent intracellular aggregates of occludin, perhaps due to self-aggregation or association with the cytoskeleton, which is also disrupted after ATP depletion (38, 52). Indeed, when cells were treated with genistein during ATP repletion, detergent-inextractable occludin, as well as ZO-1 and ZO-2, did not move back to the extractable pool even after 3 h of ATP repletion (Fig. 5, A and C). This was seen with both standard extractions in buffers

\[ \text{Fig. 5. Effect of genistein on extractabilities of TJ proteins after ATP repletion in MDCK cell monolayers.} \]

A: cells were extracted with CSK-1 buffer [0.5% Triton X-100, 10 mM Tris·HCl (pH 7.5), 100 mM NaCl, 300 mM sucrose, plus a protease inhibitor cocktail] in presence (“G”) or absence (“R”) of 100 µM genistein after 3 h of ATP repletion, then separated into two parts (“E” and “R”). B: blots were quantified, and measurements were expressed as percentage of total density of both fractions (E/E + R). C: cells were solubilized with a buffer that contains 1% Triton X-100, 0.5% deoxycholate, 0.2% SDS, 10 mM HEPES (pH 7.5), 100 mM NaCl, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 2 mM EDTA, 2 mM sodium orthovanadate (NaV), plus a protease inhibitor cocktail upon incubation in presence (“G”) or absence (“R”) of 100 µM genistein after 3 h of ATP repletion. Samples were separated into two parts (“S” and “P”). The same volumes of each fraction were separated by 7.5% SDS-PAGE and subjected to Western blotting. D: blots were quantified, and measurements were expressed as percentage of total density of both fractions (S/S + P). Note the differences in shape of occludin in the insoluble (“P”) fraction. Data are representative of 4 separate experiments.
containing 0.5% Triton X-100 (Fig. 5A) and those which included 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS (Fig. 5C). After ATP repletion, in untreated cells the predominant form of occludin was a high-molecular-mass form with few intermediate forms, whereas in genistein-treated cells not only was a high-molecular-mass form present, but there were bands of intermediate-molecular mass (Fig. 5, A and C). This was always best seen in the pellet fraction (P) present after solubilization with buffers containing ionic detergents (Fig. 5C), and both patterns were clearly different from those in control cells. The results indicate that tyrosine phosphorylation is involved in the movement of occludin and other TJ proteins between inextractable/insoluble and extractable/soluble pools after ATP depletion-repletion and suggest that a variety of occludin intermediates are formed during TJ reassembly prior to acquisition of a stable phosphorylation state. (The question of phosphorylation is analyzed further in more detail below.)

Occludin fractionates into a large macromolecular complex after ATP depletion. The insolubility of occludin after ATP depletion suggested that it might associate with itself or with other proteins, and the relative resistance of its insolubility to buffers containing ionic detergents or high salt (Figs. 4 and 5) suggested that these associations might be different than those for ZO-1 and ZO-2, which can be almost completely solubilized after ATP depletion and are known to exist in high-molecular-mass complexes (52). We therefore sought to analyze the size of soluble occludin by isopycnic centrifugation. Cells were lysed in a buffer containing 100 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS, which was able to solubilize nearly all of ZO-1 and ZO-2, as well as 80% of occludin in control and ATP-replete cells, and also 95% of ZO-1 and ZO-2 but only 50% of occludin in ATP-depleted cells (Fig. 5D). The lystate from control, ATP-depleted, ATP-replete, and ATP-replete/genistein-treated cells was subjected to rate-zonal centrifugation through 5–20% sucrose gradients. As shown in Fig. 6, more occludin and ZO-1 were found in higher density fractions (fractions 10–17) after ATP depletion. Densitometric analyses showed an approximately twofold increase of occludin and ZO-1 in higher density fractions (fractions 10–17) (Fig. 6C). After 3 h of ATP repletion, this movement of soluble occludin and ZO-1 into high-density fractions was almost completely reversed. Genistein did not retard this movement of occludin and ZO-1 back to lower density fractions after ATP repletion. Thus, even though they exhibit some differences in their solubility behavior, occludin as well as ZO-1 form high-molecular-mass complexes after ATP depletion (consistent with their decreased solubility) (Figs. 4 and 5), and the dissolution of these TJ protein-containing complexes that could be solubilized after ATP repletion is not sensitive to genistein, despite the fact that the movement of ZO-1 and occludin from the inextractable/insoluble to the extractable/soluble fraction is clearly sensitive to genistein (Fig. 5, A and C). Nevertheless, it is worth noting here this approach was limited by the fact that it was not possible to solubilize intact occludin complexes from the residue fraction in ATP-depleted and genistein-treated cells; more than 0.5% of SDS necessary to solubilize Triton X-100-insoluble occludin resulted in monomeric occludin found almost exclusively in fractions 2–5 on sucrose gradients (data not shown).

