Oncogenic Raf-1 Disrupts Epithelial Tight Junctions via Downregulation of Occludin

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Abstract. Occludin is an integral membrane protein of the epithelial cell tight junction (TJ). Its potential role in coordinating structural and functional events of TJ formation has been suggested recently. Using a rat salivary gland epithelial cell line (Pa-4) as a model system, we have demonstrated that occludin not only is a critical component of functional TJs but also controls the phenotypic changes associated with epithelium oncogenesis. Transfection of an oncogenic Raf-1 into Pa-4 cells resulted in a complete loss of TJ function and the acquisition of a stratified phenotype that lacked cell-cell contact growth control. The expression of occludin and claudin-1 was downregulated, and the distribution patterns of ZO-1 and E-cadherin were altered. Introduction of the human occludin gene into Raf-1–activated Pa-4 cells resulted in reacquisition of a monolayer phenotype and the formation of functionally intact TJs. In addition, the presence of exogenous occludin protein led to a recovery in claudin-1 protein level, relocation of the zonula occludens 1 protein (ZO-1) to the TJ, and redistribution of E-cadherin to the lateral membrane. Furthermore, the expression of occludin inhibited anchorage-independent growth of Raf-1–activated Pa-4 cells in soft agarose. Thus, occludin may act as a pivotal signaling molecule in oncogenic Raf-1–induced disruption of TJs and regulates phenotypic changes associated with epithelial cell transformation.

Key words: occludin • Raf-MEK-ERK signaling • tight junction • claudin-1 • epithelium transformation

Introduction

Epithelia exhibit specialized structures involved in cell-cell contacts known as tight junctions (TJs) and adherens junctions (AJs). AJ complexes are involved in maintaining cell-cell adhesions between adjacent epithelial cells, whereas TJ structures provide the barrier to uncontrolled paracellular permeability. The positioning of these junctions is coordinated and stabilized through associations with a continuous band of bundled actin filaments known as an adhesion belt (Madara, 1998). Occludin is a transmembrane protein located at TJ complexes (Saitou et al., 1997) and appears to be involved in the tight coupling between adjacent epithelial cells (Wong and Gumbiner, 1997; Lacaz-Vieira et al., 1999). Interactions of occludin with several other components of the TJs have been proposed (Fanning et al., 1999). In particular, an association with intracellular protein zonula occludens 1 (ZO-1) (Furuse et al., 1994) has led to the possibility that occludin may act to organize the annular ring of actin present in the cytosol at the neck of epithelial cells (Madara, 1998). Many studies suggest that occludin is involved in the barrier and fence functions of the TJs: overexpression of occludin in MDCK cells increases the number of TJ strands and their transepithelial electrical resistance (TEER) (McCarthy et al., 1996); the COOH terminus of occludin is required for the correct assembly of TJ barrier function (Chen et al., 1997); small synthetic peptides homologous to the external loops of occludin impair TJ resealing (Wong, 1997; Lacaz-Vieira et al., 1999); and a dominant mutant of occludin can disrupt TJ structure and function (Bamforth et al., 1999). However, there is also evidence indicating that occludin is not the only integral membrane protein involved in establishing the paracellular barrier of the TJs. When occludin was overexpressed in mouse L fibroblasts, only short and poorly developed TJ strand–like structures were formed (Furuse et al., 1998a). In primary cultured rat hepatocytes, disruption of circumferential actin filament caused disappearance of occludin from the cell borders without distinct changes in TJ strands (Kojima et al., 1999). Most directly, occludin knockout embryonic stem cells formed well-developed TJ structures (Saitou et al., 1998). These findings have led to the recent identification of claudins, a family of transmembrane proteins located at epithelial TJs (Furuse et al., 1998b). Although the exact physiological function of occludin in TJs remains unresolved, it is likely...
that claudins and occludin can interact in a collaborating way in order to achieve the full function of TJ's as a paracellular barrier and transmembrane fence.

