

VAP-33 localizes to both an intracellular vesicle population and with occludin at the tight junction

Lynne A. Lapierre^{1,3,*}, Pamela L. Tuma², Jennifer Navarre³, James R. Goldenring³ and James M. Anderson¹

¹Departments of Internal Medicine and Cell Biology and the Yale Liver Center, Yale University, New Haven, CT, USA

²Department Of Cell Biology and Anatomy, John Hopkins University, School of Medicine, Baltimore, MD, USA

³The Institute of Molecular Medicine and Genetics, Medical College of Georgia and the Augusta VA Medical Center, Augusta, GA 30912, USA

*Author for correspondence (e-mail: lynnel@immg.mcg.edu)

Accepted 16 August; published on WWW 18 October 1999

SUMMARY

Tight junctions create a regulated intercellular seal between epithelial and endothelial cells and also establish polarity between plasma membrane domains within the cell. Tight junctions have also been implicated in many other cellular functions, including cell signaling and growth regulation, but they have yet to be directly implicated in vesicle movement. Occludin is a transmembrane protein located at tight junctions and is known to interact with other tight junction proteins, including ZO-1. To investigate occludin's role in other cellular functions we performed a yeast two-hybrid screen using the cytoplasmic C terminus of occludin and a human liver cDNA library. From this screen we identified VAP-33 which was initially cloned from *Aplysia* by its ability to interact with VAMP/synaptobrevin and thus was implicated in vesicle docking/fusion. Extraction characteristics indicated that VAP-33 was an integral membrane protein. Antibodies to

the human VAP-33 co-localized with occludin at the tight junction in many tissues and tissue culture cell lines. Subcellular fractionation of liver demonstrated that 83% of VAP-33 co-isolated with occludin and DPPIV in a plasma membrane fraction and 14% fractionated in a vesicular pool. Thus, both immunofluorescence and fractionation data suggest that VAP-33 is present in two distinct pools in the cells. In further support of this conclusion, a GFP-VAP-33 chimera also distributed to two sites within MDCK cells and interestingly shifted occludin's localization basally. Since VAP-33 has previously been implicated in vesicle docking/fusion, our results suggest that tight junctions may participate in vesicle targeting at the plasma membrane or alternatively VAP-33 may regulate the localization of occludin.

Key words: Tight junction, Occludin, VAP-33, Vesicle trafficking

INTRODUCTION

The tight junction (zonula occludens) forms a regulated barrier to the movement of solutes and cells across both epithelial or endothelial layers (Reuss, 1992; Anderson and Van Itallie, 1995). It is positioned at the apical extreme of the lateral surface where it also functions to maintain the apical/basolateral polarity of the cell (Dragsten et al., 1981; Van Meer and Simons, 1986). Three currently known transmembrane proteins within the tight junction are JAM, claudin and occludin. JAM (junctional adhesion molecule) is an Ig-superfamily protein that influences immune cell migration through the tight junction (Martin-Padurs et al., 1998). The claudins (Furuse et al., 1998a) are a family of proteins that appear to play a major role in forming the strands of the tight junction and in barrier function (Furuse et al., 1998b). Occludin (Furuse et al., 1993) has also been shown to have some involvement in cell-cell adhesion (Van Itallie and Anderson, 1997) and in creating the paracellular barrier (McCarthy et al., 1996). The tight junction also contains members of the membrane-associated guanylate kinase

(MAGUK) family, including ZO-1, which are involved in scaffolding of proteins at the membrane (Anderson et al., 1995). Some of the small GTPases, including members of the Rho and Rab families (Braga et al., 1997; Goldenring et al., 1996; Zahraoui et al., 1994) have also been localized to the apical junctional complex thus implicating the tight junction in nucleotide-regulated protein interactions. Despite the tight junction's role in many cellular functions including the intercellular seal, the intramembranous polarity barrier, and potentially in cell signaling and growth regulation it has yet to be directly implicated in vesicle targeting.

Some of the tight junction proteins have been shown to interact with each other; ZO-1 binds both ZO-2 and the cytoplasmic C-terminal tail of occludin (Gumbiner and Simons, 1986; Furuse et al., 1994). In fact, the contribution of occludin to cell adhesiveness correlates with the ability of occludin to interact with ZO-1 (Chen et al., 1997; Van Itallie and Anderson, 1997). To understand better how the cytoplasmic C-terminal tail of occludin might contribute in other cellular functions, we employed the yeast two-hybrid screen to search for binding partners. From this screen we

isolated the human homologue of VAP-33 (VAMP associated protein of 33 kDa), a protein previously isolated from *Aplysia* by its ability to interact with VAMP (vesicle associated membrane protein: Skehel et al., 1995), also known as synaptobrevin.

The work presented here demonstrates that VAP-33, previously implicated in vesicle docking in neurons of *Aplysia*, binds the tight junction protein occludin. VAP-33 is expressed ubiquitously, both immunolocalization and subcellular fractionation demonstrates that VAP-33 is present in a cytoplasmic vesicle pool and at the plasma membrane with occludin. These observations lead us to speculate that VAP-33 is functioning as a component of the cellular vesicle docking machinery specifically involved in targeting of vesicles to the tight junction, whether these are general trafficking vesicles or vesicles specific to occludin is yet to be determined.

MATERIALS AND METHODS

Yeast two-hybrid screen

To produce the 'bait' vector, pGB-cOcc, a cDNA encoding the C-terminal 146 amino acids (corresponding to amino acids 358-504; (Furuse et al., 1993) of the chicken occludin cytoplasmic tail was cloned into a modified version of the yeast vector, pGBT9 (Clontech) containing a new multi-cloning site (5'-*NotI*, *Bam*HI, *Sma*I, *Eco*RI, *Pst*I-3'). This construct was then used as 'bait' to screen an adult, human liver cDNA library cloned into the yeast vector pACT2 (Clontech). The screen was performed according to the manufacturer's protocol with the following modifications. The co-transformation was performed sequentially by first transforming the yeast strain Y190 with the pGB-cOcc vector then transforming with the library. The co-transformed yeast were grown for 5 days on the Trp/Leu/His dropout plates supplemented with 20 mM 3-aminotriazole (Sigma). The β -galactosidase filter assay, binary assays and plasmid isolation from yeast were performed according to the manufacturer's protocol. The activation domain vectors were recovered by electroporation into HB101 cells (Bio-Rad) following the manufacturer's instructions. To enhance for bacteria transfected with the pACT2 vector the bacteria were grown on minimal medium plates according to the Clontech protocol. Three plasmids containing the full-length hVAP-33 were isolated from this screen and sequenced by the Keck Foundation Biotechnology Resource Laboratory at the Yale School of Medicine.

Database searches, alignment and sequence analysis

The GenBank search was performed using dbblast of the Wisconsin GCG program. The multiple sequence alignment and the motif search were performed using Lasergene (DNASTAR Inc. Madison, WI).

