

# Symplekin, a Novel Type of Tight Junction Plaque Protein

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**Abstract.** Using a monoclonal antibody we have identified and cDNA-cloned a novel type of protein localized, by light and electron microscopy, to the plaque associated with the cytoplasmic face of the tight junction-containing zone (*zonula occludens*) of polar epithelial cells and of Sertoli cells of testis, but absent from the junctions of vascular endothelia. The ~3.7-kb mRNA encodes a polypeptide of 1142 amino acids (calculated molecular weight 126.5 kD, pI 6.25), for which the name "symplekin" (from Greek *συνπλέκειν*, to tie together, to weave, to be intertwined) is proposed. However, both the mRNA and the protein can also be

detected in a wide range of cell types that do not form tight junctions or are even completely devoid of any stable cell contacts. Careful analyses have revealed that the protein occurs in all these diverse cells in the nucleoplasm, and only in those cells forming tight junctions is it recruited, partly but specifically, to the plaque structure of the *zonula occludens*. We discuss symplekin as a representative of a group of dual residence proteins which occur and probably function in the nucleus as well as in the plaques exclusive for either tight junctions, adherens junctions, or desmosomes.

**T**HE tight junction (*zonula occludens*) is a fundamental type of intercellular junction that occurs in polarized epithelia and certain endothelia and is believed to provide a permeability barrier to prevent paracellular transport of molecules and particles and to restrict the lateral diffusion of membrane lipids and proteins (for reviews see Pinto da Silva and Kachar, 1982; Madara, 1988; Simons, 1990; Citi, 1993; Gumbiner, 1993; Hirsch and Noske, 1993). Ultrastructurally, tight junctions represent a system of linear close contacts between the two plasma membranes which on freeze-fractures along the hydrophobic plane appear as a network of anastomizing strands. Unlike the diverse adhering junctions, however, information on the molecular composition, the assembly and the functional regulation of tight junctions is still scarce, and only recently the first transmembrane protein, occludin, has been described (Furuse et al., 1993; Ando-Akatsuka et al., 1996).

In addition, a number of cytoplasmic proteins have been reported to be associated with the loosely woven plaque structure undercoating the *zonula occludens*. Some of these components have only been identified as antigens whereas others have been characterized by cDNA-cloning and biochemical studies:

Protein ZO-1 ( $M_r$  220 K; 1,745 amino acids (aa)<sup>1</sup>;  $M_r$  195 kD; Itoh et al., 1993; Willot et al., 1993), appearing in two isoforms, is a component of the plaque of the apical *zonula*

*occludens* of polar epithelia as well as of the junctional belt of vascular endothelia (Stevenson et al., 1986; Anderson et al., 1988; Kurihara et al., 1992; Balda and Anderson, 1993) and in the "junctional specializations" of Sertoli cells of testis (Stevenson et al., 1986; Byers et al., 1991), but has also been described in the plaques of the small junctions connecting the basal processes of podocytes of kidney glomeruli (Schnabel et al., 1990), in certain adherens junction plaques (e.g., Itoh et al., 1991) and as a membrane-associated or "free" cytoplasmic protein not incorporated into junctions (Howarth et al., 1992, 1994; Miragall et al., 1994; Petrov et al., 1994; Van Itallie et al., 1995). On the basis of amino acid sequence comparisons this protein has been grouped into the MAGUK (membrane-associated guanylate kinases) family of proteins (for reviews see Tsukita et al., 1993; Kim, 1995).

Protein ZO-2 ( $M_r$  160 K; Gumbiner et al., 1991; Jesaitis and Goodenough, 1994) has been identified in *zonula occludens* plaques of polar epithelia and vascular endothelia, is highly homologous to ZO-1 and also a member of the MAGUK protein family.

Cingulin (two forms of  $M_r$  108 K and 140 K; Citi et al., 1988) was originally isolated from an avian intestinal brush border fraction and immunolocalized specifically to the *zonula occludens* plaque of polar epithelia and vascular endothelia (Citi et al., 1989). Biochemical analyses of the purified, heat-stable  $M_r$  108 K protein and partial cDNA cloning have revealed that cingulin is a coiled-coil dimer with structural similarities to certain cytoskeletal proteins (Citi et al., 1990).

Antigen 7H6 ( $M_r$  155 K or 175 K, depending on the species; 1,125 aa in the rat protein; Zhong et al., 1993, 1994; Satoh et al., 1996) is an antigen which has been localized specifically to the *zonula occludens* plaque of polar epithe-

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1. *Abbreviations used in this paper:* aa, amino acid(s); mAb, monoclonal antibody; NFD, non-fat dry milk powder; PVDF, poly(vinylidene difluoride); TBST (TBS, 0.5% Tween).

lia and to the junctional zone of brain capillary endothelial cells.

Antigen TJ1 (Kapprell et al., 1990) occurs at the *zonula occludens* plaque specifically in polar epithelia but not in endothelial cells.

Additionally, several other proteins have been localized to the *zonula occludens* plaque, including actin (Stevenson and Begg, 1994; Nusrat et al., 1995) and the GTP-binding proteins, rab3B (Weber et al., 1994) and rab13 (Zahraoui et al., 1994).

In the course of our studies on plaque proteins of the junctional complexes of epithelial cells, we obtained a mAb reactive with a single protein distinctly located at tight junctions of diverse epithelia but not endothelia. Using this mAb, we have cDNA-cloned and sequenced the corresponding mRNA and identified a novel type of plaque protein, named symplekin. In addition, we show that symplekin is not synthesized in relation to the differentiation of polar epithelial cells, but is rather a house-keeping protein in a wide range of diverse cells, including many that do not form tight junctions, and that in all these cells symplekin can be detected in the nucleoplasm.

## Materials and Methods

### Tissues and Cultured Cells

Specimens of human colon, duodenum, liver, stomach, pancreas, epidermis, esophagus, tongue, heart, testis, lymph nodes, and brain, as well as colon and liver carcinomas were obtained and snap-frozen in isopentane cooled by liquid nitrogen to about  $-140^{\circ}\text{C}$  as described (Moll et al., 1982, 1986; Schäfer et al., 1994, 1996). Cell culture lines used included human colon adenocarcinoma Caco-2 and HT29, primary liver carcinoma PLC, mammary carcinoma MCF-7, vulvar squamous cell carcinoma-derived SV80 fibroblasts, glioma U 333 CG/343 MG, erythroleukemia K562, keratinocyte HaCaT, various cultures of endothelial cells of umbilical cord veins (HuVEC cells), Burkitt lymphoma Raji cells, calf lens-forming cells, rat vascular smooth muscle-derived RVF-cells, and mouse 3T3-L1 preadipocytes (for sources of cell lines, see American Type Culture Collection, Rockville, MD, and previous reports from this laboratory, e.g., Franke et al., 1980, 1987; Achtstätter et al., 1986b; Knapp and Franke, 1989; Schäfer et al., 1994). Cells were maintained under standardized conditions and routinely grown to confluence if not indicated otherwise.

