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Occludin confers adhesiveness when expressed in fibroblasts

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SUMMARY

Occludin is an integral membrane protein specifically associated with tight junctions. Previous studies suggest it is likely to function in forming the intercellular seal. In the present study, we expressed occludin under an inducible promotor in occludin-null fibroblasts to determine whether this protein confers intercellular adhesion. When human occludin is stably expressed in NRK and Rat-1 fibroblasts, which lack endogenous occludin and tight junctions but do have well developed ZO-1-containing adherens-like junctions, occludin colocalizes with ZO-1 to points of cell-cell contact. In contrast, L-cell fibroblasts which lack cadherin-based adherens junctions, target neither ZO-1 nor occludin to sites of cell contact. Occludin-induced adhesion was next quantified using a suspended cell assay. In NRK and Rat-1 cells, occludin expression induces adhesion in the absence of calcium, thus independent of cadherin-cadherin contacts. In contrast, L-cells are nonadhesive in this assay and show no increase in adhesion after induction of occludin expression. Binding of an antibody to the first of the putative extracellular loops of occludin confirmed that this sequence was exposed on the cell surface, and synthetic peptides containing the amino acid sequence of this loop inhibit adhesion induced by occludin expression. These results suggest that the extracellular surface of occludin is directly involved in cell-cell adhesion and the ability to confer adhesiveness correlates with the ability to colocalize with its cytoplasmic binding protein, ZO-1.

Key words: Tight junction, Occludin, ZO-1, Intercellular junction, MDCK cell, Rat-1, NRK, L-cell fibroblast

INTRODUCTION

Tight junctions are a characteristic feature of epithelial cells, forming an apical intercellular barrier that both regulates paracellular permeability (Reuss, 1991; Anderson and Van Itallie, 1995) and delimits the apical and basolateral cell surfaces (Dragsten et al., 1981; van Meer and Simons, 1986). Structurally, this intercellular junction is composed of a continuous network of intramembrane strands, presumably rows of transmembrane proteins (Staehelin, 1973) with associated cytoplasmic components. Recent studies have begun to enumerate and characterize the proteins that make up this complex structure. A number of proteins associated with the cytoplasmic surface of tight junctions have been identified, including ZO-1 (Stevenson et al., 1986), ZO-2 (Gumbiner et al., 1991), cingulin (Citi et al., 1988), 7H6 (Zhong et al., 1993), symplekin (Keon et al., 1996), rab3B (Weber et al., 1994), as well as one transmembrane protein, occludin (Furuse et al., 1993) which has been suggested to function in creating the intercellular seal.

Sequence analysis of occludin predicts four hydrophobic alpha helical transmembrane spanning segments (Furuse et al., 1993) and immunoelectron microscope analysis demonstrated that a monoclonal antibody, recognizing an epitope near the C terminus of the protein, labeled the cytoplasmic surface of the junctional membrane. These observations led Furuse et al. (1993) to suggest a model for occludin structure in which both the N- and C-termini are intracellular and two short loops, 46 and 48 residues in length, are positioned on the outer cell surface. Although this topology has not been unambiguously proven, these authors noted its similarity to both the transmembrane proteins synaptophysin and the gap junction channel protein connexin. Recently, Ando-Akatsuka et al. (1996) reported the full length sequences of occludin from several mammalian species, all of which are strongly predicted to share a similar transmembrane topology. Although the mammalian occludin amino acid sequences share a high degree of homology, it is of some interest that one of the least well conserved regions in amino acid sequence is the putative first extracellular loop, where there is only 78% identity between human and mouse, compared with 90% for the protein as a whole. The human and chicken sequences are only 53% identical in this region. The most striking feature of this first putative extracellular loop is not conservation of primary sequence but its unusual amino acid composition. Specifically, it lacks charged residues and has a very high percentage of tyrosine plus glycine (63% in human occludin). This unusual composition has led to speculation that these residues may be involved in sealing the intercellular space (Ando-Akatsuka et al., 1996). Although the nature of occludin's extracellular interactions remains to be determined, there is indirect evidence that occludin might form an adhesive homopolymer, since overexpression of occludin in Sf9 insect cells results in multilamellar inclusions formed by

sheets of occludin-containing membrane (Furuse et al., 1996). Occludin was not, however, targeted to the plasma membrane in invertebrate Sf9 cells and changes in cell-cell adhesion could not be assessed.

Two recent studies (Balda et al., 1996; McCarthy et al., 1996) have begun to examine the contribution of occludin to tight junction function. In both studies, chicken occludin was overexpressed in MDCK epithelial cells, and tight junction structure was assessed by immunohistochemistry and freeze fracture analysis and function was assessed by transepithelial electrical resistance and paracellular solute flux. Although there were some differences in the findings in these two studies, both demonstrated that forced overexpression of occludin increases the transepithelial resistance as well as increases paracellular solute flux. Increases in transepithelial electrical resistance after occludin transfection are consistent with the idea that occludin is the sealing protein of the tight junction. The increase in paracellular permeability that accompanied expression of chicken occludin suggests either that occludin is also involved in forming aqueous paracellular channels or that the increased occludin level in the transfected cells may have somehow disrupted normal occludin function (Balda et al., 1996; McCarthy et al., 1996). One possibility is that extracellular loops of transfected chicken and endogenous canine occludin are not completely compatible, since the amino acid sequences within the first extracellular loops are quite different (Ando-Akatsuka et al., 1996).