Fusion protein and anti-human VAP-33 production

A His-tagged fusion protein of human VAP-33 missing the hydrophobic C-terminal tail (hVAP Δ C) was produced by subcloning a cDNA encoding amino acids 1-226 of human VAP-33 into pTrcHisC. This fusion protein was insoluble. To produce a semi-soluble fusion protein, harvesting and binding of the hVAP Δ C fusion protein to the nickel column was performed following the Xpress system (Invitrogen) denaturing protocol. After washing the column with the denaturing wash buffer (8 M urea, 20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) the column buffer was slowly switched to the native wash buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH 6.0) in a step-wise manner (1:1, 1:4, 1:8 ratio of denaturing wash buffer to native wash buffer). The fusion protein could then be eluted off in a soluble form using 500 mM imidazole in native wash buffer. This fusion protein was then used as

an immunogen to produce the rabbit antisera DE and RO. All antisera were produced in rabbits by the Yale Veterinary Clinical Services using NIH-approved protocols.

Other antibodies used

Rabbit (Zymed Laboratories) and guinea pig (Anderson and Van Itallie, 1995) polyclonal antisera and a mouse monoclonal antibody (Zymed) against human occludin were used for western blotting, immunofluorescence, and immunogold, respectively. A rat anti-ZO-1 (Chemicon) was used for immunofluorescence, while a rabbit anti-ZO-1 (Zymed) was used for western blotting. As controls for the One Step Golgi Flotation rabbit antisera to dipeptidyl peptidase IV (DPP-IV; Bartles et al., 1987), syntaxin 3 (Fujita et al., 1998), and to sialyl transferase (a kind gift from Gerald Hart, John Hopkins University) were used.

GFP-VAP-33 construction and transfections

The entire VAP-33 cDNA was subcloned from the pACT2 vector into the pEGFP-C2 (Clontech). The resulting chimera protein will have the GFP protein fused to the N terminus of the VAP-33 protein. Transfection into Madin-Darby canine kidney (MDCK) cells was then performed using Effectene (Qiagen) following the manufacturers protocol. The DNA (1 μ g/60 mm plate) complex was incubated with the cells overnight, the cells were then washed, refed and allowed to recover 48 hours before selection. Cells were then trypsinized and replated to limiting dilution under selection with 0.5 mg/ml G-418 (Cellgro). Individual colonies were identified and colonally expanded based on their GFP-fluorescence.

Cell culture

All cell lines were grown in D-MEM supplemented with penicillin-streptomycin (100 units/ml and 100 mg/ml, respectively), L-glutamine (2 mM), MEM non-essential amino acids (0.1 mM; Gibco/BRL). In addition the Caco-2 cells were supplemented with human transferrin (10 mg/ml; Boehringer Mannheim) while the GFP transfected cells were supplemented with 0.5 mg/ml G418 sulfate (Cellgro). Caco-2 cells were split 1:5 and NRK cells were split 1:10 onto glass coverslips in 12-well plates (Corning) for immunofluorescence or 6-well plates (Corning) for protein preparation and grown at 37°C in 5% CO₂ for 5 days. MDCK and MDCK/GFP-VAP were split 1:20 onto 0.4 μ M transwell filters (Costar) and grown as above for 4 days for confocal microscopy and immunogold TEM.

Protein sample preparation and western blots

Rat tissue from brain, colon, kidney, liver and pancreas were homogenized in a bicarbonate buffer without detergents (10 ml of buffer for every gram of tissue), this crude homogenate was then mixed with 1/3 volume of 10 \times Laemmli sample buffer. Tissue culture cells were washed with PBS, then scrap harvested into microcentrifuge tubes with PBS, spun 5 minutes at 3,000 g and resuspended in 2 \times Laemmli sample buffer. All samples were heated for 5 minutes at 100°C and separated on a 12% SDS-PAGE gels according to the method of Laemmli (1970).

To test the extraction properties of VAP-33 rat liver was homogenized in HKB buffer (50 mM Hepes, 65 mM KCl and 10 mM NaHCO₃, pH 7.6) containing a protease inhibitor cocktail (aprotinin, leupeptin, antipain and trypsin-chymotrypsin inhibitor all at 0.1 mg/ml and benzamidine and phenylmethylsulfonyl fluoride at 0.2 mM). The crude homogenate was spun at 20 rpm in a refrigerated table top microcentrifuge for 30 minutes at 4°C; an aliquot of the supernatant was saved for western analysis. The pellet was resuspended and divided equally among the extraction treatments (see the legend of Fig. 2). The mixtures were incubated for 60 minutes at 0°C, then centrifuged as above with the exception of the Triton X-114 treated sample. This sample was placed over a sucrose cushion (6% sucrose, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton X-

114), warmed for 3 minutes at 30°C then spun for 3 minutes at 3,000 g, RT. The supernatant was re-extracted with 0.5% Triton X-114 (0°C), incubated for 3 minutes at 0°C, layered over the same cushion and treated as above. For all treatments the final supernatants were transferred to a new tube and the remaining pellets were resuspended in the original volume of extraction solution. All samples were separated on 4-12% gradient SDS-PAGE gel as above.

Gels were transferred to Immobilon-P (Milipore) and blocked for 1 hour at RT with DMP/PBS-T (10% dry milk powder, PBS, 0.1% Tween-20). The blots were cut into thirds with the top third probed for ZO-1, the middle for occludin and the bottom for VAP-33. The primary antibodies were diluted in DMP/PBS-T (all at 1:1000) and incubated with the blots for 1 hour at RT. The blots were washed 4× 10 minutes with PBS-T, then incubated for 1 hour at RT with horseradish peroxidase-labeled donkey anti-rabbit secondary antibody (Amersham) diluted in DMP/PBS-T, washed as above and detected with ECL (Amersham) and Kodak XAR film.

Immunofluorescence

Rat tissues were quick frozen with liquid nitrogen cooled freon and then stored in liquid nitrogen until needed. The blocks were mounted in Tissue-tek, 6 µm sections were cut with a Cryostat (Lab-Tek Instruments), the sections were mounted onto gelatin or VectaBond (Vector laboratories) coated slides and stored at -80°C until needed. At the time of staining the sections were allowed to air dry for 10 minutes, then fixed with methanol (prechilled to -20°C) for 30 minutes at 4°C and extracted with acetone (prechilled to -20°C) for 3 minutes at -20°C. The sections were rehydrated with PBS prior to blocking for 1 hour at RT with IF block (2% normal goat serum, PBS-T). Primary antibodies were diluted with IF block and incubated on the sections overnight at 4°C. Washing was done 4× 15 minutes in PBS-T at RT with gentle agitation. Secondary antibodies, FITC-donkey anti-rabbit and Texas Red-donkey anti-guinea pig were diluted in PBS-T and incubated for 1 hour at RT. Both secondary antibodies used were affinity purified (Jackson ImmunoResearch Laboratories) and tested for species specificity. The secondary antibodies were also tested to rule out bleed through of the FITC fluorescence into the Texas Red channel, and also for the reverse. Washing was performed as above. Sections were rinsed in sterile water just prior to mounting and then mounted using VectaShield (Vector Laboratories). Tissue culture cells grown on coverslips were washed with PBS containing 1 mM CaCl₂ and then processed as above starting with the methanol incubation. All staining, was observed using a Nikon Microphot-FX (Tucson, AZ), the images were captured either with a CCD camera (Sensys) controlled by Image-Pro Plus software (Media Cybernetics, Silver Springs, MD) or with 35 mm film that was then digitized with SprintScan (Polaroid) into Adobe Photoshop.