### Antibodies and Antibody Production

The mouse hybridoma line secreting mAb Sym-TJ-E150 was obtained according to previously described methods in the course of a program designed to raise antibodies against junctional proteins (see Schäfer et al., 1996). The resultant hybridoma supernatants were screened by immunoblotting of proteins of fractions from human Caco-2 cells, separated by SDS-PAGE, and by immunofluorescence microscopy of Caco-2 cell monolayers on cover slips (see below). Guinea pig antibodies to synthesized peptides (Schnölzer et al., 1992; Nuber et al., 1996) derived from the amino acid sequence of the antigen of Sym-TJ-E150 (see below) were prepared by Drs. H.-R. Rackwitz and M. Schnölzer in our laboratory essentially as described (see Demlehner et al., 1995). In the experiments reported here, antibodies against the sequence of aa 532-548 (one-letter code) FTKVVLEAPLITESALE were routinely used. For comparison we also used mAb ZA-TJ1 described earlier (Kapprell et al., 1990).

Murine mAbs as well as rabbit and guinea pig antibodies to desmoplakin were as described (e.g., Franke et al., 1982; Cowin et al., 1985). Rabbit antibodies to E-cadherin,  $\alpha$ -catenin, and  $\beta$ -catenin were generously provided by Dr. R. Kemler (Max Planck Institute for Immunology, Freiburg i. Br., Germany). Affinity-purified rabbit antibodies to protein ZO-1 were from Zymed Laboratories, Inc. (San Francisco, CA).

Secondary antibodies used for immunofluorescence microscopy were Texas Red- and FITC-conjugated goat antibodies to immunoglobulins of mouse and rabbit or guinea pig, respectively. For immunoblotting, HRP-

conjugated antibodies to mouse or guinea pig immunoglobulins (Dianova, Hamburg, Germany) were used.

### cDNA Library Screening and DNA Sequencing

A  $\lambda$ gt11 human colon carcinoma cDNA expression library (cf. Franke et al., 1989) was screened using mAb Sym-TJ-E150. Approximately  $1 \times 10^6$  plaques were evaluated using a protocol from Stratagene (La Jolla, CA). Expression of the desired antigen was detected by ECL (Amersham Buchler GmbH, Braunschweig, Germany)-enhanced immunoblotting under stringent conditions. Under the conditions used a single positive 2.1-kb cDNA clone (2a-1-2B) was isolated and exonuclease deletion fragments were generated using a "nested deletion" kit (MBI Fermentas, Frankfurt, Germany), subcloned into pBluescript II (SK-) (Stratagene), and sequenced in both directions. A 700 bp deletion fragment corresponding to the 5-prime end of clone 2a-1-2B was used to generate randomly primed probes for rescreening the same library from which three clones (Sym-3a, -5b, and -9a) were isolated, sub-cloned and partially sequenced along both strands. Clone Sym-5b was determined to be a full-length clone, fully overlapping with clone 2a-1-2B and containing an additional 1.5-kb segment, including a "Kozak sequence" (Kozak, 1989) before the start codon. Clone Sym-5b was sequenced completely in both directions after the generation and subcloning of PstI endonuclease restriction fragments. Additionally, the entire clone was sequenced along both strands, using internal oligonucleotide primers. Sequence alignment, analyses, and data base searches were accomplished using the software program package HUSAR (Heidelberg Unix Sequence Analysis Resources).

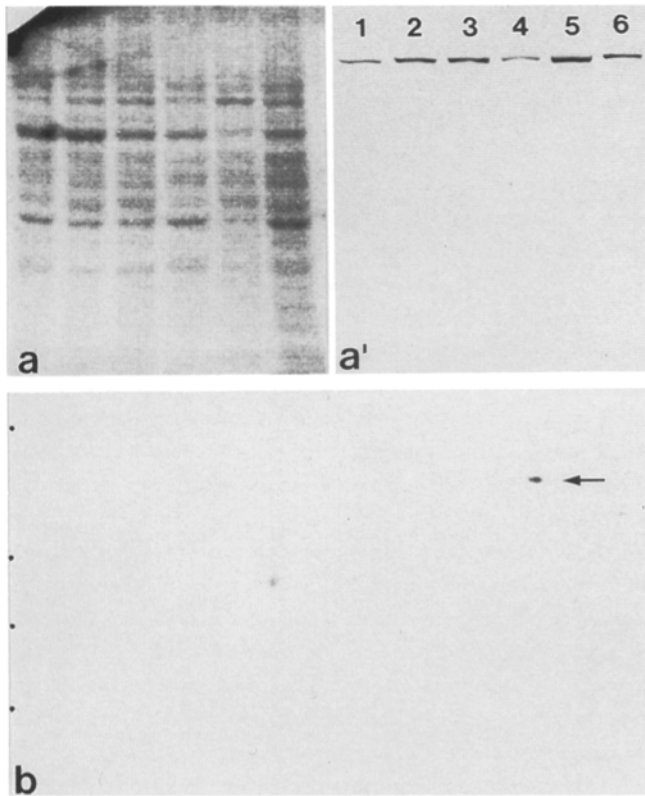
### RNA Isolation, Northern Blot Analysis, and In Vitro Transcription and Translation

Total RNA from diverse human cultured cell lines and mouse 3T3 cells, and total or poly(A)<sup>+</sup> mRNA from various human tissues as well as colon and hepatocyte carcinomas were isolated essentially as described (Schäfer et al., 1994, 1996). Northern blot analyses were also as described (Schäfer et al., 1994) using <sup>32</sup>P-labeled antisense riboprobes derived from clone 2a-1-2B.

Clone Sym-5b was transcribed and translated in vitro in the presence of <sup>35</sup>S-methionine in a rabbit reticulocyte lysate system (Promega, via Serva, Heidelberg, Germany) according to manufacturer's instructions. Products were separated by SDS-PAGE, stained with Coomassie blue (Sigma Chem. Co., St. Louis, MO), destained, soaked for 15 min in Amplify solution (Amersham), dried, and exposed to X-ray film (Kodak, via Siemens, Stuttgart, Germany) for 4 h at  $-80^{\circ}\text{C}$ .