On the cytoplasmic surface of occludin, a domain within the C-terminal 150 residues has been shown to bind directly to the well-characterized tight junction protein ZO-1 (Furuse et al., 1994). Recently, Rajasekaran et al. (1996) has demonstrated that ZO-1 forms a transient complex with the cadherin-binding protein β-catenin in MDCK cells immediately after Ca^{2+} is added back to cells grown without Ca^{2+}. These data suggest a mechanism for occludin targeting to cadherin-based junctions, via its interaction with ZO-1; in epithelial cells, these early points of contact subsequently differentiate into distinct tight and adherens junctions. However, Balda et al. (1996) also demonstrated that removal of the entire internal carboxy terminus of occludin still allowed for efficient targeting of occludin to the tight junction, implying that binding to ZO-1 is not necessarily required for proper targeting in this epithelial cell type.

It remains unclear whether occludin would be appropriately localized or functional in cells which lack preformed tight junctions. To investigate occludin targeting and to test whether occludin is directly involved in cell-cell adhesion, we expressed human occludin in mammalian fibroblast cell lines that lack occludin. We observed that occludin confers calcium-independent adhesion in cells where it colocalizes at cadherin-based cell contacts with ZO-1, but not in cells which lack cadherin and do not concentrate ZO-1 at cell contacts. These data suggest that occludin confers adhesiveness only when clustered on the cell surface or when bound on the cytoplasmic surface to other tight junction plaque proteins. In addition, after confirming that the first putative extracellular loop of occludin is in fact exposed on the cell surface, we used peptides whose sequences were contained within this loop to specifically abolish the occludin-dependent cell adhesion, presumably through competitive inhibition of its binding function.

MATERIALS AND METHODS

cDNAs, antibodies, peptides and cell lines

The 675 nucleotide occludin sequence found in the untranslated region of the human neuronal apoptosis inhibitory gene (Roy et al., 1995) was used to design PCR primers, and reverse transcription-PCR was performed using poly(A)$^+$ mRNA from Caco-2 cells as template. The resulting cDNA fragment was used to screen a human liver library (Clontech) and a full length cDNA was isolated and sequenced (GenBank accession no. U53823). A similar protocol was recently reported by Ando-Akatsuka et al. (1996) to clone the full-length human occludin, which demonstrates an exact match at the amino acid level to our sequence. The full length sequence was subcloned into the pCB6 expression vector with and without a 15 amino acid tag at the C terminus. This tag represents the carboxy terminus of the vesicular stomatitis virus glycoprotein (VSV-G).

A cDNA encoding the last 150 amino acids of human occludin was subcloned into the pGEX-1N vector and the resulting glutathione-S-transferase (GST) fusion protein used to generate anti-human occludin antibodies in guinea pigs (Yale Animal Care and Use Facilities). The same GST-fusion protein was also used to generate rabbit polyclonal antibodies using an accelerated immunization program developed by and performed at Zymed Laboratories (South San Francisco, CA) referred to as PolyQuik™. The resultant rabbit anti-human occludin polyclonal anti-sera was affinity purified using a GST-occludin coupled gel and was kindly provided by Zymed. Rabbit polyclonal anti-peptide antibodies raised against amino acids 90-112 of human occludin were also supplied to us by Zymed along with two contiguous peptides: peptide #1 (amino acids 90-112; CDRGYGTSLLGGSVGYPYGGSGFG) and peptide #2 (amino acids 113-135; CSYGSGYGYGYGYGYGGYTDPR). Together these contiguous peptides compose the putative first extracellular loop of the occludin protein. Amino-terminal cysteine residues are not part of the occludin sequence but were added to allow conjugation for antibody production. Because of the highly repetitive nature of the amino acid sequence of loop #1, it was difficult to design a control peptide by 'scrambling' the sequence. Instead, a peptide from the putative cytoplasmic N-terminal region of occludin (amino acids 18-38; NHYAPSNDIYGGEMVHRPML), with the same isoelectric point (pI=6.2) as peptide #1, was used (Keck Foundation Biotechnology Resource Laboratory, Yale University). Anti-ZO-1 antibody was from Zymed, secondary antibodies (FITC and Texas Red labelled) for immunofluorescence are affinity-purified, species-specific from Jackson Immunoresearch Laboratories (Westover, IA) and for immunoblots from Amersham Corp. (Arlington Heights, IL) and Chemicon International, Inc. (Temecula, CA). Anti-VSV-G antibody was from MBL (Nagoya, Japan).

MDCK cells, NRK and Rat-1 cells were from ATCC. Caco-2 cells were kindly supplied by Dr Mark Mooseker (Department of Biology, Yale University) and L cells (lacking cadherin) were generously supplied by K. Siemers and W. J. Nelson (Beckman Center, Stanford University).

Tissue culture and cell transfection

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA) and antibiotics in 5% CO_2. Cells were transfected by calcium phosphate coprecipitation (Chen and Okayama, 1988); transient transfectants were induced with 5 mM sodium butyrate for 16-20 hours before immunofluorescence analysis. Occludin localization experiments were originally attempted after transient transfections, but results were variable and only results from stable cell lines are reported here. Stable cell lines were selected with 600 μg/ml G418 (Gibco BRL) for 10 days, at which time resistant clones were analyzed for occludin expression by immunofluorescence. Occludin-positive cells were maintained in 250 μg/ml G418.

Immunoblotting and immunofluorescence

For immunoblot analysis, confluent Caco-2 cells were rinsed in PBS and lysed in SDS sample buffer and heated to 95°C for 10 minutes. Control and stable occludin-expressing cell lines were plated at subconfluent density, allowed to attach and spread for 8 hours, and induced with 5 mM sodium butyrate for 16-20 hours. Cells were rinsed with PBS and samples prepared as above. Protein samples were separated by SDS 10% PAGE (Laemmli, 1970) and transferred to nitrocellulose (Towbin et al., 1979). Nonspecific protein binding was blocked with 10% nonfat dry milk, 0.1% Tween-20 in PBS for at least 1 hour at room temperature. Anti-occludin antibody (rabbit anti-human, Zymed) was used at 1:1,000 dilution, others as indicated in figure legends. Detection was by enhanced chemiluminescence (ECL, Amersham).