Loss of TJ and AJ structures is frequently observed in epithelium-derived cancers (Quinonez and Simon, 1988). E-cadherin at AJ complexes signals through interactions involving α-, β-, and γ-catenins (Weiss et al., 1998) and loss of functional coupling through E-cadherin has been shown to induce a transformed phenotype in epithelial cells (Takeichi, 1993). Although epithelial-derived cancers appear to be lacking both AJ and TJ complexes (Quinonez and Simon, 1988), a role for functional coupling through TJ structures similar to that observed through AJ structures has not been described. It is possible that anti-neoplastic signaling through TJ structures is similar to that observed for AJ structures. This is supported by the finding that several tumor-promoting agents can induce TJ disruption (Soler et al., 1993), and that neoplastic progression can be correlated with an increase in paracellular permeability across epithelia, suggesting a loss of TJ function (Mullin et al., 1997). If signaling through cell–cell contacts at AJ and TJ structures is important for growth and differentiation events in epithelial cells, then similar mechanisms may control the activity of these two systems.

Sequence mutations that result in a constitutively active state of the small GTP-binding protein Ras as are commonly associated with epithelium oncogenesis (Yu and Poirier, 1988). Also, the cell activation characteristics of ras mutations make it an important component in the cascade of genetic alterations described for cancers derived from epithelial cell sheets (Vogelstein et al., 1988). Thus, Ras and the intracellular pathways driven by Ras would be prime candidates for possible regulatory function if TJ signaling was a component of epithelial cell transformation. A constitutively active RAf-1 stimulates multiple intracellular signaling pathways (Marshall, 1996). In particular, RAf-1 is considered to be a primary and central downstream effector of Ras (Marshall, 1996). A well-established signaling pathway activated by RAf-1 involves sequential phosphorylation and activation of mitogen-activated protein kinase (MEK) and extracellular-regulated kinase (ERK) kinases (Cobb et al., 1994). A constitutively active RAf-1 kinases regulate a variety of cellular processes, including proliferation, differentiation, and transformation (Cobb et al., 1994). We manipulated the activity of RAf-1 in Pa-4 cells, an immortalized rat parotid gland cell line, which grows as a monolayer with high TEER on semipermeable supports similar to normal epithelia. Stable transfections of Pa-4 cells were carried out with an oncogenic RAf-1, ΔRAf-1:ER, which was constructed by fusing the kinase domain of RAf-1 with the hormone-binding domain of estrogen receptor (Samuels et al., 1993). We have shown that a constitutively active RAf-1 induces the transition of Pa-4 cells from a high-resistance monolayer to a low-resistance multilayer phenotype characteristic of oncogenic conversion. These modified cells demonstrate a redistribution of the actin cytoskeleton along with a downregulation of occludin expression. In addition, introduction of an exogenous occludin gene into RAf-1–activated Pa-4 cells results in reacquisition of normal epithelial monolayer phenotype and functionally intact TJs. Furthermore, the expression of exogenous occludin appeared to stabilize and coordinate membrane localization of other junctional proteins in RAf-1–activated Pa-4 cells, and the presence of occludin also inhibited anchorage-independent growth of PA4ΔRaf:ER cells. Based on these results, we suggest that occludin plays a critical role in the disruption of epithelial TJ's induced by oncogenic RAf-1.

Materials and Methods

Reagents and Antibodies

An antibody that specifically recognizes the phosphorylated form of ERK1 and ERK2 was purchased from Santa Cruz Biotechnologies, Inc. An antibody to occludin, ZO-1, and claudin-1 were purchased from Zymed Laboratories, Inc. An antibody to E-cadherin was from Transduction Laboratories. PD98059, a selective inhibitor of MEK, was purchased from Calbiochem. Rhodamine-phalloidin was obtained from Molecular Probes, Inc.

Constructs and Plasmids

A constitutively active RAf-1 (ΔRAf-1:ER), comprised of the catalytic domain of human RAf-1 fused with the hormone-binding domain of human estrogen receptor was constructed as described (Samuels et al., 1993). A constitutively active form of RAf-1 containing only the catalytic domain of the kinase, was kindly provided by Dr. J. Jakob Tropmair, University of Wurzburg, Wurzburg, Germany (Bruder et al., 1992). A constitutively active MEK mutant, pFC-MEK1, was obtained from Stratagene. Human occludin cDNA was cloned using a TB4 cell library (Stratagene). The probe used in cloning and Northern blotting was obtained by reverse transcription-PCR. Human occludin cDNA was then subcloned into pcDNA vector, which has a cytomegalovirus promoter.