### Cell Fractionation

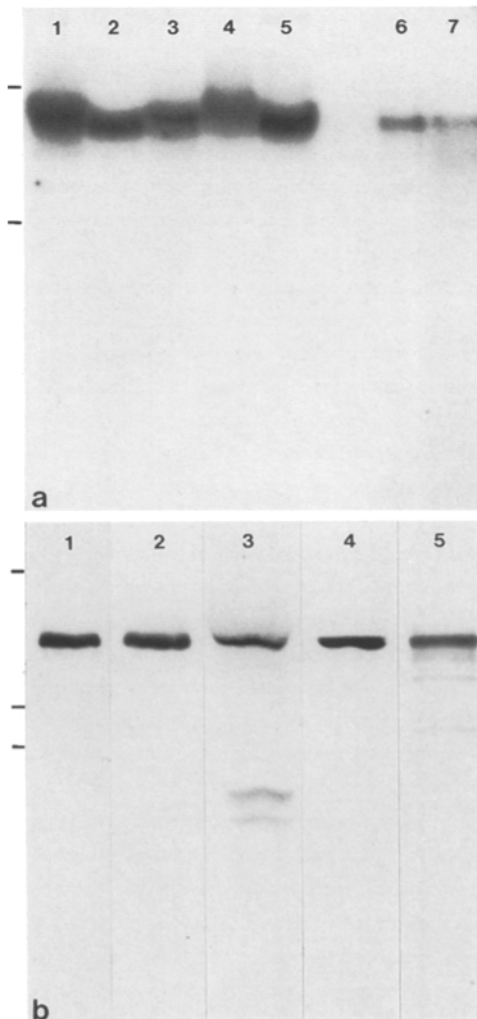
Human carcinoma cells of lines PLC or Caco-2 grown to confluence in 10 cm diameter sterile culture dishes were washed three times with PBS and either fractionated or dissolved directly in detergent buffer for gel electrophoresis as described below. Typically 10 or 20 dishes were used for a fractionation. 2 ml of ice-cold hypotonic buffer (1 mM NaHCO<sub>3</sub> adjusted to pH 7.0 and containing 0.1 mM PMSF) were added per dish and cells were left on ice 15–30 min to swell. Cells were then collected by scraping with a rubber policeman and manually homogenized in a glass-glass homogenizer by 15 return strokes. An aliquot was removed for analysis as "total cell homogenate" and the disrupted cell homogenate was centrifuged at 1500 g for 40 min at  $\sim 4^{\circ}\text{C}$ . The supernatant obtained was removed, and an aliquot taken for analysis (1,500-g supernatant). The pellet (1,500-g pellet), containing the bulk of the molecular mass 150 K protein, was either directly extracted with detergent (see below), or after removal of a small aliquot for analysis was washed by resuspension in an equal volume of homogenization buffer followed by recentrifugation for 15 min at 13,000 g and  $4^{\circ}\text{C}$  in a microcentrifuge. The supernatant was recovered and an aliquot removed for analysis (wash supernatant). The washed pellet material was again resuspended in a small defined volume of homogenization buffer, and 100 mM bicarbonate buffer (pH 9) was added to a final concentration of 10 mM. After resuspension and centrifugation for 15 min at 13,000 g and  $4^{\circ}\text{C}$ , the supernatant was removed (pH 9 extract). In some cases, the pellet was further extracted with a 500 mM NaCl solution and centrifuged as specified above to recover a high salt extract. The final pellet was then extracted with non-ionic detergent as described below, or directly solubilized in SDS-containing buffer.



**Figure 1.** Immunoblotting reaction of the  $M_r$  150 K protein with mAb Sym-TJ-E150 in diverse cultured human cell lines. (a) Coomassie brilliant blue-stained SDS-PAGE of total cellular proteins, and (a') the corresponding radiogram showing immunochemiluminescence detection of the antigen in cells of various lines: SV-40-transformed fibroblasts "SV80" (lane 1), colon carcinoma Caco-2 (lane 2), liver carcinoma PLC (lane 3), glioma U 333 CG/343 (lane 4), erythroleukemia K562 (lane 5), and colon carcinoma HT29 (lane 6). Note positive reactions of comparable intensities in all cells, independent of whether they form stable cell-cell junctions (lanes 2, 3, and 6) or not (lanes 1, 4, and 5). (b) Immuno-chemiluminescence detection of the same antigen (arrow) after two-dimensional non-equilibrium pH gradient electrophoresis (NEPHGE) separation of a Triton X-114-soluble fraction (see Materials and Methods) from PLC cell homogenates (NEPHGE, from left to right; SDS-PAGE from top to bottom). SDS-PAGE reference proteins (dots along the left margin) were of  $M_r$  200 K, 116 K, 97 K, and 66 K, respectively.

### Detergent Extraction and Phase Separation

For detergent affinity analysis of proteins (for review see Pryde, 1986), 1 ml of ice-cold Triton X-114 buffer (25 mM Tris-HCl, pH 7.5, 0.5% TX114, 150 mM NaCl, 1 mM EDTA, 0.1 mM PMSF) was added directly to each 10-cm diameter dish containing cell monolayers briefly washed with PBS and cells were then collected by scraping with a rubber policeman. Alternatively, cells were disrupted by hypotonic swelling and centrifuged as described above, and the resultant 1,500-g pellets were solubilized in approximately equal volumes of ice-cold twice concentrated Triton X-114 buffer. Cells or pellet material were homogenized by 10 return strokes with a glass-glass homogenizer (see above) and centrifuged for 15 min at 13,000 g and 4°C to remove the detergent insoluble pelletable material ("TX114 insoluble"). The supernatant was transferred first to fresh tubes on ice and then to a 30°C water bath for 3 min to induce phase separation of detergent micelles. A detergent-poor aqueous phase ("TX114 soluble aqueous") and a detergent-rich pellet ("TX114 soluble detergent") were recovered by low speed centrifugation (300 g) at room temperature for 3 min.

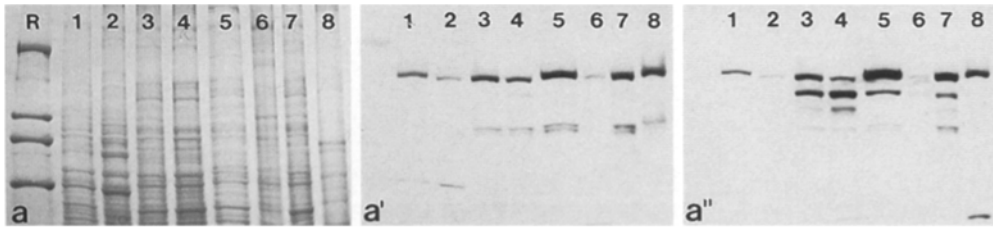


**Figure 2.** Analyses of cDNA clone Sym-5b encoding symplekin. (a) Northern blot showing the detection of an ~3.7-kb mRNA in diverse cultured human cell lines such as colon carcinoma Caco-2 (lane 1), SV-80 fibroblasts (lane 2), HaCaT keratinocytes (lane 3), glioma U 333 CG/343 (lane 4), in mouse 3T3-L1 preadipocytes (lane 5), and in human liver (lane 6) and colon (lane 7) carcinoma tissues. The widespread occurrence of a 3.7-kb symplekin mRNA in tight junction-forming cells as well as in cells that do not form any stable cell-cell contacts, is consistent with the immunoblot results shown in Fig. 1 a. Size markers were 4.4 kb and 2.37 kb from the top, respectively. (b) Immuno-chemiluminescence detection of symplekin as a  $M_r$  150 K polypeptide upon SDS-PAGE (8%), in PLC cell extracts by both mAb Sym-TJ-E150 (lane 1) and guinea pig antibodies raised against an aa sequence derived from cDNA clone Sym-5b (lane 2), in a bile canaliculi-enriched fraction from mouse liver (lane 3; lower bands represent degradation products; lane 4, cell extract from PLC cells, used here for reference) isolated according to Itolyet et al. (1991). Lane 5, autoradiogram showing the  $M_r$  150 K polypeptide as the product of cDNA clone Sym-5b, upon combined transcription and translation using [ $^{35}$ S]methionine. SDS-PAGE reference proteins (bars along the left margin) were of  $M_r$  200 K, 116 K, and 97 K, respectively.