For immunofluorescence localization studies, cells were grown on glass coverslips. To demonstrate that the human occludin construct could be expressed and targeted appropriately in tight junction-containing cells, MDCK cells were used for the initial transient transfection assays. MDCK cells were transfected and induced with sodium butyrate as described above and stable cells lines were plated and induced as described for immunoblots. Cells were washed with PBS, fixed in 1% paraformaldehyde in PBS, extracted with 0.1% Triton X-100 and quenched with 50 mM NH_4Cl in PBS. In experiments to test the accessibility to the anti-peptide #1 antibody, incubation of the primary antibody was performed without permeabilization of cells with Triton X-100. Cells were blocked for 1 hour in PBS plus 2% goat serum, incubated in primary antibodies (anti-VSV-G at 10 μg/ml, guinea pig anti-occludin at 1:250, and anti-ZO-1 at 1:300; affinity-purified anti-peptide #1 antibody at 1:10) for 1 hour, washed and incubated in affinity-purified secondary antibodies (1:100) for 1 hour. Cells were washed, dipped in H_2O and mounted in Vectashield (Vector Laboratories). The samples were examined with a Nikon Microfot-FX epifluorescence microscope; photographs were taken with TMAX400 film (Kodak, Rochester, NY) using the automatic exposure setting.

Cell adhesion assay

Adhesion of stably transfected cell lines was measured by a modification of the procedure described by Wesseling et al. (1996). Cell lines were plated and induced with sodium butyrate as described above. Cell layers were rinsed twice with Ca^{2+}-Mg^{2+}-free PBS and then incubated for 30 minutes in Ca^{2+}-Mg^{2+}-free PBS plus 1 mM EDTA and 0.1 mg/ml DNAse. After 30 minutes, cells were mechanically dissociated, counted and resuspended at a concentration of 2.5×10^5 cells/ml in Ca^{2+}-Mg^{2+}-free PBS, 1 mM EDTA and 0.1 mg/ml DNAse. For each condition, the adhesion assay was performed in duplicate in two 50 ml conical tubes on a rotating platform at 80 rpm at 25°C. At each time point, the number of particles in two 175 μl samples of each tube was determined in a Coulter Counter (Hialeah, FL). The amount of adhesion was represented by N_t/N_0, where N_0 was the initial number of particles in each sample (the starting number of single cells), and N_t was the number at each time point. All (100%) of the cells were single at the beginning of all assays, as determined by phase-contrast microscopy. Some experiments were performed in the presence of the peptides described above.

RESULTS

Anti-human occludin antibodies

As described in Materials and Methods, occludin cDNAs were isolated from a human liver library. The C-terminal 150 amino acids were subcloned into a pGEX vector and the resulting GST-fusion protein was used to generate polyclonal antibodies in guinea pigs. Use of these antibodies in western blot analysis of the human colonic cell line, Caco-2, identified an antigen (doublet) at about 65 kDa (Fig. 1, lane 1) the same size as the antigen recognized previously (Fallon et al., 1995) by our anti-chicken occludin antibody (Fig. 1, lane 4). Other antibodies used in these studies include a commercially available affinity-purified anti-human occludin rabbit polyclonal raised against the same C-terminal fusion protein (Fig. 1, lane 2) and an anti-peptide antibody raised against a 23 amino acid sequence from the putative first extracellular loop (amino acids 90-112; CDR-GYGTSLLGGSVGYPYGGSGFG) (Fig. 1, lane 3). All antibodies recognize a doublet in Caco-2 cells. Multiple bands have been reported before and may result from an uncharacterized posttranslational modification. In addition, our anti-chicken occludin antibodies recognize a smaller molecular mass protein of about 20 kDa; the nature of this cross-reacting epitope is unknown. Thus, all antibodies used in the present studies exclusively recognize occludin; results with the anti-chicken antibodies are shown to compare with our previously characterized antibodies but were not used for experiments reported here.

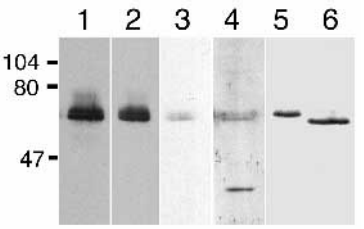

Fig. 1. Immunoblots with anti-occludin antibodies on lysates from Caco-2 cells (lanes 1-4), NRK cells induced with 5 mM sodium butyrate (lane 5) and rat kidney (lane 6). Antibodies used were guinea pig anti-human occludin (lane 1, 1:1,000); rabbit anti-human occludin (lanes 2, 5, 6, 1:1,000), rabbit anti-extracellular loop peptide #1 (lane 3, 1:10) and rabbit anti-chicken occludin (lane 4, 1:250).

Expression of transfected human occludin in MDCK cells

To confirm that transfected human occludin could localize appropriately in cells which normally expressed occludin, we transiently transfected MDCK cells with VSV-G-tagged human occludin cloned in the pCB6 vector. Expression was induced with sodium butyrate and cells stained for ZO-1 (Fig. 2a) and for VSV-G (Fig. 2b). ZO-1 immunofluorescence reveals the typical reticular pattern of tight junction staining. Immunofluorescence using the VSV-G antibody shows that in cells that expressed low levels of human occludin, the transfected protein was concentrated at sites of cell-cell contact, although higher expressing cells also expressed considerable occludin elsewhere in the cell. We concluded that the VSV-G-tagged human occludin can target appropriately to tight junctions in cultured epithelial cells which have pre-existing tight junctions.