Cell Culture, Transfections, and Treatments

Pa-4ΔRAf-1:ER cells were established as described previously by stably transfecting Pa-4 cells with the ΔRAf-1:ER construct (Li et al., 1997). Pa-4-vec and Pa-4ΔRAf-1:ER cells were cultured in DME/F12 (1:1) medium supplemented with 2.5% FBS, insulin (5 μg/ml), transferrin (5 μg/ml), EGF (25 ng/ml), hydrocortisone (1.1 μM), glutamate (5 mM), G418 (600 μg/ml), and were maintained in a humidified atmosphere containing 5% CO2 and 95% air at 35°C. Pa-4ΔRAf-1:ER-occludin cells were raised by stably transfecting Pa-4ΔRAf-1:ER cells with pcDNA-occludin and PTX-Hyg vector (Clontech) for hygromycin selection (100 μg/ml). Charcoal-stripped serum was used in maintaining Pa-4ΔRAf-1:ER and Pa-4ΔRAf-1:ER-occludin cells to minimize the estrogen level in the culture medium. Cells grown on semipermeable supports were plated at 106 cells/cm² onto collagen-coated clear polyester membrane of Costar Transwell® (0.4-μm pore size; 1-cm² surface area). The transcellular resistance of confluent epithelial sheets was measured after 1 wk in culture with a chopstick Millicell-ERS® voltmeter (Millipore). Transient transfections were performed using Lipofectamine Plus™ (Life Technologies, Inc.) and following the manufacturer’s protocol. Cells were collected and lysed 48 h after the start of transfection. Treatment of PD98059 in the transient transfection experiments was carried out by adding the inhibitor (20 μM final) 5 h after the start of transfection. Measurement was replaced every 24 h with fresh PD98059.

Western and Northern Blottings

Western Blotting. Cell lysates, prepared as Triton X-100-soluble and -insoluble fractions, were made as described previously (Li et al., 1997). In brief, cells were lysed in cold lysis buffer (Samuels et al., 1993) containing 20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 5 mM EDTA, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 μM pepstatin A, 1 mM sodium orthovanadate, 1 mM EGTA, 10 mM NaF, 1 mM tetrasodium pyrophosphate, 100 μM b-glycero phosphate at 4°C. Lysates were incubated at 4°C for 30 min, and the insoluble material was removed by centrifugation at 12,000 g for 10 min at 4°C. Triton X-100-insoluble pellets were dissolved in RIPA buffer containing 0.2% SDS. Protein concentrations were determined by Pierce BCA assay. Equal amounts of protein of cellular lysates (20 μg) were subjected to SDS-PAGE and Western blotting. Cell lysates were probed with a variety of antibodies. Equal amounts of cellular lysates (20 μg) were subjected to SDS-PAGE and Western blotting. Cell lysates were probed with a variety of antibodies.
with 5% milk solution for 1 h before incubation with primary antibodies. HRP-conjugated secondary antibodies and the enhanced chemiluminescence detection system (NEL™ Life Science Products, Inc.) were used to detect bound antibodies. The quantitative analyses of protein levels were carried out using the NIH Image 1.60/PC software.

Northern Blotting. Total cellular RNA was prepared using TRIzol™ reagent according to the manufacturer’s instructions (Life Technologies). Denatured samples were size fractionated on a formaldehyde (2.2 M)/agarose (1.5%) gel, blotted onto a ZetaProbe nylon membrane (Bio-Rad Laboratories) and hybridized with a 32P-labeled DNA probe prepared using the Ready-To-Go DNA labeling kit (Amersham Pharmacia Biotech). Northern analysis for claudin-1 message levels was carried out using a PCR-derived cDNA sequence probe (Furuse et al., 1998). Glyceraldehyde 3-phosphate dehydrogenase mRNA levels were monitored as loading controls.

Microscopy and Confocal Imaging

Phase-contrast microscopy of cells on plastic culture dishes was done using a Nikon D labophot 300 inverted microscope attached to a Nikon N6006 camera. Cells grown on Transwell® filters were fixed for 10% normal buffered formalin. Thin sections cut from paraffin-embedded samples were stained with hematoxylin and eosin and viewed by light microscopy. Characterization of filamentous actin distribution was achieved using cells fixed in formalin, permeabilized with 0.2% (vol/vol) Triton X-100, and labeled with rhodamine-phalloidin as described previously (Phillips and Tsan, 1988). Immunofluorescence staining of occludin, ZO-1, E-cadherin, and claudin-1 was carried out on cells fixed in methanol at −20°C for 10 min. Cells were then analyzed using a Leica TCS SP laser scanning confocal microscope.