### Gel Electrophoresis, Immunoblotting, and Protein Sequence Characterization

Conditions for SDS-PAGE in 8 or 10% acrylamide gels or for two-dimensional electrophoresis and for immunoblotting have been given elsewhere





**Figure 4.** Extraction of symplekin from human liver carcinoma PLC cells. (a) Coomassie brilliant blue-staining of proteins separated by SDS-PAGE (a) and (a' and a'') immuno-chemiluminescence detection of symplekin by both the guinea pig polyclonal antibodies (a') and

mAb Sym-TJ-E150 (a''), showing the distribution of symplekin in the following fractions from PLC cell extracts (for designations see Materials and Methods): total cell homogenate (lane 1), 1,500-g supernatant (lane 2), 1,500-g pellet (lane 3), wash supernatant (lane 4), pH 9 extract (lane 5), Triton X-114 insoluble (lane 6), Triton X-114 soluble aqueous phase (lane 7), and Triton X-114 soluble detergent phase (lane 8). Upon hypotonic cell disruption, symplekin was detected in the low-speed pellet from which it could be partially extracted at elevated pH and ionic strength, but was only fully released by solubilization with non-ionic detergent, the removal of which, however, did not affect its solubility. Note proteolytic susceptibility of symplekin under certain conditions, resulting in the appearance of lower bands (a', a''). Coelectrophoresed molecular mass markers (lane R in a) were of 200 K, 116 K, 97 K, and 66 K, respectively.

nol, 30% chloroform, 10 acetic acid), followed by a 10-min ethanol wash; (C) 15 min in 2% formaldehyde in PBS, freshly made from paraformaldehyde, followed by a quick water rinse, 5 min quenching in 50 mM  $\text{NH}_4\text{Cl}$  in PBS, and another PBS wash.

For immunocytochemistry of cell cultures, cells grown on poly-lysine coverslips were washed three times briefly in PBS containing 2 mM  $\text{MgCl}_2$  and then fixed at  $-20^\circ\text{C}$  in methanol (7 min) and acetone (30 s).

Cell and tissue specimens were incubated with the antibodies according to one of the following procedures: (a) Primary antibodies were directly applied for either 15, 30 or 120 min, followed by three 5 min PBS washes before application of secondary antibodies for either 15 or 30 min. (b) Specimens were either treated as in (a) or were incubated in PBS containing 0.2% Triton X-100 for 2, 3, or 5 min, and then briefly washed in PBS before application of antibodies. (c) As under (b), except that all PBS solutions used contained 0.5 or 2.0 mM  $\text{MgCl}_2$ . After antibody binding, specimens were then washed again in PBS, rinsed in water, fixed in ethanol for 5 min, dried, and mounted. Immunofluorescence was observed and documented with a Zeiss Axiophot photomicroscope (Carl Zeiss, Jena, Germany).

Confocal laser-scanning immunofluorescence microscopy was done on a Zeiss LSM 410 UV (Carl Zeiss). For simultaneous double-label fluorescence, an Argon ion laser operating at 488 nm and a Helium-neon laser operating at 543 nm were used together with a band-pass filter combination of 510-525 and 590-610 for visualization of FITC and Texas Red fluorescence, respectively. RGB images were taken in high resolution mode using  $1,024 \times 1,024$  image points (pixels) and 2 s scan times. Regularly noise levels were reduced by several line averagings of the scans. Projection images were created from 0.8- $\mu\text{m}$  optical sections of tissue preparations or cell layers. To verify the distribution of symplekin and ZO-1, the individual images were separated electronically by a 20 pixel off-set along the abscissa.

### Immunoelectron Microscopy

Frozen tissue sections (5 mm) were prepared with a Jung CM300 cryostat (Leica), mounted on coverslips, air-dried, and fixed in acetone at  $-20^\circ\text{C}$  for 10 min. Cells grown on coverslips were fixed with 2% formaldehyde for 5 min and then permeabilized with 0.1% saponin in PBS for 5 min. The hybridoma supernatant containing mAb Sym-TJ-E150 was applied for 2 h. As secondary antibodies, anti-mouse IgG coupled to 5-nm colloid gold particles (Amersham) was applied overnight or anti-mouse IgG from Nanogold (Bio Trend, Cologne, Germany), was applied for 3 h, followed in some experiments by silver enhancement for 3 min. Fixation with glutaraldehyde, dehydration, and embedding were as described (for references see Rose et al., 1995), and electron micrographs were taken using a Zeiss electron microscope EM 910 (LEO Electron Microscope Optics, Oberkochen, Germany).

## Results

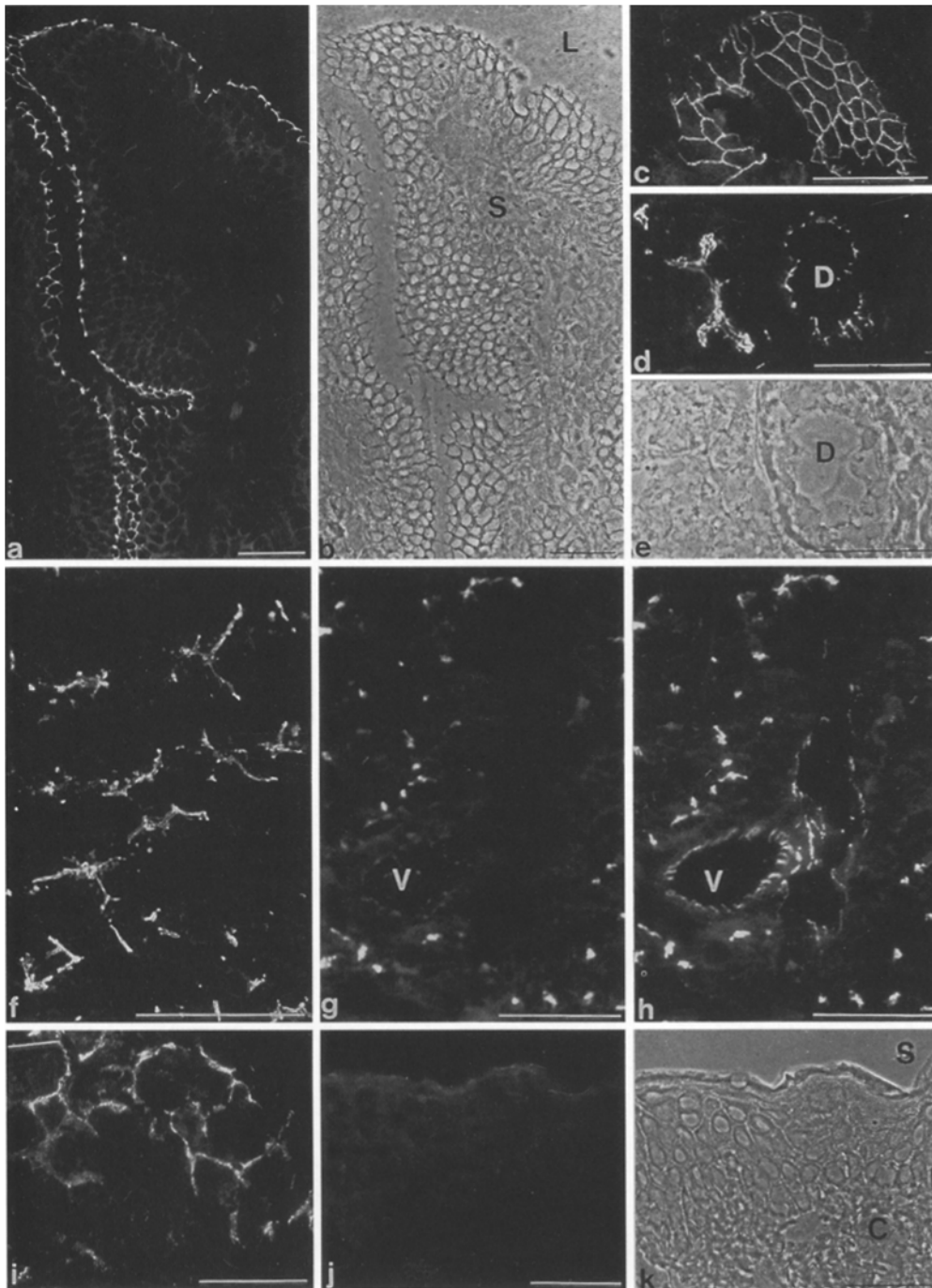
### Immunoblot Analyses of the Sym-TJ-E150 Antigen