For confocal microscopy, MDCK and MDCK cells transfected with GFP-hVAP-33 were grown on transwell filters fixed, extracted and stained as above, with the following exceptions. The fix/extracted cells were incubated overnight at 4°C with both rabbit anti-VAP-33 and guinea pig anti-occludin for the MDCK cells or just the anti-occludin for the GFP-VAP-33 transfected cells. The next day rat anti-ZO-1 was added to both and further incubated for an additional 2 hour at RT. The secondaries used were Cy2-donkey anti-rabbit, Cy3-donkey anti-rat and Cy5-donkey anti-guinea pig (Jackson ImmunoResearch), for the MDCK cells or just the latter two for the transfected cell lines. All secondaries used were affinity purified for species specificity. The filters were then cut out and mounted with Prolong Antifade solution (Molecular Probes). The cells were imaged with a scanning confocal fluorescence microscope (Molecular Dynamics, Sunnyvale, CA). Section series (thirty 0.29 µm optical sections) were performed twice, first using dual imaging with the 488 and 647 nm excitation laser lines, 530 DF30 and 660 EFLP emission filters and a 650 beam splitter to visualize the Cy2 or GFP with the Cy5 fluorochromes. The cells were then reimaged with a 568 nm excitation laser line, a 570 EFLP emission filter and a 565 beam splitter to visualize the Cy3

fluorochrome. Section series were rendered in look through projections using ImageSpace software (Molecular Dynamics) on a Silicon Graphics workstation.

One-step Golgi flotation/liver fractionation

The fractionation was performed according to the method of Bergeron et al. (1982) with minor modifications (Schell et al., 1992). Briefly, livers from male Sprague-Dawley rats (CD strain; 125-150g; Charles River Breeding Labs, Wilmington, MA) were homogenized in 5.5 volumes of ice-cold 0.25 M SKTM (0.25 M sucrose, 50 mM Tris, 25 mM KCl, 5 mM MgCl₂, pH 7.4). The homogenates were then mixed with 2 M SKTM until the sucrose concentration was 1.02 M (refractive index = 1.3827), and 19 ml aliquots were distributed into centrifuge tubes. Linear sucrose gradients (0.2-1.02 M, 18 ml) were poured on top of the homogenates and centrifuged 3.5 hours at 85,300 g in a SW28 swinging bucket rotor (Beckman). Fractions were collected from the top and designated as follows: A, top of the tube to the floated band; B, the floated band enriched in Golgi membranes; C, between the band and load; D, the load fraction; E, the pellet. All steps were performed at 4°C.

The fractions were assayed for the presence of VAP-33, occludin and specific organelle and plasma membrane markers by quantitative immunoblotting. The samples were mixed with Laemmli sample buffer and analyzed by SDS-PAGE and western blotting as described above. The relative levels of the proteins in each fraction were determined by densitometric analysis (Microcomputer Imaging Device (MCID), Imaging Research Inc., Ontario, Canada) of immunoreactive species relative to the total recovered and indicated as percentage distribution.

Electron microscopy and immunogold labeling

MDCK cells were grown on 0.4 µm transwells filters as for the immunofluorescence staining. The cells were washed with PBS, then fixed with 4% paraformaldehyde, 0.1% glutaraldehyde and 0.1% saponin for 30 minutes at room temperature, washed in PBS, the transwell filters were cut out and rolled for lateral sectioning. The rolled filters were rinsed in deionized water, dehydrated stepwise in ethyl alcohol and then infiltrated and embedded in LR White (Ted Pella, Inc).

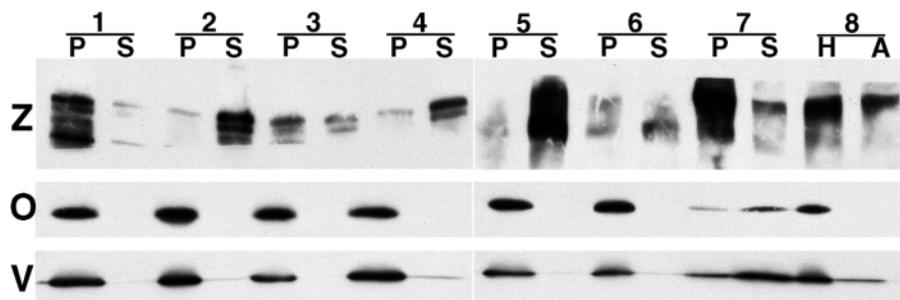
The sections were stained for VAP-33 and occludin following a modified procedure of Smith and Jarett (1993). Briefly the grids were blocked with 2% normal donkey serum in PBS-T (PBS, 0.05% Tween-20) for 1 hour at RT. Mouse anti-occludin and rabbit anti-VAP-33 (RO) were diluted (1:500 and 1:250, respectively) in the blocking buffer and incubated overnight at 4°C with the sections. The primaries were washed off with PBS-T and then incubated for 1 hour at room temperature with a 18 nm colloidal gold tagged anti-mouse secondary for occludin and a 6 nm colloidal gold tagged anti-rabbit secondary for VAP-33 (Jackson ImmunoResearch) diluted 1:25 in 0.2% NDS/PBS-T. The sections were again washed with PBS-T followed by deionized water, counter stained with 1% aqueous, neutralized uranyl acetate for 5 minutes at RT and washed with deionized water. The sections were visualized with a JEOL CM 2100 transmission electron microscope.

RESULTS

Human VAP-33: interaction with occludin, cloning, sequence analysis and antibody production

A yeast two-hybrid screen of an adult human liver library was performed using the last 146 amino acids of the carboxyl-terminal, cytoplasmic tail of chicken occludin (aa 358-504) as the probe. This screen yielded three identical cDNAs that were positive in both the nutritional and β-galactosidase assays. These clones were retested in binary yeast two-hybrid assays

Fig. 2. VAP33 is an integral membrane protein that can only be extracted by detergent. A crude membrane fraction of rat liver was subjected to differential extraction protocols as indicated in the figure using the following buffers: 1, HKB; 2, pH 11; 3, pH 2.8; 4, 0.5 M KCl; 5, 10 mM EDTA; 6, 6 M urea; 7, 2% TX-100; 8, 1% TX-114. P is pellet, S is supernatant, H is hydrophobic phase and A is aqueous phase. The top was probed for ZO-1, the middle for occludin and the bottom for VAP-33. Molecular mass markers are indicated on the right.



To characterize further the mammalian VAP-33, we produced antibodies against a fusion protein encoding the human VAP-33 cDNA corresponding to the MSP-like domain and the variable domain (see Materials and Methods). Two other anti-human rabbit polyclonal antisera were produced to a different His-VAP-33 fusion protein containing just the MSP-like domain of VAP-33, all four anti-human VAP-33 antisera recognize a 33 kDa protein from rat liver. Two anti-mollusc polyclonal rabbit sera, PAS3 and PAS5, also recognized a 33 kDa protein from rat liver (data not shown).