Measurement of Cloning Efficiency in Soft Agarose

Pa-4-vec, Pa-4ARaf-1:ER, and Pa-4ARaf-1:ER-occludin cells were plated at 10,000 cells per 35-mm culture dish in 1 ml of 0.35% (wt/vol) low melting temperature (LMT) agarose solution diluted with medium in the absence or presence of 1 μM estradiol. The dishes were coated with 1 ml of 0.7% (wt/vol) LMT agarose before cell plating, and 1 ml of overlay medium was added after cell plating. The overlay medium was changed every 3 d and fresh estradiol was added. A flask of d, the cells were stained with 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.05 mg/ml). The stained plates were photographed, and colonies >0.4 mm in diameter were counted and analyzed.

Results

Activated Raf-1 Modulates Epithelial Cell Phenotype and Downregulates Occludin Expression

Raf-1 is a serine/threonine kinase composed of two regulatory and one catalytic domain (Morrison and Cutler, 1997). Deletion of the regulatory domains of Raf-1 results in a constitutively active form capable of driving M E K - E R K pathway kinase activities. Pa-4 is an immortalized epithelial cell line derived from rat parotid gland, which grows as polarized monosheet in vitro with high transepithelial resistance. Stable transfections of Pa-4 cells were generated with a constitutively active construct of Raf-1 (Li et al., 1997). The expression of ΔRaf-1:ER protein and activation of the Raf-M E K - E R K kinase pathway in the stably transfected cells, Pa-4ΔARaf-1:ER, have been characterized previously (Li et al., 1997). Here, we verified an increased Raf-1 activity in Pa-4ΔARaf-1:ER cells by measuring the phosphorylation levels of ERK1 and ERK2 (Fig. 1 A). When grown on plastic, Pa-4ΔARaf-1:ER cells displayed significant morphological changes compared with vector-transfected control cells (Pa-4-vec) (Fig. 1 B), and had prominent stress fibers instead of pericellular actin rings (Fig. 1 C). When cultured on semipermeable filter supports, Pa-4ΔARaf-1:ER cells lost their ability to form high-resistance monolayers and acquired a stratified, low-resistance phenotype (Fig. 1 D). Immunofluorescence staining of occludin showed normal peripheral distribution pattern in control cells, but there was only background staining of occludin in Pa-4ΔARaf-1:ER cells (Fig. 1 E). Western analyses revealed that Pa-4-vec cells had high levels of occludin protein in both Triton X-100-soluble and -insoluble lysates (Fig. 1 F). In the Triton X-100-insoluble fraction, where cytoskeleton-associated proteins are enriched (Wong, 1997), a large percentage of occludin was hyperphosphorylated and probably represented a functional component of the TJ (Fig. 1 F). By comparison, Pa-4ΔARaf-1:ER cells completely lost their expression of occludin (Fig. 1 F, arrow). Northern analysis demonstrated that the downregulation of occludin protein in Pa-4ΔARaf-1:ER cells correlated with a complete loss of occludin mRNA (Fig. 1 G).

A recent study on the actions of vascular permeability factor on endothelial cell function has suggested that the loss of occludin at cell junctions occurs via the E R K -involved pathway (Kevil et al., 1998). A iso, K-i-R as activation has been shown to alter cell–cell contacts in polarized M D C K cells, inducing them to lose their monolayer phenotype and grow as multilayers (Schoenenberger et al., 1991). We examined the possible involvement of the Ras-Raf-M E K - E R K signaling module in regulating occludin expression. Pa-4 cells were transiently transfected with either an oncogenic k-ras (Capon et al., 1983), another active mutant of Raf-1, Raf BXB (Bruder et al., 1992), or a constitutively active M E K 1, pFC-M E K 1. Northern blots revealed decreases of 31, 39, and 44% in occludin mRNA levels, respectively (Fig. 2). The partial changes of occludin mRNA levels observed in this experiment were likely due to the limitation of transfection efficiency. In addition, downregulation of occludin expression induced by pFC-M E K 1 was blocked by PD 98059 (Fig. 2), a selective inhibitor of ER K activation (Dudley et al., 1995). In another cell system, A 549, which has high Ras-Raf signaling activity due to an oncogenic K-ras mutation, we observed an upregulation of occludin by transfecting a dominant negative Raf-1 construct or by treating the cells with PD 98059 (Li, D., and R. J. Mursny, manuscript in preparation). Taken together, it is likely that active Raf-1 downregulated occludin expression in Pa-4 cells through the M E K -E R K signaling pathway.