The antigen to antibody Sym-TJ-E150 was found by im-

munoblotting of SDS-PAGE-separated proteins as a 150-K molecular mass polypeptide occurring in a wide range of cultured human cell lines, including colon adenocarcinoma Caco-2 and HT29, liver carcinoma PLC, HaCaT keratinocytes, SV-80 fibroblasts, U333 CG/343 glioma, and erythroleukemia 562 (Figs. 1, a and a' and 2 b). On two-dimensional gel electrophoresis, the antigen appeared with a  $\text{pI} \sim 6.5$  (Fig. 1 b, shows NEPHGE). As we had, in our preliminary immunofluorescence microscopy experiments, localized the antigen to apical rings of cell-cell contacts in polarized epithelia, the detection of the antigenic polypeptide in non-epithelial cells, including some which never form any kind of stable cell-cell junctions, suggested that this protein, which we named symplekin, was widespread and might not be an exclusively junctional component.

### Isolation and Sequencing of cDNA Encoding Symplekin

To elucidate the identity and nature of the 150-K molecular mass polypeptide, we used mAb Sym-TJ-E150 to screen  $1 \times 10^6$  plaques from a  $\lambda\text{gt}11$  cDNA expression library derived from cultured cells of a human adenocarcinoma line as described in Materials and Methods. We initially cloned a positive phage recombinant (2a-1-2B) with a 2.1-kb open reading frame which included a consensus polyadenylation signal. To detect mRNA represented by clone 2a-1-2B in various human cell lines and tissues, we performed Northern blot analyses using a riboprobe (Fig. 2 a). An mRNA of  $\sim 3.7$  kb was detected in cultured polarized epithelial cells as well as in adenocarcinomas but also in non-epithelial cells such as fibroblasts and glioma cells, thus corroborating our immunoblot findings. We were able to confirm that the Sym-5b cDNA clone encoded the  $M_r$  150 K antigen present in the tight junction plaques by several independent criteria: (a) Polyclonal guinea pig antibodies raised against a 20-aa sequence derived from the cDNA clone also recognized the 150-K band on immunoblots of total PLC cell proteins (Fig. 2 b, lanes 1 and 2). (b) This protein was also recovered in the bile canaliculi-rich fraction from murine liver (Fig. 2 b, lane 3). (c) In vitro transcription and translation of the cDNA clone resulted in a polypeptide electrophoretically identical to that recog-



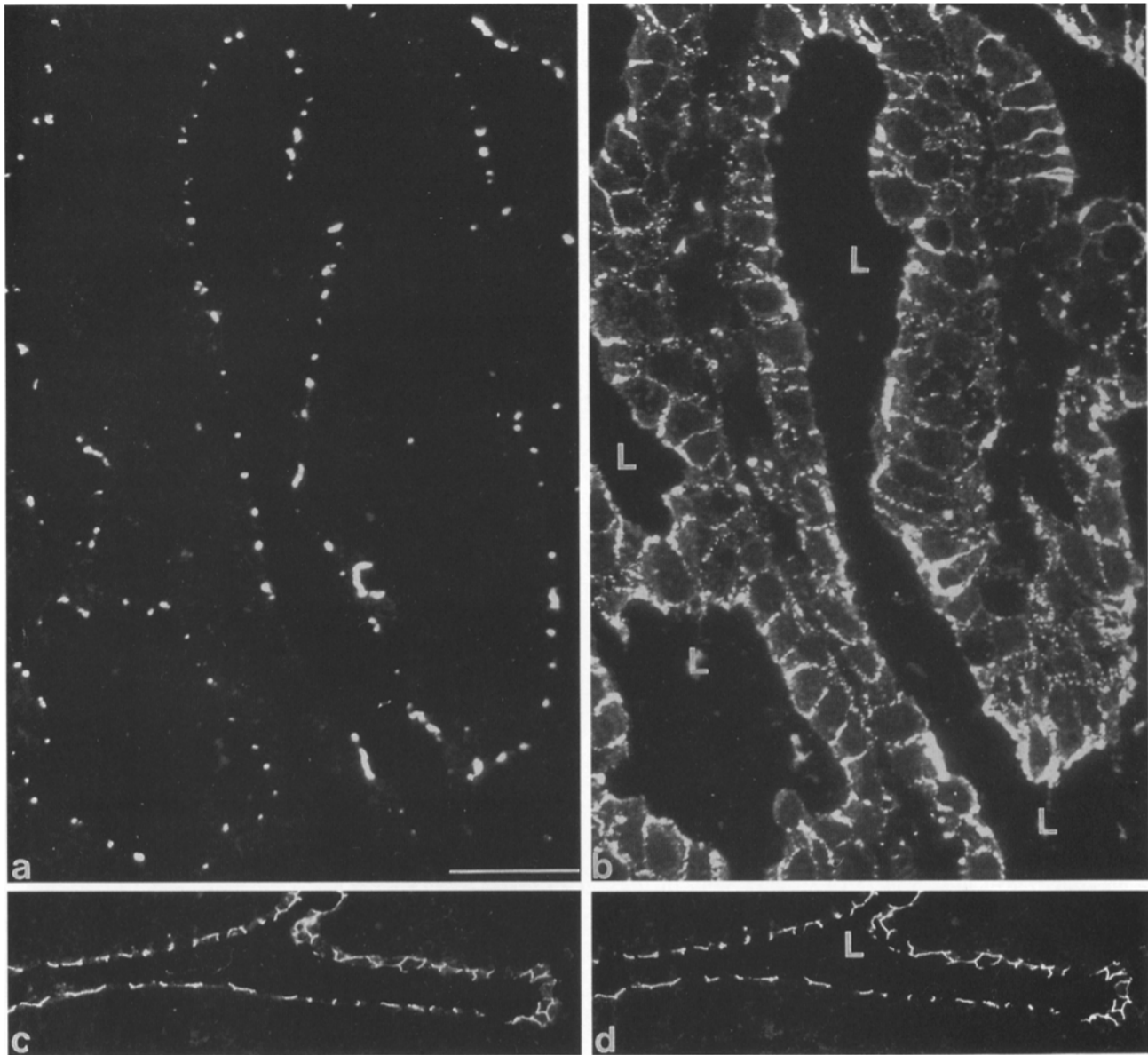
**Figure 5.** Immunofluorescence microscopy of frozen sections through human tissues, demonstrating the specific localization of symplekin by mAb Sym-TJ-E150 to the tight junction-containing apical belt (*zonula occludens*) of polarized epithelia, under the specific reaction conditions. (a) Stomach epithelium (b, corresponding phase contrast; L, lumen; S, submucosa). (c) Near-apical cross-section of the apical region in a differentiated colon carcinoma, showing directly the belt-like immunostaining in this plane. (d) Pancreatic tissue (e, phase contrast), showing near-apical staining of symplekin in acinar alveoli (left) and a duct (D). (f-h) In liver, symplekin immunostaining is exclusively along bile canaliculi where the tight junctions are (f, g). In double-label immunostaining, symplekin (g) and protein ZO-1 (h) colocalize at tight junctions in epithelial cells whereas in vascular endothelial cells only protein ZO-1 is detected (V, blood vessel). (i) Symplekin at the specialized junctions in Sertoli cells of testis. (j) Under the conditions used, symplekin has not been detected in the epidermis (k, phase contrast image of the same field; S, surface; C, connective tissue; the bracket demarcates the epidermis). Bars, 10  $\mu$ m.