VAP-33 is an integral membrane protein

The hydrophobic sequence at the carboxyl terminus of VAP-33 suggested it was a type 2 integral membrane protein (Fig. 1C). To determine biochemically if VAP-33 is associated with the membrane as an integral protein, a crude membrane fraction of rat liver was prepared and subjected to differential extraction procedures (Fig. 2). Neither high nor low pH, high salt, a chelating agent for divalent cations, nor a denaturing agent solubilized VAP-33 from the membrane pellet. Inclusion of detergent (Triton X-100 shown here) was the only condition able to solubilize VAP-33 from membranes. When another detergent, Triton X-114 was used and separated into the hydrophobic and aqueous phases, VAP-33 partitioned into the hydrophobic phase similar to other integral membrane proteins. Occludin, a transmembrane protein at the tight junction behaved similarly to VAP-33 during the extraction procedures. While ZO-1, a cytoplasmic, tight junction protein, has a non-detergent extractable sub-population similar to VAP-33 and occludin it is extractable to varying degrees by high pH, high salt and urea characteristic of a peripherally associated protein (as previously published by Anderson et al., 1988). These data confirmed the sequence motif prediction that VAP-33 is an integral membrane protein.

VAP-33 is ubiquitously expressed

VAP-33 was found in all tissues so far tested including brain, colon, kidney, liver and pancreas, as was occludin (Fig. 3). The smear of higher bands seen when the blot is probed for occludin are due to different phosphorylation states of occludin (Sakakibara et al., 1997). We believe the lower molecular mass band seen in some of the VAP-33 lanes is a proteolyzed form of VAP-33 as is the lower molecular mass band seen in some occludin lanes. While it appeared all tissue expressed both VAP-33 and occludin the ratio between them varied. For example, in colon the VAP-33 band appeared to be more intense than the occludin band while in liver they were roughly

equal or the occludin band was slightly more intense. This observation led us to investigate the possibility that there are two pools of VAP-33 in cells with potentially distinct functions. Support for this idea can be found in tissue culture cells. In an epithelial derived cell line that forms tight junctions, such as Caco-2, both VAP-33 and occludin were detected (Fig. 3), similar results were seen with MDCK, HepG2 and T84 cells (data not shown). When a fibroblastic cell line that does not form tight junctions, such as NRK, is probed for VAP-33 and occludin only VAP-33 was detected in these cells (Fig. 3), similar results were seen with other fibroblastic cell lines (rat-1 and L-cells; data not shown). These results indicate that neither the formation of tight junctions nor the presence of occludin is necessary for the expression of VAP-33.

VAP-33 co-localizes with occludin at the tight junction

Immunolocalization of VAP-33 revealed a striking co-localization with occludin in all epithelial tissues tested (Fig. 4A). The antiserum RO stained colon and co-localized with occludin at the apical edge of crypt cells (Fig. 4A, b,g), while the pre-immune sera of RO exhibited no staining in the colon (Fig. 4A, a,f) or any other tissue (data not shown). In brain VAP-33 and occludin co-localized at the tight junction in the choroid plexus, a secretory epithelium that lines the ventricles

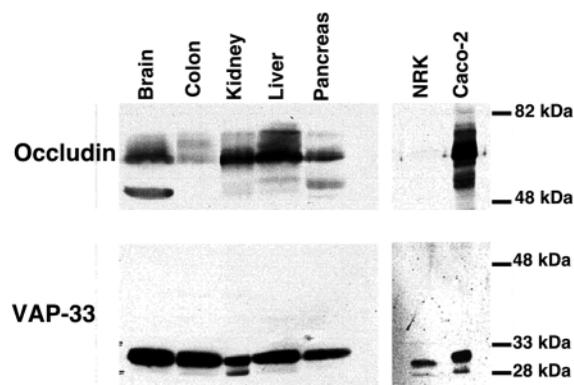
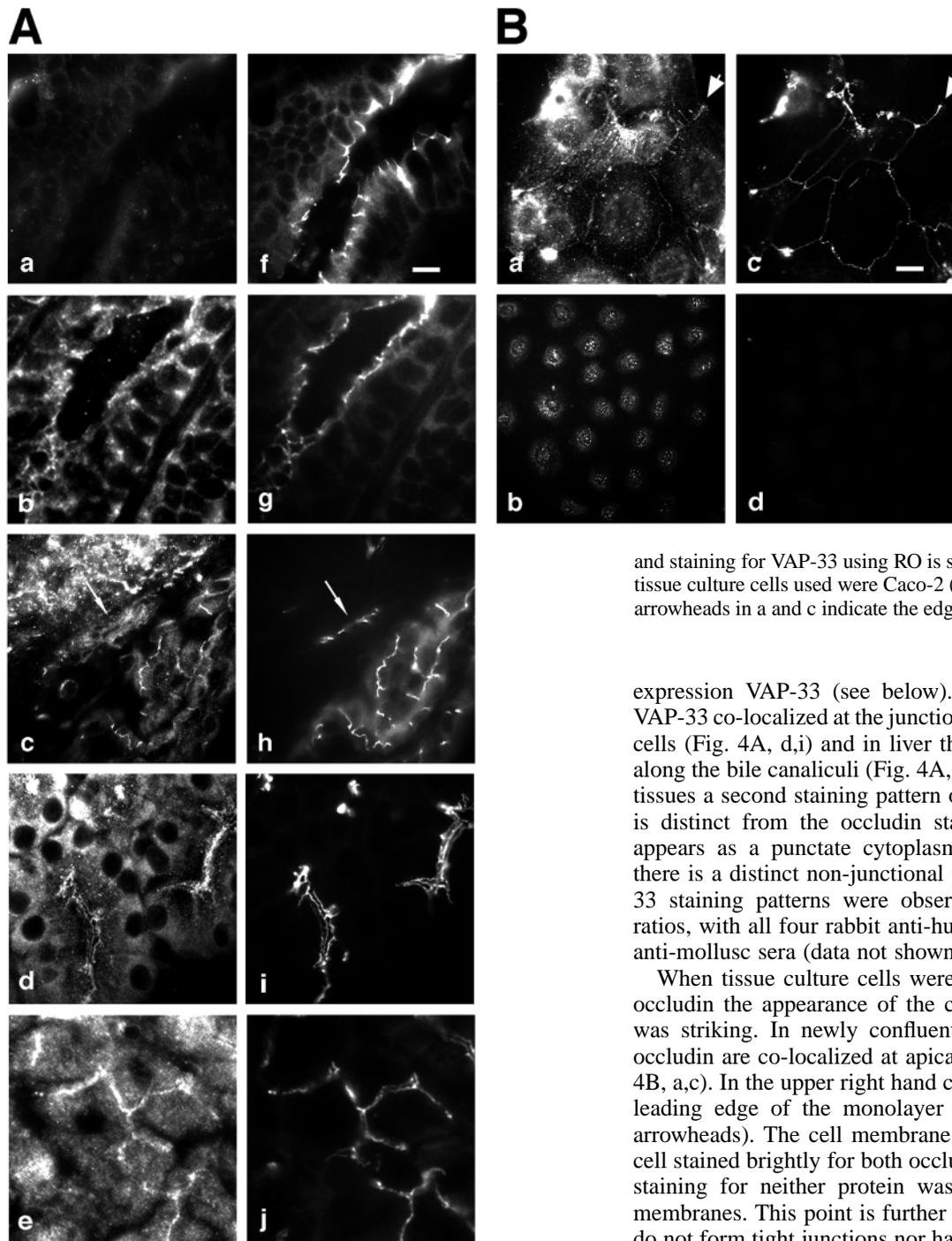


Fig. 3. Western blot of rat tissue and tissue culture cell lines indicates that VAP-33 is ubiquitously expressed. Western blot of rat tissue homogenates and tissue culture cell total lysate were first probed for VAP-33 using DE serum (lower panels, V) then stripped and re-probed for occludin (upper panels, O). Molecular mass markers are shown on the right.