Introduction of Exogenous Occludin into Pa-4ΔARaf-1:ER Cells Resulted in Reacquisition of Normal Epithelial Phenotype and Functionally Intact TJs

Although we made the observation that occludin was downregulated in Raf-1-activated cells, this downregulation could be a side effect of Raf-1 activation and have no relevance to the disruption of epithelial TJs. To directly assess the potential role of occludin in stabilizing functional epithelial TJs, we introduced an exogenous occludin gene (human) driven by the cytomegalovirus promoter into Pa-4ΔARaf-1:ER cells, which no longer express endogenous occludin. A total of eight Pa-4ΔARaf-1:ER-occludin cell clones were isolated and analyzed. Data from a representative clone, clone No. 2, have been presented for most studies. Control transfections with pCB6 vector alone did...
not yield any clones distinguishable from Pa-4\(\text{D Raf-1:ER}\) cells. Occludin-transfected Pa-4\(\text{D Raf-1:ER}\) cells were verified to have similar levels of\(\text{D Raf-1:ER}\) protein and phosphorylated ERK1 and ERK2 compared with Pa-4\(\text{D Raf-1:ER}\) cells (Fig. 3 A), indicating that elevated activity of the Raf-MEK-ERK kinase pathway was maintained. Immunoblotting of protein lysates of Pa-4\(\text{D Raf-1:ER-occludin}\) cells confirmed the presence of occludin in both Triton X-100–soluble and –insoluble fractions (Fig. 3 B), and hyperphosphorylated occludin in the Triton X-100–insoluble fraction only (Fig. 3 B, arrow). Immunostaining of Pa-4\(\text{D Raf-1:ER-occludin}\) cells verified the normal distribution of occludin at the periphery of cells (Fig. 3 C). To demonstrate that the occludin protein detected was exogenous, PCR primers were designed using unique sequences in the 5'-
untranslated regions of rat and human occludin cDNAs. Reverse transcription–PCR results revealed that rat occludin mRNA was only present in Pa-4-vec cells, whereas human occludin mRNA was only detectable in Pa-4ΔRaf-1:ER-occludin cells (Fig. 3D). When cultured on plastic, Pa-4ΔRaf-1:ER-occludin cells displayed morphology indistinguishable from that of Pa-4-vec cells (Fig. 3E), and had similar annular rings of actin (Fig. 3F). When cultured on semipermeable filter supports, Pa-4ΔRaf-1:ER-occludin cells formed monolayers (Fig. 3G) with TEER values of ~900 Ω·cm² (Fig. 3H), demonstrating the assembly of functional TJs. It is not surprising that the TEER of Pa-4ΔRaf-1:ER-occludin cells did not recover fully to control levels, because these cells still have elevated Raf-1 activity, which is likely to affect other components responsible for the fine-tuning of epithelial TJs. A another possible reason is that human occludin protein may not work perfectly in a rat cell line. But our results clearly demonstrated that occludin played a crucial role in oncogenic Raf-1-induced disruption of epithelial TJs.

**Occludin Protein Assists in the Membrane Localization of ZO-1 and E-Cadherin in Raf-1–activated Pa-4 Cells**

To investigate the potential role of occludin in coordinating other junctional proteins, we examined cellular distribution and expression of ZO-1, a TJ-associated protein, and E-cadherin, an AJ-associated protein. In Pa-4-vec cells, ZO-1 colocalized with occludin at the TJs (Fig. 4A), whereas E-cadherin localized next to occludin towards the basolateral side (Fig. 4B). However, the distribution patterns of ZO-1 and E-cadherin were disrupted in the occludin-absent Pa-4ΔRaf-1:ER cells. Although ZO-1 protein was no longer exclusively located at the cell–cell contact points in Pa-4ΔRaf-1:ER cells, there was still a substantial amount of ZO-1 appearing as plaques along the cell border, suggesting its membrane localization is independent of occludin expression and functional TJs. Introduction of exogenous occludin restored the distribution patterns of these two proteins to those observed in control cells (Fig. 4A and B). Raf-1 activation did not significantly affect the overall protein level of ZO-1, but slightly reduced the ZO-1 level in Triton X-100–insoluble fractions. The level of Triton X-100–insoluble ZO-1 recovered after introduction of occludin (Fig. 4C). This is consistent with our immunofluorescence results, where we observed a decrease in ZO-1 levels at the lateral membrane in Pa-4ΔRaf-1:ER cells, and a relocalization at the TJs in Pa-4ΔRaf-1:ER-occludin cells. A similar scenario also occurred for E-cadherin distribution, although Raf-1 activation seemed to have decreased the total protein level of E-cadherin (Fig. 4C), consistent with other reports that E-cadherin is downregulated in transformed epithelial cells (Gulliford, 1999).