nized by both mAb Sym-TJ-E150 and the polyclonal guinea pig antibodies (Fig. 2 b, lane 5).

With a randomly primed 700-bp cDNA probe, synthesized using a nested deletion fragment representing the most 5' sequence of clone 2a-1-2B, we rescreened the same cDNA library and cloned three positive recombinants, one of which was a full-length cDNA clone of ~3.66 kb (Fig. 3) which overlapped completely at the 3'-end of clone 2a-1-2B. This clone contained a consensus initiation codon (Kozak, 1989) at position 111, a 3.32-kb open reading frame, followed by a 135-bp non-coding region and a consensus polyadenylation signal. This cDNA clone encoded a 1142-aa polypeptide, i.e., symplekin, with a pre-

dicted mass of 126.5 kD and a pI of 6.25. We were able to identify by electrospray mass spectrometry 13 internal tryptic fragments of symplekin, enriched by cell fractionation, detergent extraction and ion exchange chromatography, that were identical to segments of the aa sequence derived from the cDNA clone (Fig. 3).

On sequence comparisons symplekin has not shown significant homology to any of the known proteins present in the data bases, including the tight junction-associated proteins cingulin, ZO-1, ZO-2, and other MAGUK proteins (Fig. 3). Symplekin also does not contain the extended sequence motif which the tight junction "7H6 antigen" (Zhong et al., 1993; Mori et al., 1996) shares with several



**Figure 6.** Double-label immunostaining showing the location of symplekin (*a* and *d*) relative to  $\beta$ -catenin (*b*) or protein ZO-1 (*d*) in cryosections of human intestinal epithelia such as colon (*a* and *b*) or duodenum (*c* and *d*). Symplekin is restricted precisely to the *zonula occludens* (*a* and *c*), colocalizing with protein ZO-1 (*d*) whereas the adherens junction protein  $\beta$ -catenin shows a different subapical zonular staining and also complex punctate arrays along the lateral cell–cell contact borders. *L*, lumen. Bars, 10  $\mu$ m.

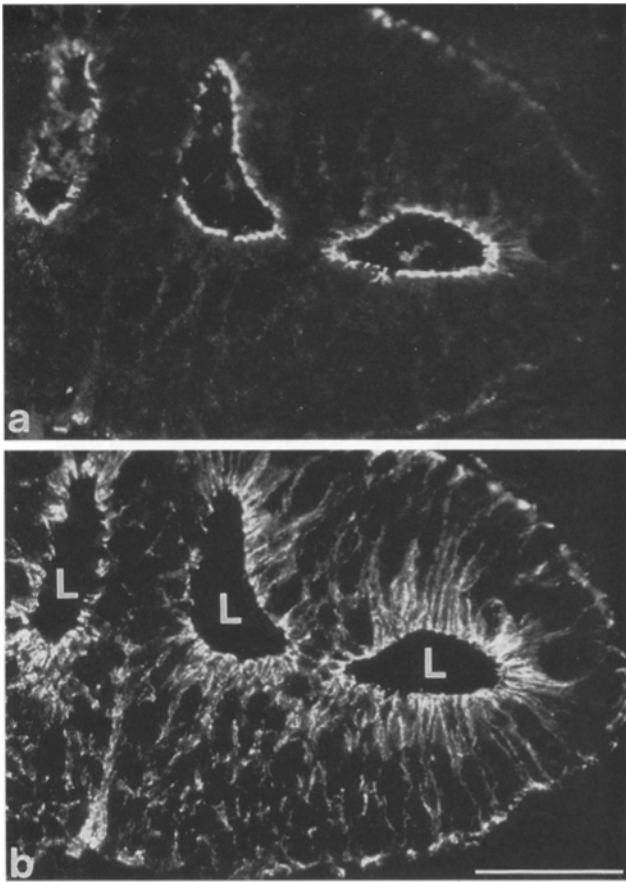
of the “chromosomal segregation proteins” of the yeast Smc1p family (Strunnikov et al., 1993).

The aa sequence of symplekin displays several extended hydrophilic segments in the amino-terminal half whereas the carboxy-terminal half is characterized by alternating short hydrophobic and hydrophilic aa stretches. A possible nuclear localization signal is located at aa positions 213–228, and several putative phosphorylation sites are spread throughout the sequence.

#### **Solubility and Stability Properties**

To determine the solubility properties of symplekin we performed cell fractionation and extraction experiments

using either PLC (Fig. 4, *a–a'*) or Caco-2 (not shown) cells. After low speed centrifugation of cells gently lysed in hypotonic buffer, the bulk of symplekin remained in the low speed pellet, although some of the protein was released from the pellet material upon resuspension in the same buffer. Symplekin could be partially extracted in alkaline low salt (10 mM sodium bicarbonate, pH 9) or in high salt (0.5 M NaCl) buffers. However, complete extraction of the protein into a soluble state was only obtained by further non-ionic detergent solubilization of the extracted pellets. Symplekin then remained soluble, after the removal of detergent by temperature-induced phase separation and subsequent buffer exchange, although in the absence of detergent and/or divalent cation chelators it displayed its susceptibility to proteolytic degradation (Fig. 4).