Expression of transfected human occludin in fibroblast cell lines

We next examined the expression and localization of occludin in three fibroblast cell lines that do not form recognizable tight junctions or electrically resistive monolayers in culture. NRK cells, Rat-1 cells and L-cells do not normally express

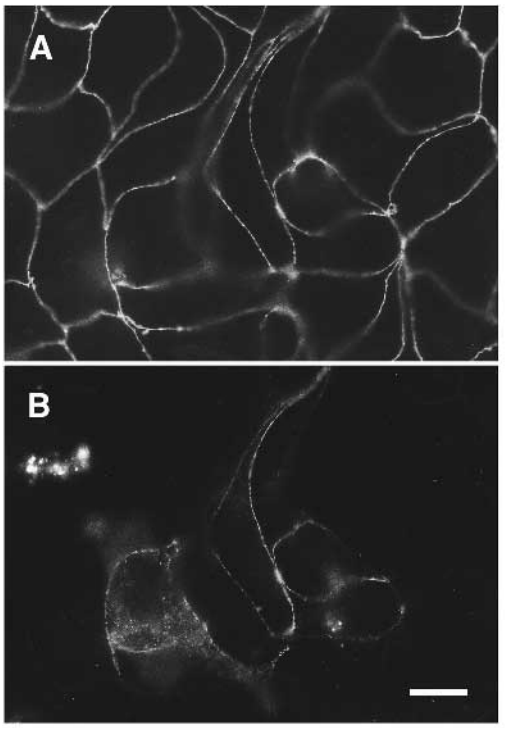

Fig. 2. Immunolocalization of VSV-G-tagged human occludin in MDCK cells. Transiently transfected MDCK cells were induced for 16 hours with 5 mM sodium butyrate and processed for double immunofluorescence. (A) ZO-1 was detected using anti-rabbit ZO-1; (B) VSV-G tagged occludin was detected with a rabbit polyclonal anti-VSV-G antibody. Bar, 10 μm.

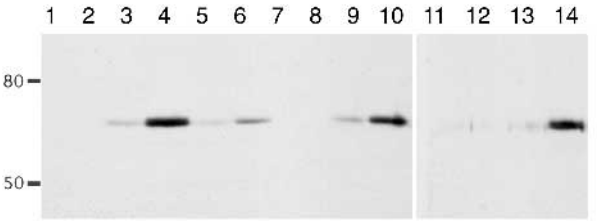

Fig. 3. Immunoblot analysis of human occludin expressed in fibroblast cell lines, detected with rabbit anti-human occludin antibody. Lanes 1 and 2, lysate from rat-1 cells; lanes 3 and 4, occludin-transfected rat-1 cells clone r9occ; lanes 5 and 6, occludin transfected rat-1 cells clone r11occ; lanes 7 and 8, L-cells; lanes 9 and 10, occludin-transfected L-cell clone L5occ; lanes 11 and 12, NRK cells; lanes 13 and 14, occludin-transfected NRK cell clone N2occ. Odd-numbered lanes, (–) butyrate; even-numbered lanes, (+) 5 mM sodium butyrate induction.

detectable occludin by western blot analysis. This apparent lack of expression is not due to an inability of the anti-human occludin antibody to recognize rodent occludin, since a slightly smaller form of occludin is easily detected in immunoblots from whole rat kidney (Fig. 1, lane 6). Fibroblast cells lines were transfected with the pCB6 occludin vector and stable cell lines were selected on the basis of antibiotic resistance with G418. G418-resistant clonal cell lines were screened by immunofluorescence and of the small number of occludin-expressing cell lines, none had more than about 40% of the cells expressing detectable occludin. Occludin was readily detectable by western blot analysis in some stable cell lines, and was inducible in all cell lines after 18 hours exposure to 5 mM sodium butyrate. As can be seen in Fig. 1, lane 5, the transfected human occludin runs at the higher apparent molecular mass characteristic of human occludin when compared with rat occludin. The three fibroblast cell clones used for most of the following experiments could be induced to express approximately equal amounts of the occludin transgene. The inducible nature of occludin expression allowed comparison of uninduced with induced cell lines, as well as comparison to non-transfected cells. In addition, a Rat-1 cell clone (R11occ) expressing about one-third less occludin was used in some studies to look at the effect of expressing a lower level of occludin (Fig. 3).

Yonemura et al. (1995) previously showed that in NRK cells, ZO-1 localizes to sites of cell-cell contact, along with other proteins normally associated with adherens junctions. We also noted this distribution of ZO-1 in NRK cells (Fig. 4a) as well as in another fibroblast line, Rat-1 cells, although the latter cells are not as flat and tend to have less regularly spaced cell contacts (Fig. 4c). In both NRK cells (Fig. 4b) and in rat-1 cells (Fig. 4d), human occludin colocalized with ZO-1 at sites of cell-cell contact, as well as showing a diffuse and lower level of expression over the entire plasma membrane. Occludin did not appear to be more concentrated between two neighboring cells when both expressed occludin, suggesting that in these transfected cells, intercellular occludin-occludin interactions do not appear to appreciably stabilize occludin localization. In fact, occludin appears to concentrate with ZO-1 even when the adjacent cell does not contain detectable occludin. Both transfected cell lines also had small amounts of occludin expressed elsewhere in the cell, possibly in intracellular vesicles and plasma membrane aggregates. In contrast, mouse L-cells, which lack E-cadherin and adherens-like junctions (Angres et al., 1996) concentrated neither ZO-1 (Fig. 4e) nor occludin at sites of cell-cell contact (Fig. 4f). Both appear diffusely distributed over the plasma membrane, although ZO-1 but not occludin, is concentrated in puncta on the apical surface.