(Fig. 4A, c,h). This co-localization appeared to be restricted to epithelial cells, since no VAP-33 staining was observed in the endothelia of the vascular tissue (Fig. 4A, c,h, arrow). Presumably, this indicates that either VAP-33 is not present in endothelial cells or it is present, but at a level below detection by immunofluorescence. We believe that the latter is more likely, since a low level of VAP-33 can be detected on a western blot of passaged human endothelial cells (a kind gift from J. S. Pober, data not shown). Staining for VAP-33 was also detected in neural cells as a punctate staining pattern (Fig. 4A, c, upper left corner). Neural cells neither form tight junctions nor express occludin, yet similar to fibroblasts they appear to

Fig. 4. VAP-33 co-localizes with occludin at the plasma membrane and is found in a second cytoplasmic pool. (A) Staining for occludin is shown on the right for all tissue (f-j). Staining for VAP-33 using the anti-human antibody RO is shown in b-e. The pre-immune serum of the RO rabbit is shown in a. The rat tissues used colon (a,f,b,g), brain (c,h), pancreas (d,l) and liver (e,j). The arrows in c and h indicate vascular endothelia cells. (B) Localization of VAP-33 in tissue culture cells. Staining for occludin is shown on the right (c,d)

and staining for VAP-33 using RO is shown on the left (a,b). The tissue culture cells used were Caco-2 (a,c) and NRK (b,d). The arrowheads in a and c indicate the edge of the monolayer. Bar, 5 μ m.

expression VAP-33 (see below). In pancreas, occludin and VAP-33 co-localized at the junction between ductular epithelial cells (Fig. 4A, d,i) and in liver they co-localized at junctions along the bile canaliculi (Fig. 4A, e,j). Interestingly, in all four tissues a second staining pattern of VAP-33 was detected that is distinct from the occludin staining. This second pattern appears as a punctate cytoplasmic staining, indicating that there is a distinct non-junctional pool of VAP-33. Both VAP-33 staining patterns were observed, with varying intensity ratios, with all four rabbit anti-human sera and the two rabbit anti-mollusc sera (data not shown).

When tissue culture cells were co-stained for VAP-33 and occludin the appearance of the cytoplasmic pool of VAP-33 was striking. In newly confluent Caco-2 cells VAP-33 and occludin are co-localized at apical cell-cell contact sites (Fig. 4B, a,c). In the upper right hand corner of the micrographs the leading edge of the monolayer can be seen (Fig. 4B, a,c, arrowheads). The cell membrane in contact with an adjacent cell stained brightly for both occludin and VAP-33, in contrast staining for neither protein was seen on the free edge of membranes. This point is further illustrated in NRK cells that do not form tight junctions nor have occludin; here only VAP-33's cytoplasmic staining pattern was observed (Fig. 4B, b,d).

VAP-33 partitions with two fractions, one containing plasma membrane and a second vesicle containing fraction

To biochemically confirm the existence of VAP-33 in two pools within cells, at the plasma membrane and with a putative, cytosolic vesicle pool, we examined its distribution in subcellular fractions of liver. In these preparations, more than half of the sialyl transferase (a trans-Golgi network marker) was recovered in fraction B representing a 50-fold enrichment (Table 1). In contrast, only 2% of either VAP-33 or occludin were detected in this fraction. Rather, the majority of both

Table 1. Distribution of VAP33 and occludin in fractionated rat liver

Fraction	Sialyl transferase*	DPPIV	Syntaxin 3*	Occludin	VAP33
A	5.0±6.1	0.2±0.06	2.0±1.9	0.2±0.3	0.03±0.06
B	58.5±9.2	0.9±0.7	2.3±0.9	2.4±0.3	1.9±1.3
C	21.9±8.6	0.3±0.3	3.5±0.4	2.0±0.5	2.8±2.1
D	10.8±14.2	1.5±1.8	25.2±11.7	7.5±2.0	14.2±3.9
E	3.8±6.6	97.1±1.8	67.3±10.8	87.8±2.3	81.1±4.2

Fractions from the one-step flotation were assayed by quantitative immunoblotting. Percentage are expressed as the mean ± s.d. from three separate fractionations. Fractions were collected from the top and designated as follows: A, top of the tube to the floated band; B, the floated band enriched in Golgi membranes; C, between the band and load; D, the load fraction; E, the pellet.

*The results for sialyl transferase and syntaxin 3 were previously published (Fujita et al., 1998). They are from the same preparations used for the rest of the table and were included here to indicate the properties of the fractions.

occludin (88%) and VAP-33 (81%) partitioned into fraction E, which also contained the majority of the apical plasma membrane marker dipeptidylpeptidase IV (DPPIV; Table 1). Fraction E also contains other membrane populations, most notably, the endoplasmic reticulum (ER). To ensure that the enrichment of VAP-33 and occludin in this fraction was not due to their association with the ER, we prepared highly purified plasma membrane sheets (protocol as in Hubbard et al., 1983) and assayed them for VAP-33 and occludin immunoreactivity. As predicted, substantial levels of both proteins were detected in these preparations (data not shown), allowing us to place the majority of VAP-33 and occludin found in Fraction E with the plasma membrane and not the ER. A significant fraction of VAP-33 (14%) was also detected in fraction D, a fraction that contains many uncharacterized vesicles, including vesicles involved in trafficking to the apical membrane as indicated by the presence of syntaxin 3 in this fraction. Interestingly syntaxin 3 was also enriched into both Fractions D and E similar to VAP-33. In contrast, occludin was relatively depleted in Fraction D (7.5%). The results of the fractionation studies support the immunofluorescence observation that VAP-33 is located in two pools, one at the plasma membrane and a second consisting of cytoplasmic vesicles. Whether the VAP-33 within the plasma membrane containing fraction (E) is actually on the plasma membrane or is located on vesicles in tight association with the plasma membrane is not known

VAP-33 is located near the tight junction using immunogold labeling

Light level localization suggested that VAP-33 co-localized with occludin (Fig. 4). To further resolve the relative locations of occludin and VAP-33 we performed ultrastructural localization using double immunogold labeled MDCK cells. As seen in Fig. 5, the occludin labeling (18 nm gold particles) was found concentrated at the apical end of lateral cell-cell contacts. Labeling for VAP-33 appeared throughout the cell as loose clusters (70-90 nm) of 6 nm gold particles. The arrow indicates such a cluster of cytoplasmic staining for VAP-33. Tighter (50-60 nm), highly concentrated clusters of VAP-33 staining were also seen near occludin and up to 400 nm basal to the occludin staining (arrowheads). The LR White embedding and post-fixation staining method preserves membrane structures poorly and we were unable to determine whether the clustered particles indicated VAP-33 in vesicles near the tight junction or along the lateral plasma membrane. Thus at this time we cannot determine precisely if the VAP-33 is on a vesicle population or the plasma membrane.