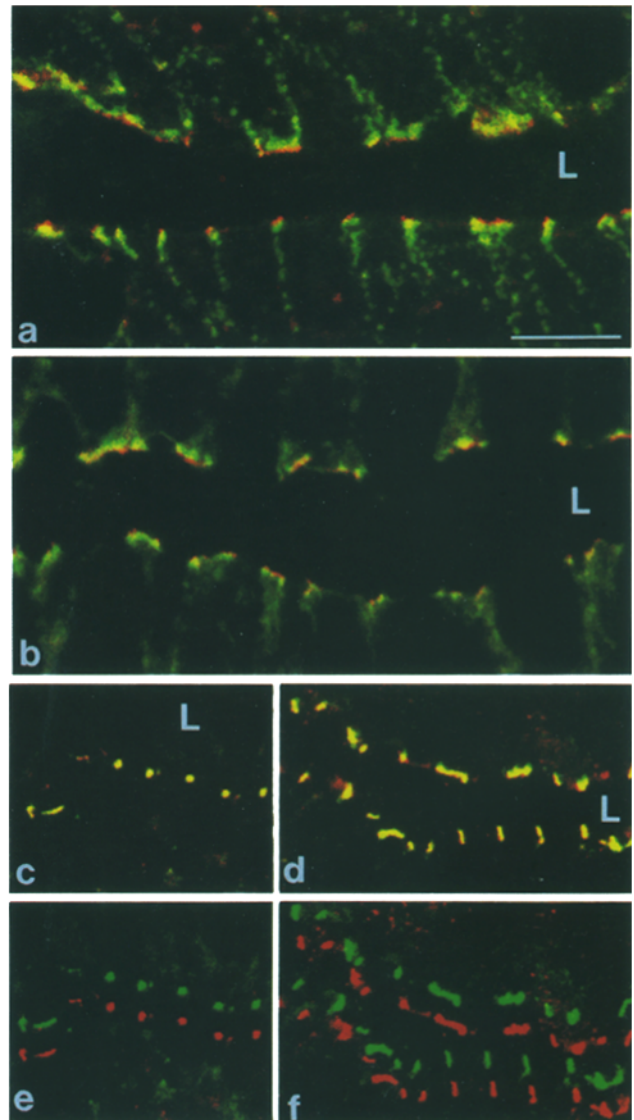


**Figure 7.** Double-label immunofluorescence microscopy, showing the maintenance of symplekin's apical assembly at the *zonula occludens* in a cryosection through a human colon carcinoma comparing symplekin (*a*, mAb Sym-TJ-E150) and E-cadherin (*b*, rabbit antibodies). Note that also in the carcinoma symplekin is restricted to a polar zonule (*a*) whereas E-cadherin shows the display of the zonular as well as punctate adherens junctions along the lateral walls. *L*, lumen. Bar, 10  $\mu$ m.

### Immunofluorescence Localization of Symplekin at Tight Junctions in Tissues

To define the localization of symplekin in diverse cell types we performed immunostaining experiments on cryosections of normal human tissues, including stomach, duodenum, pancreas, liver, testis, epidermis, lymph nodes, and fetal brain, as well as carcinomas (e.g., Figs. 5 and 6). In polarized epithelia, mAb Sym-TJ-E150 reacted specifically with the tight junction-containing peripheral ring at the immediate sub-apical level (Fig. 5, *a-c*, *f*, and *g*), and such a reaction was also seen in alveolar as well as ductal (Fig. 5, *d* and *e*) cells of various glands, in adenocarcinomas (e.g., Fig. 5 *c*), and in the "junctional specializations" of Sertoli cells of testis (Fig. 5 *i*). No reaction was detected in endothelia of vessels of various types and calibers (e.g., Fig. 5, *g* and *h*), including brain capillaries, in stratified epithelia as in epidermis (Fig. 5, *j* and *k*), in the intercalated disks and the Purkinje fiber cells of the heart and in lymph nodes (not shown).

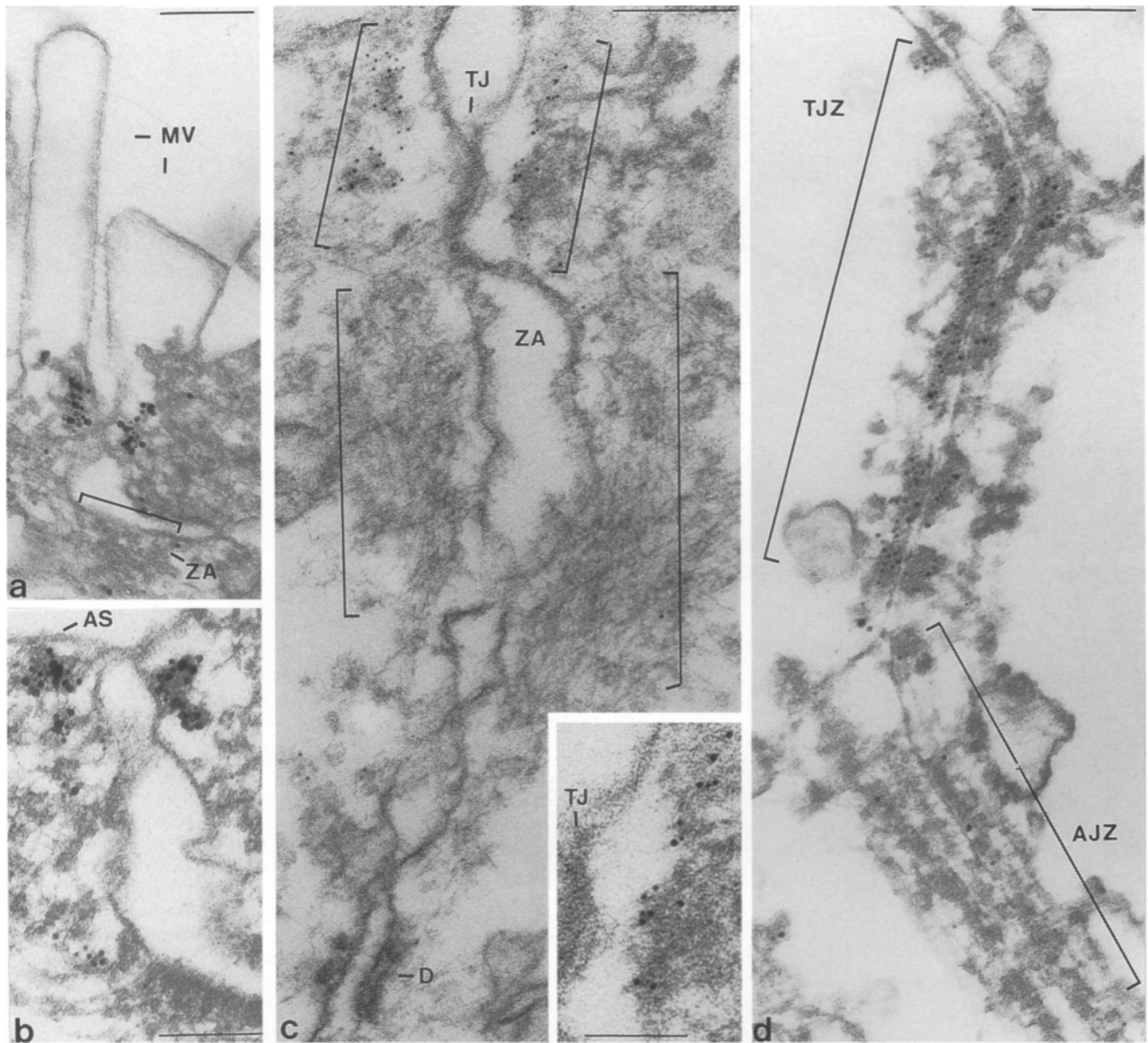
We further determined symplekin's location with respect to other plaque proteins specific for tight junctions,