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**Figure 3.** Expression of exogenous occludin induced the formation of functional epithelial cell TJs in Pa-4ΔRaf-1:ER cells. (A) Western blots using antibodies to human estrogen receptor (top), phosphorylated ERK1 and ERK2 (middle), or actin (bottom). Lanes 1, 2, and 3 represent Pa-4-vec, Pa-4ΔRaf-1:ER, Pa-4ΔRaf-1:ER-occludin, respectively. Similar results were seen in all eight Pa-4ΔRaf-1:ER-occludin clones. (B) Pa-4ΔRaf-1:ER-occludin lysates were immunoblotted with an antioccludin antibody. S, Triton X-100–soluble; I, Triton X-100–insoluble. An arrow indicates hyperphosphorylated occludin. (C) Immunofluorescence staining (Cy5) showing occludin was concentrated at the cell borders. (D) Total RNA was isolated from Pa-4-vec (lane 1), Pa-4ΔRaf-1:ER (lane 2), and Pa-4ΔRaf-1:ER-occludin (lane 3) cells. Reverse transcription–PCR was performed using primer sets specific for rat occludin, human occludin, or glyceraldehyde 3-phosphate dehydrogenase (G3PDH) (as control). (E) Pa-4ΔRaf-1:ER-occludin cells grown on plastic displayed similar phenotype to that of Pa-4-vec cells. (F) Rhodamine-phalloidin labeling of actin showed the reappearance of pericellular actin rings in Pa-4ΔRaf-1:ER-occludin cells. (G) Pa-4ΔRaf-1:ER-occludin cells cultured on semipermeable filters grew as monolayers. (H) Formation of functional epithelial TJs in Pa-4ΔRaf-1:ER-occludin cells. TEER of Pa-4-vec (square), Pa-4ΔRaf-1:ER (triangle), and Pa-4ΔRaf-1:ER-occludin (circle) cells grown on filters were measured using a chopstick voltmeter. Data represent the mean ± SEM of six filters from each cell type. Bars, 10 μm.
Our observation implies that occludin may play a role in the localization of these junctional proteins.

**Occludin Protein Stabilized Claudin-1 Protein in Raf-1--activated Pa-4 Cells**

Claudins have been implicated in the structure and function of TJ strands (Tsukita and Furuse, 1999). So far 16 members of the claudin gene family have been reported. Claudin-1 was found in TJ strands associated largely with the P-face in freeze-fractured images (Furuse et al., 1998), and the amount of P-face TJ strand particles has been shown to correlate with the tightness of TJs (Wolburg et al., 1994), suggesting its involvement in tight TJs. Since Pa-4 cells have relatively tight TJs with TEER >2,000 Ω·cm², we examined claudin-1 as a representative of the claudin gene family in our system. Unlike occludin, the majority of claudin-1 protein was Triton X-100–soluble in control Pa-4 cells and only a small percentage was detected in the Triton X-100–insoluble fraction (Fig. 5 A). This suggests that claudin-1 may not be as directly associated with the actin cytoskeleton as occludin. Claudin-1 protein was almost undetectable in Pa-4ΔRaf-1:ER cells, but recovered to the levels seen in Pa-4-vec cells after the reexpression of occludin (Fig. 5 A). Although there was a decrease of claudin-1 mRNA level in Pa-4ΔRaf-1:ER cells, surprisingly, Pa-4ΔRaf-1:ER-occludin cells showed a level of claudin-1 mRNA comparable to that of Pa-4ΔRaf-1:ER cells (Fig. 5 B) even with the apparent difference in protein levels. To further investigate this observation, we examined the protein levels of occludin and claudin-1 in eight different Pa-4ΔRaf-1:ER-occludin cell clones. The levels of occludin protein varied significantly among the clones (Fig. 5 C). This is likely due to the location effects of gene insertion during stable transfection. We observed a general correlation between the protein levels of claudin-1 and those of occludin in the Pa-4ΔRaf-1:ER-occludin clones (Fig. 5 C), but the levels of claudin-1 mRNA among the clones did not change significantly (Fig. 5 D). Therefore, it seems possible that claudin-1 protein was stabilized by the presence of occludin protein in Raf-1--activated Pa-4 cells. Costaining of claudin-1 and occludin revealed some colocalization between these two proteins in Pa-4-vec and Pa-4ΔRaf-1:ER-occludin cells (Fig. 5 E). Thus, active Raf-1 can completely downregulate the expression of occludin (no detectable mRNA or protein) and decrease the expression of claudin-1, although to a lesser extent compared with occludin (50% decrease of mRNA, 95% decrease of protein). It is possible that the loss of claudin-1 protein in Pa-4ΔRaf-1:ER cells was due to accelerated protein degradation, implicating a role for occludin in the stabilization of claudin-1. The mechanism of this stabilization is unknown, but may result from protein–protein interactions between occludin and claudin-1 at the TJs. We also noticed that the TEER among the Pa-4ΔRaf-1:ER-occludin clones did not correlate with the expression levels of occludin or claudin-1 (data not shown), suggesting that occludin and claudins are not the only players in regulating epithelial TJ function. A delicate balance between claudins and occludin levels and other regulatory components of TJs might be necessary to achieve the maximum barrier function.