Occludin expression confers adhesion on NRK and Rat-1 cells, but not L-cells

The ability of transfected occludin to confer adhesion onto fibroblast cell lines was assessed using a suspended cell aggregation assay (Wesseling et al., 1996). All cells were counted as single particles at the beginning of the assay and any decrease in the fraction of particles over the 80 minute assay was the result of aggregates which are excluded by the Coulter Counter. Aggregation was qualitatively confirmed by light microscopic inspection and correlated with results quantified by the Coulter Counter. Immunofluorescence confirmed that ZO-1 and occludin are clustered between adherent cells after the 80 minute assay (Fig. 5).

As shown in Fig. 6, human occludin promoted cell aggregation in the absence of calcium in both Rat-1 cells and in NRK fibroblasts, but not in L-cells. Expression of occludin in NRK and Rat-1 fibroblasts induced a steeper slope ($[N_t/N_0]$/time) and lower final number of particles per unit volume at 80 minutes, the longest time assayed. In addition, when two Rat-

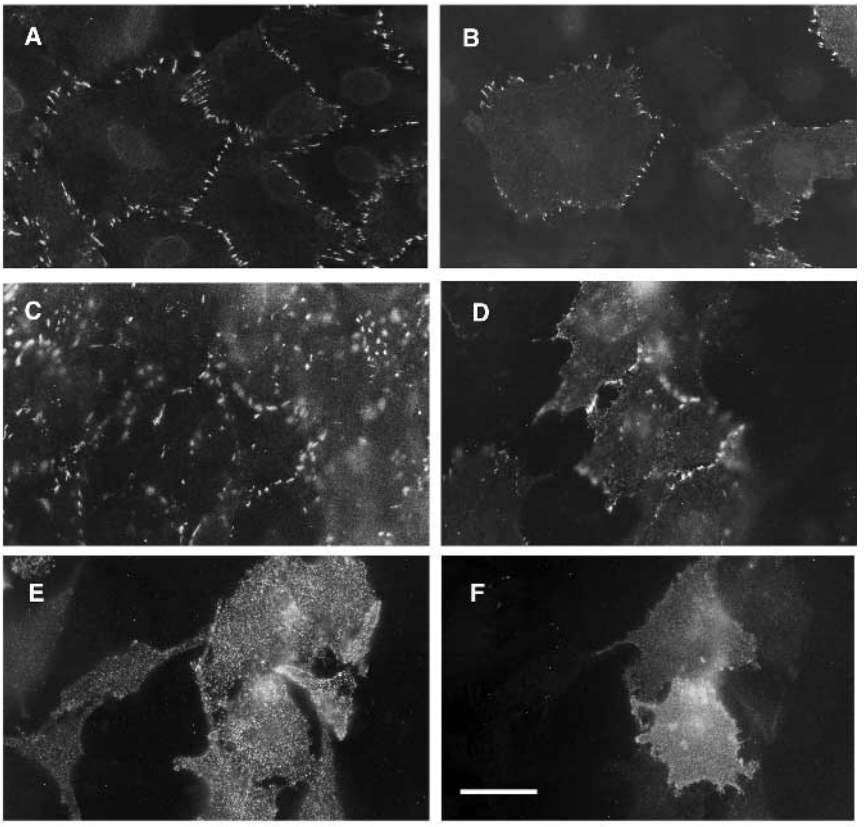

Fig. 4. Double immunofluorescence localization of ZO-1 (A,C,E; rabbit anti-ZO-1 polyclonal antibody) and transfected human occludin (B,D,F; guinea pig anti-human occludin antibody) in fibroblast cell lines. (A,B) NRK clone N2occ; (C,D) rat-1 clone r9occ; (E,F) L-cell clone L5occ. Bar, 20 μm.

1 clones with differing levels of occludin expression (Fig. 3) were tested in this assay, the degree of adhesion at 80 minutes correlated in a positive way with the level of occludin expression (Fig. 7). We have not determined whether the degree of adhesion measured in this assay correlates with the level of occludin expressed per cell or the percentage of cells expressing occludin. The effect of adhesion was not due to treatment of cells with butyrate, since adding butyrate to fibroblasts transfected with pCB6 without the occludin cDNA did not result in increased adhesion. The value of $[N_t/N_0 \times 100]$ never dropped below 40% suggesting some cells were nonadhesive. Although immunofluorescence studies showed not all cells express occludin, we were unsuccessful in attempts to separate aggregated from single cells and determine whether nonaggregating cells were those showing less or no occludin expression.

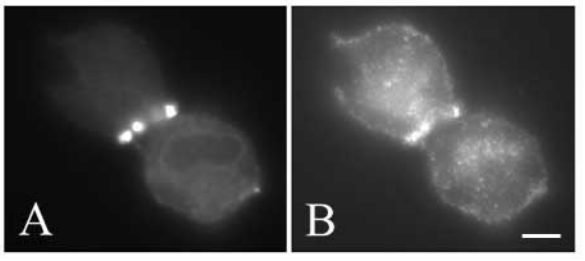

Fig. 5. Immunofluorescence colocalization of ZO-1 (A) and occludin (B) in a pair of N2occ cells at the end of an adhesion assay (80 minutes) performed in the absence of calcium and with 1 mM EDTA. Bar, 10 μm.