Occludin, ZO-2, and p130/ZO-3 are tyrosine phosphorylated after ATP repletion in a genistein-sensitive fashion. To analyze the total phosphorylation and tyrosine phosphorylation of occludin, we metabolically labeled MDCK monolayers with inorganic $^{32}$P, separated them into two pools with CSK-A and CSK-B buffers (the extractability of occludin in CSK-A buffer, which contains 0.5% Triton X-100 as the sole detergent, was approximately the same as in CSK-1 buffer; however, CSK-B buffer, which contains 1% SDS, was necessary to solubilize the Triton X-100-insoluble occludin that accumulated in ATP-depleted and genistein-treated cells; data not shown), and subjected the extracts to immunoprecipitation. Although longer incubations (more than 22 h) with $^{32}$P were performed to maximize $^{32}$P labeling of occludin as has been done in previous studies by others (43, 54), we chose 3 h for metabolic labeling with $^{32}$P because the functional recovery of the TJ (TER) occurred within 3 h after ATP repletion (Fig. 1). Under these conditions, the incorporation of $^{32}$P into occludin at the TJ (Triton X-100-insoluble occludin) appeared to be less than that of Triton X-100-soluble occludin (data not shown). In the case of ZO-1 immunoprecipitates, cells were lysed with a detergent-rich buffer (containing 1% Triton X-100, 0.5% deoxycholate, and 0.2% SDS), which could solubilize more than 95% of ZO-1 and ZO-2 in all conditions (Fig. 5, C and D).

 Autoradiography of $^{32}$P-labeled occludin immunoprecipitates revealed higher-molecular-weight occludin in the Triton X-100-insoluble (CSK-B-soluble) fraction (Fig. 7; in contrast to Fig. 5, one volume of E fraction and one volume of R fraction were analyzed) (43, 54). Occludin in both Triton X-100-soluble (CSK-A; E) and -insoluble (CSK-B; R) pools was dephosphorylated after ATP depletion and rephosphorylated after ATP repletion. The loss of occludin phosphorylation correlated with the collapse of occludin bands detected on immunoblots to a single 65-kDa band (Fig. 7). Immune complexes containing ZO-1 showed similar rephosphorylation (Fig. 8A). Moreover, an immunoblot of the same filter with anti-phosphotyrosine antibody revealed that the higher-molecular-weight occludin (in the Triton X-100-insoluble fraction) and ZO-2 and p130/ZO-3 (but not ZO-1) were tyrosine phosphorylated after ATP repletion (Figs. 7 and 8B). Incorporation of inorganic $^{32}$P into Triton X-100-insoluble occludin (R) was found to be increased in ATP-replete MDCK cells compared with steady-state cells, suggesting that incorporation of $^{32}$P into Triton X-100-insoluble occludin after ATP repletion is higher than that in control cells. Since Triton X-100-insoluble occludin is located at the TJ in both steady-state and ATP-replete MDCK monolayers, this
indicates that the rephosphorylation of occludin, including its tyrosine phosphorylation, is enhanced at the TJ after ATP repletion.

Genistein, which inhibited functional, immunocytochemical, and biochemical correlates of TJ reassembly after ATP repletion, inhibited the total as well as tyrosine phosphorylation of Triton X-100-insoluble occludin, leading to lower-molecular-weight forms of phosphorylated occludin (Fig. 7). Genistein also inhibited tyrosine (though not total) phosphorylation of ZO-2 and p130/ZO-3 (Fig. 8B). Although several additional tyrosine phosphorylated proteins (of molecular masses ~250 and 110 kDa) could be detected in ZO-1 immunoprecipitates and their tyrosine phosphorylation was inhibited by genistein as well, the identity of these proteins remains unclear (Fig. 8B). NaV treatment of cells, a specific inhibitor of tyrosine phosphatases, did not increase or decrease the total phosphorylation content of either occludin- or ZO-1-containing complex (Figs. 7 and 8A). However, occludin (in both Triton X-100-soluble and -insoluble pools), as well as ZO-1, ZO-2, and p130/ZO-3, were highly tyrosine phosphorylated in NaV-treated cells (Figs. 7 and 8B). Together, these results suggest that TJ proteins are differentially phosphorylated on tyrosine and possibly other residues during TJ reassembly after ATP repletion. The tyrosine
phosphorylation appears to be mediated by a genistein-sensitive kinase.