**Occludin Expression Inhibited Clonal Formation of Pa-4ΔRaf-1:ER Cells in Soft Agarose**

Cancer cells grow aggressively and invasively. In a cell culture system, this is represented by their ability to grow anchorage-independently in soft agarose. Although the basal activity of ΔRaf-1:ER fusion kinase was sufficient to disrupt TJ function and suppress occludin expression in Pa-4 cells, the kinase activity of ΔRaf-1:ER can be further induced in the presence of added estradiol. Further induction of Raf-1 activity can greatly increase the ability of Pa-4ΔRaf-1:ER cells to form colonies in soft agarose plates (Li et al., 1997). We examined the effect of occludin reintroduction on the growth characteristics of Raf-1--activated Pa-4 cells. We found that expression of occludin in...
Pa-4ΔR af-1:ER cells significantly decreased their ability to grow in soft agarose in the absence or presence of estradiol (Fig. 6). Thus, there is a possibility that upregulation of occludin expression could be a potential approach to treat Raf-1–induced epithelial cancers.

**Discussion**

Mechanisms that control functional aspects of cell–cell contacts are not yet fully understood. Events commonly associated with the oncogenic transformation of epithelial cells, however, include the loss of cell–cell contacts and the acquisition of more migratory and invasive phenotypes (Birchmeier et al., 1993). Deregulation or loss of function of AJ and gap junction communications has been shown to occur in epithelial-derived cancers (Bulkmol et al., 1998; Ruch et al., 1998). However, the impact of TJ function on oncogenic transformation of epithelial cells has not been clearly established. Many studies have focused on Rac and Rho, two small GTPases downstream of Ras, as regulators of TJ function. Neither Rac nor Rho appears to have a direct effect on expression levels of occludin and ZO-1 (Jou et al., 1998), although Rho signaling induces posttranslational modification on these two TJ components. (Gô-
Inactivation of Rho function by ADP ribosylation with C3 exoenzyme from C. botulinum leads to a disruption of TJ function through a loosening of the adhesive band of actin in intestinal epithelial cells (Nusrat et al., 1995). Constitutively active forms of Rho and Rac can also disrupt TJ function through a disorganization of occludin, ZO-1, and actin in MDCK cells (Jou et al., 1998). These studies suggest that these GTPases must be finely controlled for the normal structure and function of TJ complexes.