Antibody accessibility reveals exposure of occludin on the extracellular surface

The model for occludin topography predicts a region rich in glycine and tyrosine is positioned as the first, or more N-terminal, of the two extracellular loops. To determine whether these sequences are exposed on the outside of the cell, we assessed their availability in nonpermeabilized cells to an antibody raised against a synthetic peptide corresponding to the first 23 residues of this loop. As determined by western blotting, these antibodies have very low affinity (data not shown) and consistent results were only obtained using human Caco-2 cells which contain many fold higher levels of occludin than did the transfected cells. The C-terminal ZO-1 binding domain of occludin was previously shown to be intracellular (Furuse et al., 1993) and consistent with this, immunofluorescence analysis of nonpermeabilized Caco-2 cells stained with an antibody to this region reveals no specific staining (Fig. 8a). After detergent permeabilization, the same antibody reveals a typical reticular occludin staining pattern (Fig. 8b). Nonpermeabilized Caco-2 cells incubated in 1 mM EDTA and antibody raised against the putative extracellular sequence (Fig. 8c) shows a similar pattern of staining to that seen in Fig. 8b suggesting this sequence is in fact exposed on the cell surface. Less labeling was observed when cells were not exposed to EDTA, suggesting the sequences are not available to bind antibodies unless contacts are first disrupted by chelating divalent cations. However, even after exposure to EDTA the antigen is not uniformly accessible (Fig. 8c). The punctate apical staining likely represents background from the high concentration of antibody required, since the same punctate staining was observed in untransfected fibroblasts.

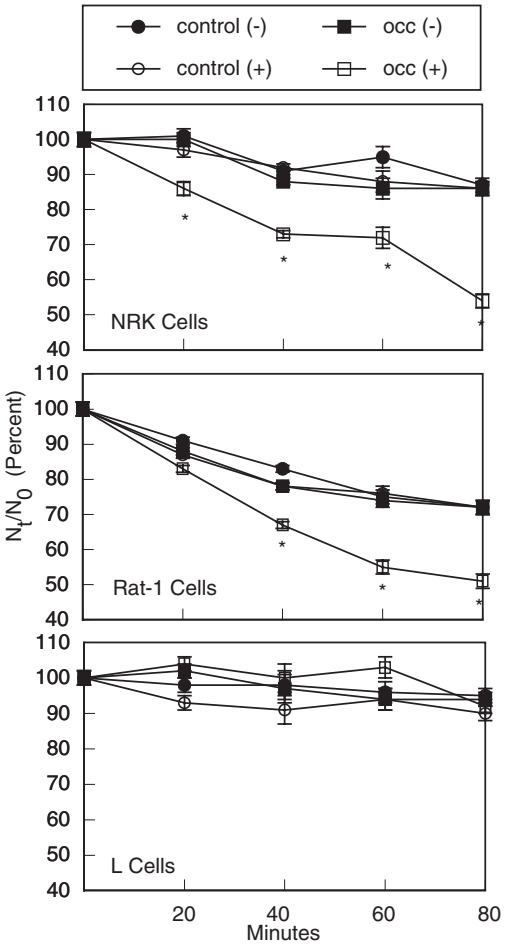

Fig. 6. Aggregation of human occludin transfected cell lines. Aggregation kinetics of the various fibroblast cells lines (control untransfected NRK, rat-1 and L-cells) and occludin-transfected cell lines (N2occ, R9occ, L5occ) without (–) and with (+) 16 hours of butyrate induction as measured with the Coulter counter. The decrease in the relative percentage of particles ($N_t/N_0 \times 100$) as a function of time indicates the extent of aggregation. The results from at least three separate experiments are combined (*P<0.01, ANOVA).

Occludin peptides inhibit occludin-induced adhesion

To determine if the extracellular sequences of occludin are directly involved in the adhesion observed in the occludin-transfected fibroblasts, we attempted to compete for the adhesive function using synthetic peptides corresponding to both halves of the first putative extracellular loop (Fig. 9). Peptides were tested at 1, 10 and 100 μM. Both peptides #1 and #2 (Materials and Methods) completely inhibited adhesion in both the Rat-1 and NRK cell clones at 100 μM (Rat-1 cell data not shown). Peptide #1 reversed approximately half the adhesion at 10 μM, suggesting an apparent K_i in this range. The apparent K_i for peptide #2 was reproducibly somewhat higher, in the range between 10-100 μM. These apparent inhibition constants suggest this region of occludin participates in a relatively low affinity binding interaction. An internal occludin peptide added over the same concentration range was ineffective at inhibiting occludin-dependent cell adhesion.

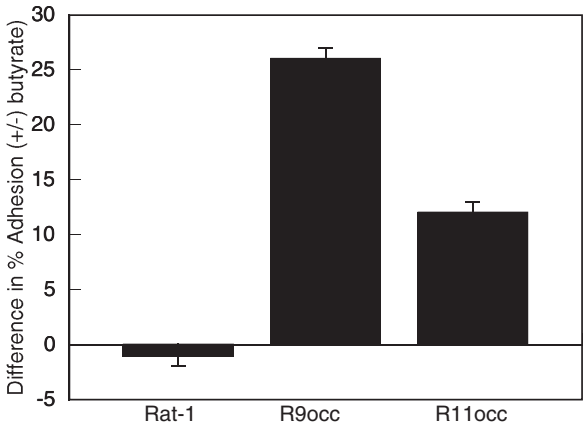

Fig. 7. Comparison of aggregation in rat-1 cell clones. The difference in the percentage adhesion ($N_t/N_0 \times 100$) at 80 minutes in rat-1 cells without (–) and with (+) butyrate treatment is compared for the parent cell line (rat-1) and two occludin-transfected clones (R9occ and R11occ) which express different levels of human occludin. Results from three separate experiments are combined.