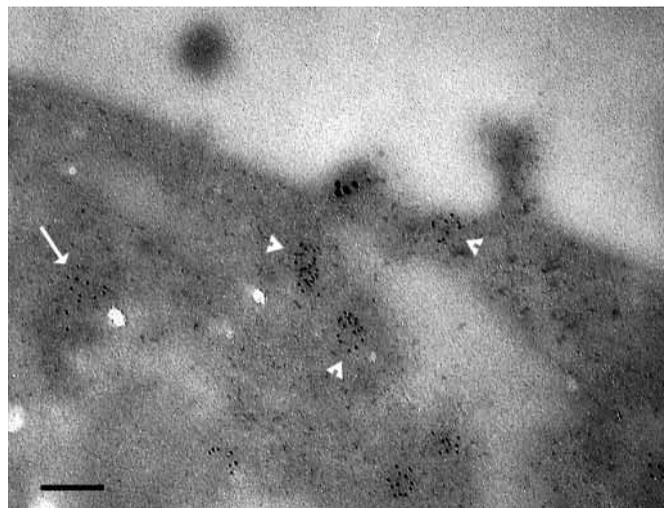


Fig. 5. VAP-33 is located adjacent to the plasma membrane at the tight junction. Thin sections were co-stained for VAP-33 (6 nm gold) and occludin (18 nm gold). The arrowheads indicate clusters of 6 nm gold particles labeling VAP-33 that appear to be basal to occludin at the tight junction. The arrow indicates potential, cytoplasmic vesicles containing VAP-33. Bar, 100 nm.

GFP-VAP-33 relocated occludin along the lateral membrane

We expressed a GFP-tagged chimera of the VAP-33 protein in MDCK cells to determine if it could locate into the same two compartments as the endogenous VAP-33, and to determine if over-expression of VAP-33 had any effect upon endogenous occludin. The GFP-tagged VAP-33 localized to both a cytosolic vesicular compartment and adjacent to the plasma membrane (Fig. 6B) similar to the endogenous VAP-33 (Fig. 6A). Three, independent stable lines of GFP-VAP-33 all exhibited this same localization pattern. The majority of the lateral endogenous VAP-33 localized with occludin and ZO-1 near the tight junction (Fig. 6A, triple z axis). Interestingly endogenous VAP-33 did not localize along the entire length of the tight junction. It appeared to be at the basal end of the junctional plaque (white area in Fig. 6A, triple) similar to the pattern observed in Fig. 5, while ZO-1 and occludin co-localized along the entire length of the tight junction (purple area Fig. 6A, triple).

The majority of the GFP-VAP-33 was observed along the entire lateral membrane and not just in the junctional area. This might be due to the level of over-expression of the construct.

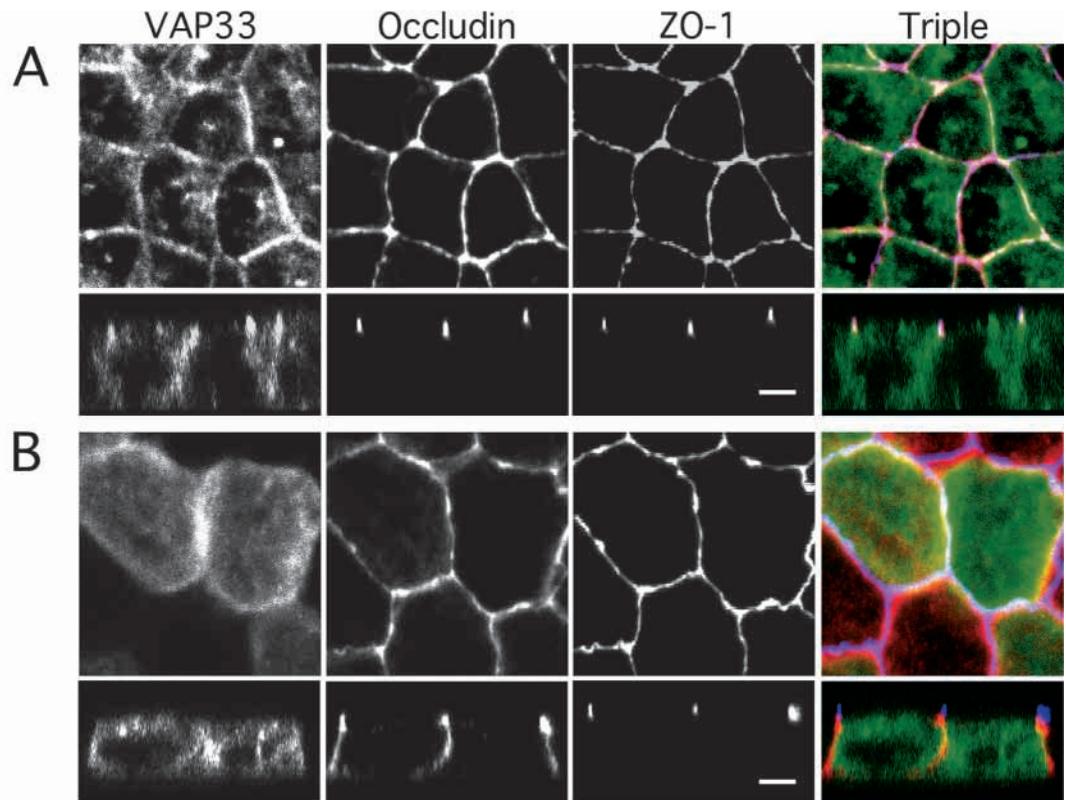


Fig. 6. GFP-VAP-33 relocates occludin along the lateral membrane. (A) MDCK cells stained for endogenous VAP-33 (RO), occludin and ZO-1. (B) GFP-VAP-33 transfected MDCK cells stained as in A for occludin and ZO-1. Here the VAP-33 panel is the over-expressed GFP-VAP-33 and not the endogenous VAP-33. Bar, 2 μ m.

At the light microscopic level the majority of the excess GFP-VAP-33 was lined up along the lateral edge of the plasma membrane. Strikingly, the endogenous occludin had relocated along the lateral membrane following the GFP-VAP-33 pattern (Fig. 6B). This was not a general shift of tight junction components, since ZO-1 was unaffected by the presence of the GFP-VAP-33. This effect on occludin's localization by GFP-VAP-33 lends further functional support to the yeast two-hybrid results demonstrating an interaction of VAP-33 and occludin.

DISCUSSION

Screening a human liver yeast two-hybrid library with the carboxy terminus of occludin we isolated a known protein, VAP-33. During the preparation of this manuscript Weir et al. (1998) published the human VAP-33 sequence cloned from a pancreatic islet cell library. Two other known proteins exhibited extensive homology with the human and *Aplysia* VAP-33, SCS-2 and MSP. The exact function of the SCS2 gene product is unknown at this time. The MSPs constitute a family of very closely related proteins found only in nematode sperm (for a review see Roberts and Stewart, 1995). MSP proteins can polymerize with themselves in a pH dependent manner to form filaments that allow rapid locomotion of the nematode sperm (King et al., 1994). Suggesting that VAP-33 may be able to associate with itself.