Using the chimeric construct ΔRaf-1:ER in a rat salivary epithelial cell system, we have shown that Raf-1 acts as a signal transducer capable of modulating TJ function. Regulation of occludin expression by Raf-1 may represent a previously unappreciated mechanism in epithelial cell transformation. Although the kinase activity of ΔRaf-1:ER can be further increased in the presence of added estradiol, the ΔRaf-1:ER fusion protein has a high level of basal activity (Li et al., 1997). This basal activity is sufficient to induce morphology changes, disruption of TJs, and downregulation of occludin and claudin-1 in Pa-4 cells. That is why most of our studies were carried out in the absence of added estradiol. We have analyzed each of the eight Pa-4ΔRaf-1:ER-occludin clones obtained through stable transfection. Although these cells showed different levels of occludin and claudin-1 expression, many other properties of these clones that we have studied were similar. For instance, they all maintained high levels of ΔRaf-1:ER expression, comparable to that of the Pa-4ΔRaf-1:ER cells. When cultured on semipermeable filter supports, all eight clones grew as monolayers. Immunofluorescence staining also revealed normal distributions of occludin, claudin-1, ZO-1, and actin in these clones. We have also examined the potential effects of added estradiol on Pa-4ΔRaf-1:ER-occludin cells. Further activation of ΔRaf-1:ER by estradiol did not change the monolayer phenotype or TEER in any of the Pa-4ΔRaf-1:ER-occludin clones (data not shown), nor did it change the inability of these cells to grow in soft agarose (Fig. 6). These results are consistent with our other observations, demonstrating that the downregulation of occludin is a downstream cellular event after Raf-1 activation, and reexpression of occludin can reverse some of the phenotypic changes induced by active Raf-1.

Although the MEK-ERK kinase pathway is likely involved in the Raf-1-controlled occludin expression, the details of this regulation remain unresolved. Raf-1 signaling may downregulate occludin at the transcription level or through accelerated protein degradation. In Pa-4ΔRaf-1:ER-occludin cells, exogenous (human) occludin protein did not appear to be degraded, even though these cells maintained elevated Raf-1 activity. Human occludin protein is highly homologous to rat occludin. If we assume that the occludin degradation machinery in rat cells can recognize human occludin, our data would suggest that the downregulation of occludin by activated Raf-1 was not through accelerated protein degradation. In addition, we have shown that constitutive activation of Raf-1 led to the complete loss of occludin mRNA in Pa-4 cells, clearly demonstrating that changes at the message level were involved. Furthermore, in A 549 cells, which have an oncogenic K-ras mutation, inhibition of the Raf-MEK-ERK ki-

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**Figure 6.** Expression of occludin suppressed anchorage-independent growth of Pa-4ΔRaf-1:ER cells in soft agarose. (A) Representative photographs of dimethylthiazol-diphenyltetrazolium bromide (MTT)-stained dishes. Pa-4-vec (panels a and b), Pa-4ΔRaf-1:ER (panels c and d), and Pa-4ΔRaf-1:ER-occludin (panels e and f) cells were plated at 10,000 cells/35-mm dish in semisolid media containing 0.35% low melting temperature agarose in the absence (panels a, c, and e) or presence (panels b, d, and f) of 1 mM estradiol. The cells were stained with 0.05 mg/ml MTT after 15 d. (B) The average number of colonies obtained from three 35-mm dishes/group ± SD is represented graphically. Colonies >0.3 mm in diameter were counted. Similar results were seen in seven of eight Pa-4ΔRaf-1:ER-occludin clones.
nase pathway by a dominant negative Raf-1 mutant or by the M E K inhibitor PD 98059 resulted in increased m R N A level of occludin (Li, D., and R. J. Msny, manuscript in preparation). A though we cannot rule out the possibility that Raf-1 modulates the stability of occludin m R N A , we think it is more likely that Raf-1 regulates occludin expression at the transcription level rather than through degra-
dation.

Raf-1 activation appeared to have broad effects on several other TJ-associated proteins, including claudin-1 and ZO-1. ZO-1 is known to directly interact with occludin (Furuse et al., 1994). A recent study reported that con-
exion-noccludin chimeras containing the ZO-binding do-
main of occludin localized at epithelial cell TJs and nonepi-
thelial cell contacts (Mitic et al., 1999). In fibroblasts, it has been shown that claudin-1 or claudin-2 recruited occludin to reconstituted TJ strands (Furuse et al., 1998). We observed that in occludin-absent Pa-4AR-1 E R cells, the TJ localization of ZO-1 was disrupted and claudin-1 protein was downregulated. The introduction of exogenous occludin into these cells led to the reappearance of ZO-1 and claudin-1 proteins in the TJs. A though the mechanisms underlying these changes are unclear, our study sug-
gests that the TJ targeting interaction among these proteins could be bidirectional. The changes of claudin-1 and ZO-1 in Pa-4AR-1 E R cells are likely to have contributed to the disruption of TJs induced by active Raf-1. H owever, the fact that reintroduction of occludin alone re-
sulted in the return of normal epithelial phenotype and es-
tablishment of functional TJs implicates occludin as a
result of normal epithelial phenotype and es-
tablishment of functional TJs implicates occludin as a
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