DISCUSSION

In the present study, we have demonstrated that transfected human occludin colocalizes with ZO-1 at sites of cell-cell contact in some fibroblast cell lines, and that cells in which occludin colocalizes acquire adhesiveness. In addition, we have shown that the putative first extracellular loop of occludin is accessible to antibodies in the absence of cell permeabilization, thus supporting the predicted transmembrane topology originally based on hydrophobicity profiles (Furuse et al., 1993). Finally, we have demonstrated that peptides with sequences matching this extracellular loop decrease cell adhesion in occludin-expressing fibroblasts in a dose-dependent fashion. These data imply that this loop participates in an adhesive interaction, and the peptides are acting as competitive inhibitors at the adhesive surface.

The human occludin used in our studies was capable of properly targeting when expressed in cultured epithelial cells which contain preformed tight junctions (MDCK cells). In contrast, when expressed ectopically in fibroblasts, occludin localized only at cell-cell contacts in lines already capable of localizing ZO-1, i.e. NRK cells and Rat-1 cells. ZO-1 is known to bind directly to occludin through a 150 amino domain at the C terminus of occludin (Furuse et al., 1994). Thus, the simplest explanation for our observations in fibroblasts is that occludin uses prelocalized ZO-1 as its predominate targeting signal. This observation is different from that of Balda et al. (1996) who expressed a truncated form of occludin without the ZO-1 binding domain in MDCK cells and found it still capable of targeting to the tight junction. Because these cells already contain preformed tight junctions, lateral interactions between occludin proteins within the same cell or between cells could account for localization. However, in the absence of endogenous occludin, de novo targeting of occludin in fibroblasts appears to require binding to ZO-1, not lateral or cell-cell association between occludin proteins.

Neither ZO-1 nor ectopically-expressed occludin was capable of localizing to cell contacts in the L-cell clone used

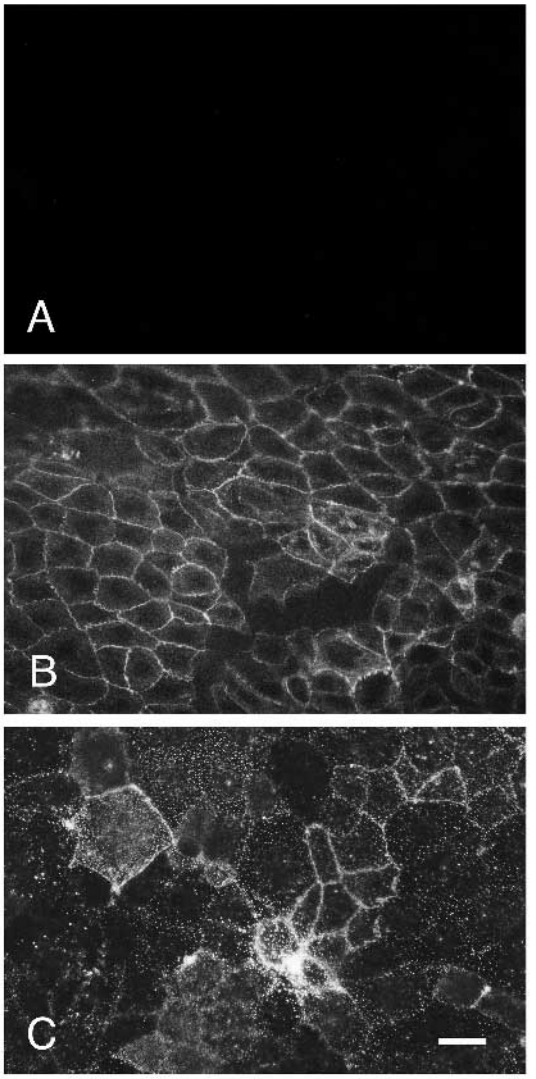

Fig. 8. Immunofluorescence localization of occludin in Caco-2 cells using an intracellular antibody (see text) in (A) nonpermeabilized cells, or (B) permeabilized cells; (C) immunofluorescence localization of occludin in nonpermeabilized cells using a rabbit polyclonal antibody raised against a synthetic peptide from the first extracellular loop of occludin.

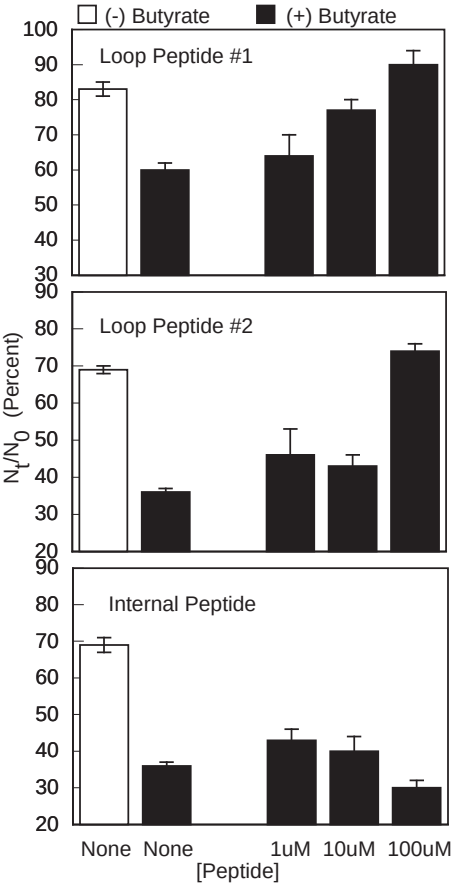

Fig. 9. Aggregation of N2occ cells in the absence or presence of various concentrations of synthetic peptides. Peptides were added in equal volumes of PBS at the start of the incubation period; extent of aggregation is shown after 80 minutes of incubation. Loop peptides represent contiguous sequences in the first extracellular loop; internal peptide (residues 18-38) with the same pI as loop peptide #1. Peptide sequences are in the Materials and Methods. One of three experiments with identical results is shown.