Western blotting and immunofluorescence indicated that VAP-33 is ubiquitously expressed in tissues and both epithelia and fibroblast cell lines. Extraction data indicated that VAP-33 is a membrane associated protein. VAP-33's sequence

suggested a type 2 integral membrane protein with a coiled coil domain at the carboxyl terminus, these structural characteristics are shared with members of the syntaxin family. Syntaxins also are type 2 integral membrane protein with a coiled coil region within the later half of the proteins that is believed to mediate binding to synaptobrevin and SNAP-25 (Calakos et al., 1994; Chapman et al., 1994).

Immunolocalization and direct interaction with the cytoplasmic tail of occludin, as revealed through the yeast two-hybrid method, indicated that VAP-33 localized near the tight junction. Although we were unable to confirm this interaction using biochemical techniques, we have shown that VAP-33 and occludin co-localize at the tight junction (Figs 4, 5, and 6A) and that over-expression of a GFP-VAP-33 chimera can influence the localization of occludin (Fig. 6B). We feel that these two results support the results of the yeast two-hybrid assay that VAP-33 and occludin interact.

Our immunofluorescence data further suggest that VAP-33 is localized in two subcellular populations within epithelial cells. One population is dispersed though out the cytoplasm with potentially a concentration in the apical end of the cell, while a second population is located in close proximity to the tight junction and occludin (Figs 4, 6). Interestingly only the first population is observed in fibroblast cell lines indicating that occludin is required for either VAP-33 localization near the plasma membrane or in focusing VAP-33 at the membrane. The fractionation of liver membranes also indicated that VAP-33 is in two pools, one fraction of cytoplasmic vesicles that does not contain occludin and a second membrane fraction that does contain occludin (Table 1: Fractions D and E, respectively). When Skehel et al. (1995) fractionated *Aplysia* tissue they also detected VAP-33 in two

fractions, a vesicular fraction and one associated with plasma membrane markers.

At the light microscopic level it is difficult to determine if the population localizing with occludin is directly in the plasma membrane or is located in small vesicles in close association with the plasma membrane. Minimal stabilization of membrane structure with the LR White embedding medium and the inability of the antibodies to bind in the presence of osmium tetroxide or tannic acid did not allow us to visualize the membranes. Nevertheless, loose clusters (70-90 nm) of VAP-33 labeling are observed within the cytoplasm of the cell along with tighter, more concentrated clusters (50-60 nm) of VAP-33 labeling basal to the occludin labeling, indicating that VAP-33 and occludin are localizing adjacent to each other at the tight junction. The lack of VAP-33 labeling directly next to occludin indicates to us that occludin and VAP-33 associate through a transient rather than a stable interaction.

The functional implication of an interaction between VAP-33 and occludin is unclear. We can speculate that VAP-33 may function to regulate the delivery of occludin to the tight junction, as the GFP-VAP-33 data seem to indicate. Still we have yet to see endogenous VAP-33 and occludin co-staining on cytoplasmic vesicles and VAP-33 is relatively abundant compared to occludin, especially in non-polarized cells where occludin is not detectable thus, we believe that VAP-33 has a more general function in vesicle trafficking. Considering the evolutionary diversity between mollusks and humans, the percentage of similarity found in VAP-33 of the two species is striking and could indicate a conserved function. *Aplysia* VAP-33 was originally identified by its ability to bind VAMP/synaptobrevin. Recently, Weir et al. (1998) have shown that hVAP-33 is also capable of interacting with both synaptobrevin/VAMP-1 and -2. Based on these observations we speculate that VAP-33 may play a role in vesicle trafficking, specifically to the tight junction plaque.

Louvard (1980) observed that endocytosed aminopeptidase recycling took place primarily in the region of cell-cell contact. Further support for a model involving vesicle trafficking through the apical junctional complex comes from Grindstaff et al. (1998). They recently identified the mammalian homologues of two yeast genes, Sec6/8, that are known to be important in targeting vesicles to specific areas of the yeast membrane. In MDCK cells Sec6/8 localized to junctional complexes and were involved in vesicle targeting to the basolateral membrane, but not to the apical membrane. We do not propose that vesicles are docking and fusing at the tight junction, rather we suggest that vesicles are using occludin located in the tight junction, as a directional indicator of polarity. In non-polarized cells there is no tight junction and the apical/basolateral boundary does not exist. In these cells there is no occludin and VAP-33 does not localize to or focus at the plasma membrane. Potentially, in non-polarized cells the trafficking of vesicles to specific areas of the plasma membrane is not as crucial and thus VAP-33 is not needed as a focused, directional indicator. As to what if any role VAP-33 actually plays in apical recycling or in general vesicle trafficking is currently under investigation.

In conclusion, we have cloned the human homologue of a protein thought to be involved in vesicle trafficking in *Aplysia*, VAP-33. In mammalian polarized epithelial cells this protein co-localizes with occludin at the tight junction and with a

potential vesicular population. While VAP-33 shares no sequence homology with any of the known SNARE proteins, it does contain the molecular characteristics of a SNARE, it is a small type 2, integral membrane protein with a coiled coil domain near its carboxyl terminus. VAP-33 could be using occludin as a marker or anchor at the apical/basolateral boundary to function as a directional indicator for vesicle trafficking. Alternatively, VAP-33 could be regulating the localization of occludin at the tight junction.

We are grateful to Paul Skehel and Kelsey Martin for the gifts of the anti-*Aplysia* VAP-33 sera. We thank Pietro DeCamilli and James D. Jamieson for their helpful conversation and members of the James M. Anderson and James Boyer laboratories for their assistance. Confocal microscopy was performed in the Imaging Core and electron microscopy was performed in the Electron Microscopy Core of the Institute for Molecular Medicine and Genetics at the Medical College of Georgia. L.A.L. received stipend support from DK45134, DK38979 and DK07356. Research was supported by NIH grants DK38979 and DK45134 (to J.M.A.), DK48370 and DK43405 and a Department of Veterans Affairs Merit Review (to J.R.G.).