In some experiments, immunoblots with antibodies against ZO-1 and ZO-2 immunoprecipitates containing tyrosine-hyperphosphorylated ZO-1 and ZO-2 (after NaV treatment) revealed that the anti-ZO-1 antibody could not recognize its antigen when it was highly tyrosine phosphorylated (Fig. 8C). This finding was not specific for cells after ATP repletion in the presence of NaV and was also observed in NaV-treated cells without ATP depletion (data not shown).

Changes in the phosphorylation status of TJ proteins during ATP depletion-repletion leads to mobility shifts on SDS-PAGE of occludin, ZO-1, and ZO-2. In addition to changes in the mobility of occludin on SDS-PAGE after ATP depletion that were reversed during ATP repletion, we observed a decrease in the apparent molecular masses of both ZO-1 and ZO-2, which increased to the original apparent molecular masses after ATP repletion (Figs. 7 and 8). To determine whether these reversible mobility shifts of TJ proteins in the ATP depletion-repletion model might correspond to dephosphorylation/rephosphorylation, TJ proteins immunoprecipitated with specific antibodies were subjected to alkaline phosphatase treatment as has been previously done with occludin (43). As shown in Fig. 9, in both ATP-replete and control cells, this treatment had the same effect on TJ protein mobilities as ATP depletion. Thus the mobility shifts of the three TJ proteins in the ATP depletion-repletion model are due to the existence of dephosphorylated and rephosphorylated forms.
Fig. 9. Alkaline phosphatase (Alk P) treatment leads to mobility shifts of ZO-1, ZO-2, and occludin on SDS-PAGE gels similar to mobility shifts observed after ATP depletion. After ATP depletion and following repletion, MDCK monolayers were lysed and subjected to immunoprecipitation with the indicated antibody. Immune complexes were then incubated with Alk P for 30 min and analyzed by Western blot. Note that the molecular weight of TJ proteins (occludin, ZO-1, and ZO-2) after Alk P treatment shows a decrease comparable to that seen after ATP depletion. Data are representative of 3 separate experiments.

**DISCUSSION**

Many of our results are summarized in Table 1. The membrane TJ protein, occludin, redistributed significantly after ATP depletion and virtually completely regained its original distribution after ATP repletion (Figs. 2 and 3), correlating with the recovery of TER (Fig. 1). A tyrosine kinase inhibitor, genistein, blocked this functional recovery of the TJ after ATP repletion, and this was associated with the incomplete recruitment of occludin to the TJ (Figs. 1–3). Although genistein did not alter the size of occludin-containing macromolecular complexes as well as ZO-1-containing complexes after ATP repletion (Fig. 6), it inhibited the movement of TJ proteins from Triton X-100-insoluble pool to the Triton X-100-extractable pool (Figs. 4 and 5), as well as the rephosphorylation of TJ proteins (Figs. 7 and 8). During the rephosphorylation, tyrosine phosphorylation of occludin as well as ZO-2, p130/ZO-3, and other proteins was observed, and this was blocked by genistein (Figs. 7 and 8).