for this study. Occludin was diffusely distributed over the cell, again suggesting that accumulation at cell contacts is not a strong intrinsic property of occludin. This L-cell clone was previously shown to express very low levels of cadherin, and consistent with this it lacks Ca^{2+}-dependent adhesion (Angres et al., 1996). It was recently shown that the cadherin-binding protein β-catenin binds to ZO-1 early after initiation of cell contacts, and that these proteins subsequently sort over time into distinct tight and adherens junctions (Rajasekaran et al., 1996). Interaction with cadherin through β-catenin provides a tentative explanation for why ZO-1 clusters at cadherin contacts in occludin-null cells and why ectopically-expressed occludin fails to cluster in cells which lack cadherin. The cell-cell contacts of NRK cells have been shown to contain several components of adherens-type junctions, including vinculin and α-actinin (Yonemura et al., 1995), thus direct or indirect interaction with any of these could conceivably also provide a mechanism to recruit ZO-1 and occludin.

Our results suggest a correlation in fibroblasts between the ability of occludin to cluster at cell-cell contacts and confer adhesiveness. One possible explanation is that occludin molecules must cluster to gain sufficient cooperativity for adhesiveness to be detected in the assay employed here. Other circumstantial evidence also suggests occludin's adhesiveness is not inherently high. For example, as judged by immunofluorescence, no more occludin accumulates between two fibroblasts which both express occludin than between null cells and expressing cells. In addition, even when expressed on two adjacent cells, occludin never promotes a continuous linear, tight junction-like, contact and its expression does not seem to morphologically alter the pre-existent ZO-1 containing contact. Another explanation for the correlation between clustering and adhesion might be that occludin must interact with cytoplasmic proteins present in the plaque to induce an adhesive conformation. Both models are consistent with the known properties of other adhesion molecules, such as the integrins, which increase adhesion through clustering as well as through con-

formational changes induced from the cytoplasmic side (Dehar and Hannigan, 1996).

McCarthy et al. (1996) demonstrated the colocalization of ZO-1 and chicken occludin in MDCK cells cultured in low calcium, both in vesicular structures within cells and occasionally between cell pairs, consistent with the idea that ZO-1-occludin interactions can be maintained in the absence of Ca^{2+}. We demonstrated that occludin is adhesive in the absence of calcium, although one possiblility is that occludin merely enhances cadherin-based adhesion, or adhesion due to other cell suface proteins, even in low calcium. It seems more likely that the longstanding observation that tight junction formation is dependent on calcium-dependent cadherin-dependent cell contact may be based on the requirement of cadherin to induce the highly organized and adhesive state of occludin within the tight junction and not on the requirement for cadherin as a co-adhesive receptor. Contrasting results in NRK and Rat-1 cells with L-cells suggests the testable hypothesis that clustering and/or interaction with ZO-1, and not cadherin per se is required to observe adhesion.

Occludin has been proposed to have two extracellular loops, based on four predicted hydrophobic transmembrane helices and immunologic evidence that the C terminus is intracellular (Furuse et al., 1993). We have confirmed that at least the first of these loops is in fact extracellular, since it is accessible in a nonpermeabilized cell to an antibody generated to a peptide sequence contained within this loop. In this paper, we have focused on the first extracellular loop because it is the least conserved and thus may provide species specific recognition. Recent work of Wong and Gumbiner (1997) has demonstrated that a peptide consisting of the chicken sequence for the second loop blocked transepithelial electrical resistance when applied to cultured monolayers of *Xenopus* A6 cells. In their assay, a peptide consisting of the first loop of the chicken sequence had no effect on transepithelial electrical resistance, consistent with the possibility for a species specific sequence requirement.

We have shown that two separate peptides containing contiguous sequences of the first extracellular loop are both capable of inhibiting adhesion in the occludin-transfected fibroblasts. Similar methods have been used to inhibit the function of other cell adhesion molecules; for example, small peptides containing extracellular loop sequences for connexins delay gap junction formation (Warner et al., 1995), and a cadherin extracellular peptide inhibits embryo compaction (Blaschuk et al. 1990) and contact-dependent granulosa cell apoptosis (Peluso et al., 1996). The simplest explanation for our results is that the extracellular loops of occludin are involved in binding a protein on the adjacent cell, either through a homophilic interaction or with some other binding partner. Consistent with the possibility that occludin is a homophilic adhesion protein is its induction of adhesion in previously occludin-null fibroblasts.

The apparent K_i in the fibroblast assay for both peptides is in the range of 10-100 μM, suggesting a relatively low affinity. The observation that both non-overlapping peptides, which together represent the entire first loop, separately inhibit adhesion suggests the protein interaction surface may include the entire loop. This would be consistent with the observation that among the five occludin sequences available, it is the unusual composition of the loop, and not necessarily its primary sequence, which is conserved (Ando-Akatsuka, 1996). Occludin has been show to be a component of the tight junction strands visualized by freeze fracture electron microscopy (Fujimoto, 1995). The ability to form linear polymers in the plasma membrane and interact over an extensive protein surface may be the mechanism by which occludin creates a molecular-level barrier across the paracellular pathway.

Our results confirm the topography of occludin, demonstrate occludin's ability to induce adhesion when expressed in cells lacking tight junctions and suggest it must be clustered or interact with cytoplasmic proteins in order to be adhesive. Together these results suggest testable models for how the occludin-based intercellular seal of the tight junction is created by both the specific chemistry of its extracellular loops and by influences of cytoplasmic plaque proteins.

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