REFERENCES

- Anderson, J. M., Stevenson, B. R., Jesaitis, L. A., Goodenough, D. A. and Mooseker, M. S. (1988). Characterization of ZO-1, a protein component of the tight junction from mouse liver and Madin-Darby canine kidney cells. *J. Cell Biol.* **106**, 1141-1149.
- Anderson, J. M. and Van Itallie, C. M. (1995). Tight junctions and the molecular basis for regulation of paracellular permeability. *Am. J. Physiol.* **269**, G467-G475.
- Anderson, J. M., Fanning, A. S., Lapierre, L. A. and Van Itallie, C. M. (1995). Membrane-associated guanylate kinase homologs of the tight junction. *Trans. Biochem. Soc.* **23**, 470-475.
- Bartles, J. R., Fercci, H. M., Stieger, B. and Hubbard, A. L. (1987). Biogenesis of the rat hepatocyte plasma membrane in vivo: comparison of the pathways taken by apical and basolateral proteins using subcellular fractionation. *J. Cell Biol.* **105**, 1241-1251.
- Bergeron, J. J. M., Rachubinski, R. A., Sikstrom, R. A., Posner, B. I. and Paiement, J. (1982). Galactose transfer to endogenous acceptors within Golgi fractions of rat liver. *J. Cell Biol.* **92**, 139-146.
- Braga, V. M. M., Machesky, L. M., Hall, A. and Hotchin, N. A. (1997). The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. *J. Cell Biol.* **137**, 1421-1431.
- Calakos, N., Bennett, M. K., Peterson, K. E. and Scheller, R. H. (1994). Protein-protein interactions contributing to the specificity of intracellular vesicular trafficking. *Science* **263**, 1146-1149.
- Chapman, E. R., An, S., Barton, N. and Jahn, R. (1994). SNAP-25, a t-SNARE which binds to both syntaxin and synaptobrevin via domains that may form coiled coils. *J. Biol. Chem.* **269**, 27427-27432.
- Chen, Y.-H., Merzdorf, C., Paul, D. L. and Goodenough, D. A. (1997). COOH terminus of occludin is required for tight junction barrier function in early *Xenopus* embryos. *J. Cell Biol.* **138**, 891-899.
- Dragsten, P. R., Blumenthal, R. and Handler, J. S. (1981). Membrane asymmetry in epithelial: is the tight junction a barrier to diffusion in the plasma membrane. *Nature* **294**, 718-722.
- Fujita, H., Tuma, P. L., Finnegan, C. M., Locco, L. and Hubbard, A. L. (1998). Endogenous syntaxins 2, 3 and 4 exhibit distinct but overlapping patterns of expression at the hepatocyte plasma membrane. *Biochem. J.* **329**, 527-538.
- Furuse, M., Hirase, T., Itoh, M., Nagafuchi, A., Yonemura, S., Tsukita, S. and Tsukita, S. (1993). Occludin: A novel integral membrane protein localizing at tight junction. *J. Cell Biol.* **123**, 1777-1788.
- Furuse, M., Itoh, M., Hirase, T., Nagafuchi, A., Yonemura, S., Tsukita, S. and Tsukita, S. (1994). Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J. Cell Biol.* **127**, 1617-1626.
- Furuse, M., Fujita, K., Hiiragi, T., Fujimoto, K. and Tsukita, S. (1998a). Claudin-1 and -2: novel integral membrane proteins localizing at tight

- junctions with no sequence similarity to occludin. *J. Cell Biol.* **141**, 1539-1550.
- Furuse, M., H. Sasaki, K. Fujimoto and Tsukita, S.** (1998b). A single gene product, Claudin-1 or -2, reconstitutes tight junction strands and recruits occludin in fibroblasts. *J. Cell Biol.* **143**, 391-401.
- Goldenring, J. R., Smith, J., Vaughan, H. D., Cameron, P., Hawkins, W. and Navarre, J.** (1996). Rab11 is an apically located small GTP-binding protein in epithelial tissues. *Am. J. Physiol.* **270**, G515-G525.
- Grindstaff, K. K., Yeaman, C., Anandasabapathy, N., Hsu, S.-C., Rodriguez-Boulan, E., Scheller, R. H. and Nelson, W. J.** (1998). Sec6/8 complex is recruited to cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* **93**, 731-740.
- Gumbiner, B. M. and Simons, K.** (1986). A functional assay for proteins in establishing an epithelial occludin barrier: identification of an ovomorulin-like polypeptide. *J. Cell Biol.* **102**, 457-468.
- Hubbard, A. L., Wall, D. A. and Ma, A.** (1983). Isolation of rat hepatocyte plasma membranes. I. Presence of the three major domains. *J. Cell Biol.* **96**, 217-229.
- King, K. L., Essig, J., Roberts, T. M. and Moerland, T. S.** (1994). Regulation of the *Ascaris* major protein (MSP) cytoskeleton by intracellular pH. *Cell Motil. Cytoskel.* **27**, 193-205.
- Laemmli, U. K.** (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.
- Louvard, D.** (1980). Apical membrane aminopeptidase appears at site of cell-cell contact in cultured kidney epithelial cells. *Proc. Nat. Acad. Sci. USA* **77**, 4132-4136.
- Martin-Padurs, I., S. Lostaglio, M. Schneemann, L. Williams, M. Romano, P. Fruscell, C. Panzeri, A. Stoppacciaro, L. Ruco, A. Villa, D. Simmons and Dejana, E.** (1998). Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J. Cell Biol.* **142**, 117-127.
- McCarthy, K. M., Skare, I. B., Stankewich, M. C., Furuse, M., Tsukita, Sh., Rogers, R. A., Lynch, R. D. and Schneeberger, E. E.** (1996). Occludin is a functional component of the tight junction. *J. Cell Sci.* **109**, 2287-2298.
- Reuss, L.** (1992). Tight junction permeability to ions and water. In *Tight junctions* (ed. M. Cereijido), pp. 49-66. Boca Raton: CRC Press
- Roberts, T. M. and Stewart, M.** (1995). Nematode sperm locomotion. *Curr. Opin. Cell Biol.* **7**, 13-17.
- Sakakibara, A., Furuse, M., Saitou, M., Ando-Akatsuka, Y. and Tsukita, Sh.** (1997). Possible involvement of phosphorylation of occludin in tight junction formation. *J. Cell Biol.* **137**, 1393-1401.
- Schell, M. J., Maurice, M., Stieger, B. and Hubbard, A. L.** (1992). 5'Nucleotidase is sorted to the apical domain of hepatocytes via an indirect route. *J. Cell Biol.* **119**, 1173-1182.
- Skehel, P. A., Martin, K. C., Kandel, E. R. and Bartsch, D.** (1995). A VAMP-binding protein from *Aplysia* required for neurotransmitter release. *Science* **269**, 1580-1583.
- Smith, R. M. and Jarett, L.** (1993). Electron microscopic immunocytochemical approaches to the localization of ligands, receptors, transducers and transporters. In *Handbook of Endocrine Research Techniques* (ed. F. de Pablo, C. C. Scanes and B. D. Weintraub), pp. 227-264. San Diego CA: Academic Press.
- Van Itallie, C. M. and Anderson, J. M.** (1997). Occludin confers adhesiveness when expressed in fibroblasts. *J. Cell Sci.* **110**, 1113-1121
- Van Meer, G. and Simons, K.** (1986). The function of tight junctions in maintaining differences in lipid composition between the apical and the basolateral cell surface domains of MDCK cells. *EMBO J.* **51**, 455-464.
- Weir, M. L., A. Klip and Trimble, W. S.** (1998). Identification of a human homologue of the vesicle-associated membrane protein (VAMP)-associated protein of 33 kDa (VAP-33): a broadly expressed protein that binds to VAMP. *J. Cell Sci.* **333**, 247-251.
- Zahraoui, A., Joberty, G., Arpin, M., Fontaine, J. J., Hellio, R., Tavitin, A. and Louvard, D.** (1994). A small rab GTPase is distributed in cytoplasmic vesicles in non polarized cells but colocalizes with the tight junction marker ZO-1 in polarized epithelial cells. *J. Cell Biol.* **124**, 101-115.