Phosphorylation is known to regulate the function of both existing and assembling TJ s (11, 40, 43, 47). In fact, in the “calcium switch”, another model for TJ assembly, it is clear that protein kinase C plays an important role in TJ biogenesis (4, 40, 47). Thus it seemed plausible that regulated protein phosphorylation might play a role in the reassembly of the TJ after ATP depletion-repletion. We have now shown, by functional, immunocytochemical, and biochemical analyses that TJ reassembly after ATP repletion is dependent upon a genistein-sensitive tyrosine kinase. Disassembly of the TJ during ATP depletion is accompanied by a marked diminution in the phosphorylation content of all the TJ proteins studied, which is reflected in changes in the electrophoretic mobility of ZO-1, ZO-2, and occludin (Figs. 8, C and D, and 9). TJ reassembly during ATP repletion led to the rapid rephosphorylation of the TJ proteins (Figs. 7 and 8A). The higher-molecular-mass bands of occludin corresponding to its hyperphosphorylated form tended to associate with the insoluble fraction after ATP repletion as well as steady-state MDCK monolayers (Figs. 4, 5, and 7), consistent with what has been previously described (43). The somewhat different profiles of phospho-occludin bands in control and ATP-replete cells raises the possibility that extensive “remodeling” of this and other TJ proteins may occur due to a series of phosphorylation and dephosphorylation steps during TJ reassembly before a stable phosphorylation state is established (Fig. 5); a genistein-sensitive tyrosine kinase is likely to be involved in this process. A lag time of recovery (~2 h) between the change in extractability of TJ proteins and the recovery of TER after ATP repletion (Figs. 1A and 4A) was observed. This may be because the reassembly of the functional TJ after ATP repletion could be a multi-step process, and the rephosphorylation and dissociation of TJ proteins from the inextractable to extractable pool, followed by the recruitment of occludin to the TJ, might be necessary for “remodeling” of the TJ for it to become fully functional.

During TJ reassembly in the ATP repletion phase of this model, we were able to detect the phosphorylation of Triton X-100-insoluble occludin, ZO-2, and p130/ZO-3 on tyrosine residues (Figs. 7 and 8B), whereas we were unable to reproducibly detect any such tyrosine phosphorylation of ZO-1 (Fig. 8B). This “differential” tyrosine phosphorylation, which may reflect a specific role for the ZO-1-containing complex in the reassembling TJ, was inhibited by the tyrosine kinase inhibitor genistein. Although a direct link remains to be established, the potential functional importance of tyrosine phosphorylation in TJ reassembly was strongly supported by the ability of genistein to inhibit the recovery of TER (Fig. 1). During ATP repletion, the tyrosine phosphorylation of other unknown proteins associated with the ZO-1 complex was observed (Fig. 8B). This raises the possibility that other “minor” proteins exist in the ZO-1 complex, and their tyrosine phosphoryla-

**Table 1. Effect of genistein on TJ reassembly after ATP repletion**

<table>
<thead>
<tr>
<th>Effect on resolution of complexes</th>
<th>Genistein Effect</th>
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<tbody>
<tr>
<td>Ocludin</td>
<td>+ (Fig. 7)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>− (Fig. 8)</td>
</tr>
<tr>
<td>ZO-2</td>
<td>±(Fig. 8)</td>
</tr>
<tr>
<td>p130/ZO-3</td>
<td>±(Fig. 8)</td>
</tr>
<tr>
<td>Tyrosine phosphorylation of occludin</td>
<td>+ (Fig. 7)</td>
</tr>
<tr>
<td>ZO-1</td>
<td>?</td>
</tr>
<tr>
<td>ZO-2</td>
<td>+ (Fig. 8)</td>
</tr>
<tr>
<td>p130/ZO-3</td>
<td>+ (Fig. 8)</td>
</tr>
</tbody>
</table>

Effect of genistein is measured from positive to negative (+, ±, and -, respectively). Relevant figures are shown in parentheses. TJ, tight junction.
tyrosine phosphorylation could be important in TJ reassembly, at least in this context.

In sucrose density gradient analyses of detergent-soluble TJ proteins, both occludin and ZO-1 sedimented in large macromolecular complexes after ATP depletion (Fig. 6). The insolubility of these proteins and their presence in very large complexes presumably reflects increased cytoskeletal association, as has previously been shown for ZO-1 after ATP depletion (52), and possibly aggregation, which are reversible after ATP repletion. Whereas genistein treatment did not affect the dissolution of the large complexes containing ZO-1 and occludin after ATP repletion (Fig. 6), it did inhibit the resolubilization of these proteins (Fig. 5). Nevertheless, it should be kept in mind that because of the extensive insolubility of occludin after ATP depletion, and because detergents that could solubilize it appeared to disrupt protein-protein interactions on sucrose gradients, it remains possible that the insoluble complex present in genistein-treated cells after ATP repletion is very large. The behavior of occludin after ATP depletion-repletion differs from that which we previously reported for the nonintegral membrane proteins of the TJ, ZO-1, ZO-2, and cingulin in several important ways (52). Whereas the striking insolubility of the latter proteins after ATP depletion could be decreased by raising the ionic strength of detergent-salt buffers, the ratio of insoluble occludin did not change when the salt concentration was increased (Fig. 4). This suggests that the biochemical basis of occludin insolubility, whether due to cytoskeletal association or self-aggregation or both, is (at least in part) different from that responsible for the insolubility of the other three nontransmembrane TJ proteins. Taken together, our data indicates that not only is tyrosine phosphorylation important for functional TJ recovery, but it is also necessary for the release of specific TJ proteins from the insoluble fraction during TJ reassembly. This may be due to a specific interaction between TJ proteins and the cytoskeleton that is dependent upon tyrosine phosphorylation (of either occludin, ZO-2, or p130/ZO-3, for example) or due to a disaggregation step that is similarly regulated.

ZO-1, ZO-2, p130/ZO-3, and occludin are all intensely tyrosine phosphorylated in the presence of NaV. This suggests the existence of a tyrosine kinase that can, at least under certain conditions, act on most TJ proteins. It had been previously been shown that Src activation can lead to tyrosine phosphorylation of ZO-1 (49, 50). The impressive effect of NaV also suggests the existence of a counteracting phosphatase that has access to the TJ (6, 39). It is worth noting that hyperphosphorylation of ZO-1 on tyrosine residues in the presence of NaV led to the disappearance of the ZO-1 antibody signal on Western blots, suggesting that the epitope recognized by the antibody can be altered by tyrosine phosphorylation somewhere on the molecule.

The fact that ZO-1 and ZO-2 remain at the lateral cell surface after ATP depletion may provide both a scaffolding and homing information for occludin and other TJ-associated proteins to quickly reassemble during ATP repletion. Interestingly, occludin has been reported to directly bind to the guanylate kinase domain of ZO-1 (17). The presence of this domain in ZO-1 and ZO-2, which (unlike occludin) remain near the TJ after ATP depletion, may be important in the recruitment of occludin and other proteins back to the TJ, and this process may be regulated by phosphorylation, including tyrosine phosphorylation (as suggested by the results in this study). It is now established that occludin is not the only membrane protein of the TJ (42). Indeed, a new set of integral membrane proteins localizing at TJs, the claudins, were reported during preparation of this report (21). The contribution of these molecules to TJ function and its regulation is currently unknown, but in the future it will be important to analyze the behavior of these proteins in TJ reassembly after ATP repletion.

Growth factors have been shown to ameliorate acute epithelial tissue injury and may soon to find their way into clinical practice (10, 20, 24, 27). However, the mechanistic basis of their protective effect remains far from clear. Most likely, growth factors act through a variety of mechanisms to exert their protective and possibly regenerative effects, including enhanced cell proliferation, motility, anti-apoptosis, and morphogenesis (9, 23, 46). Although it remains unclear whether transmembrane receptor tyrosine kinases (RTKs) are internalized after ATP depletion like several other membrane proteins, including occludin (36), if such is the case, it may induce the cell to increase their synthesis. Since, after a brief insult, both the original (previously internalized) and new RTKs would be expected to populate the plasma membrane, the cell may be “primed” to respond to growth factors. It is also possible that reactive oxygen species (ROS), which are generated during ATP depletion-repletion and during ischemia-reperfusion injury, induce tyrosine phosphorylation and inhibit protein tyrosine phosphatase activity; the latter effect is mediated through an essential sulfhydryl group that is responsible for the phosphatase activity and susceptible to oxidation (26, 45). Moreover, ROS have been demonstrated to activate tyrosine kinases such as Src (18, 30). Thus, in the setting of ATP depletion and repletion, certain ROS play a role in reassembly of the TJ through a cascade involving tyrosine phosphorylation.

The results of this study are consistent with whole organ studies that indicate that the impaired TJ barrier function provides a paracellular pathway through which back leak of a number of solutes occurs, leading to a reduction of glomerular filtration in ischemic acute renal failure. Our data begin to suggest how the reassembly of the TJ, and thus the recovery of the permeability barrier, might be regulated by tyrosine phosphorylation after acute ischemic renal tubular injury.

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