**Figure 8.** Confocal laser-scanning micrographs of double-label immunostaining of symplekin (red fluorescence) in human colon tissue relative to (in green fluorescence) either desmoplakin (*a*),  $\alpha$ -catenin (*b*), or protein ZO-1 (*c* and *d*), showing the compositional and positional distinction of the *zonula occludens* from both desmosomes and *adherens junctions*. Symplekin staining could be resolved from both desmoplakin (*a*) and  $\alpha$ -catenin (*b*) staining patterns where in both cases symplekin was located at the apical-most position of the tripartite junctional zone. In contrast, symplekin staining could not be resolved from that of protein ZO-1 (*c* and *d*), reflecting their colocalization in the tight junction-containing zone (*yellow-orange*). In *e* and *f*, a computer-assisted frame off-set (20 pixels) of the images shown in *c* and *d* along the abscissa was produced to artificially separate the individual fluorochrome emissions to demonstrate the similar relative intensities of the respective antibody reactions and to underscore their colocalization. *L*, lumen. Bar, 10  $\mu$ m.

adherens junctions, or desmosomes. Symplekin clearly colocalized with protein ZO-1 in all epithelia examined (Fig. 5, *g* and *h*, and Fig. 6, *c* and *d*), but not in endothelia (Fig. 5, *g* and *h*). The larger subapical region displayed similar immunostaining for symplekin and the adherens

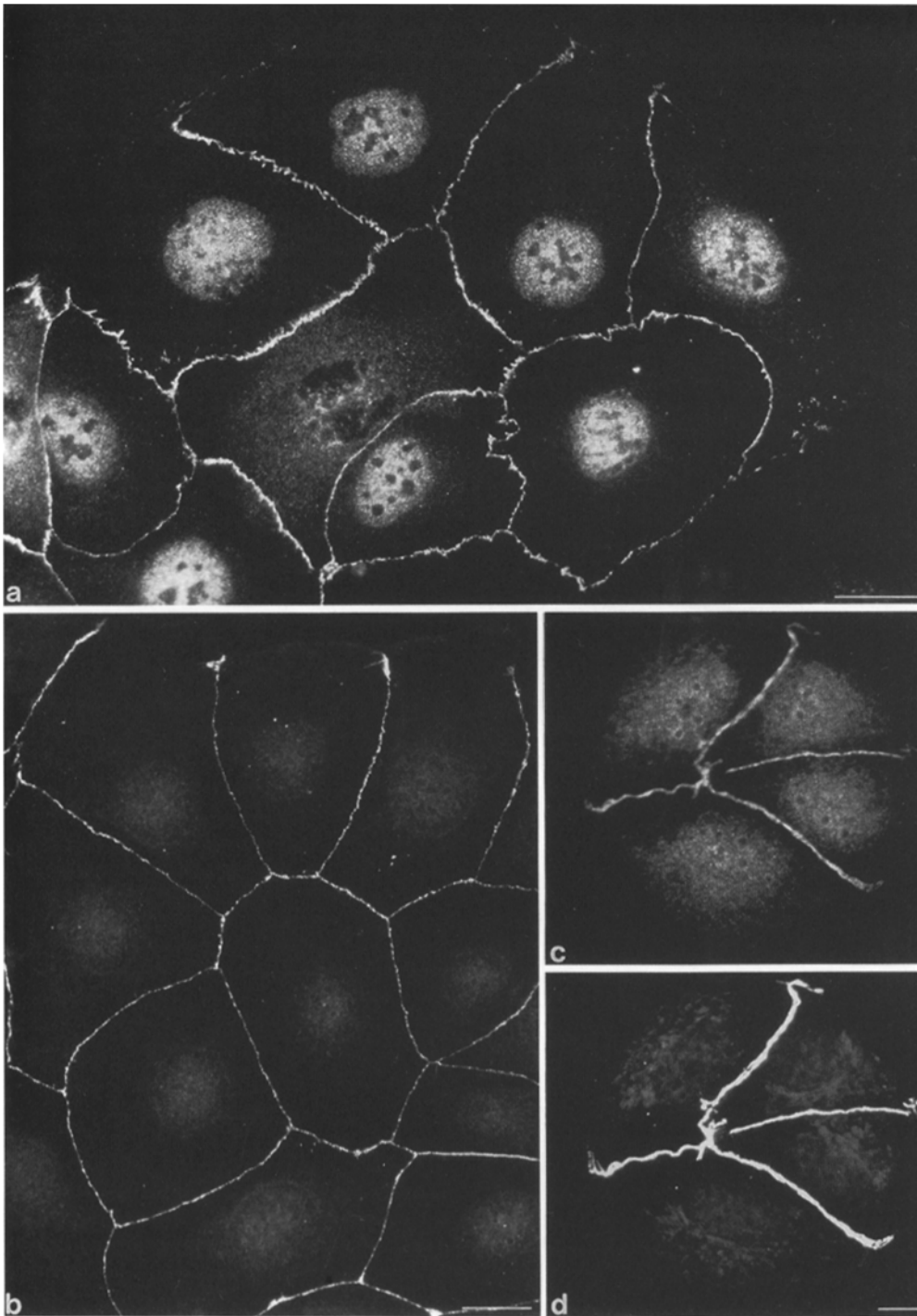




**Figure 9.** Electron microscopy localization of symplekin to the tight junction-associated plaque, using mAb Sym-TJ-E150 on cryosections from human colon (*a-c*) or detergent-permeabilized cultured PLC cells (*d*), incubated with primary and secondary 5-nm gold-coupled antibodies before fixation and embedding, shown with (*a*, *b*, and *d*) or without (*c*) the immunogold silver enhancement technique. The gold particles specifically decorated the apical tight junction (TJ)-associated cytoplasmic plaque of the *zonula occludens* (*a* and *b*), and this was also seen after retraction of the plaque from the membrane under slightly hypotonic conditions (*c*, *bracket*). Note that symplekin immunogold is practically absent from the plaque structures of the *zonula adherens* (ZA; *a-c*, *brackets*) and of desmosomes (D). The orientation of the gold particles to one side of the plaque structure in longitudinal sections (*c*, *inset* in lower right presents a magnification) suggests that symplekin, or at least this epitope, is located between the membrane and the plaque. (*d*) In PLC cell monolayers, gold particles densely decorated one plaque zone, the tight junction-containing zone (TJZ) along cell-cell contact borders, but were essentially absent from a similarly dense adjacent plaque zone representing the adherens junction zone (AJZ). MV, microvilli; AS, apical surface. Bars: (*a-d*) 0.2  $\mu\text{m}$ ; (*inset*) 0.1  $\mu\text{m}$ .

junction marker proteins  $\alpha$ -catenin,  $\beta$ -catenin, and E-cadherin. However, the symplekin staining was always restricted to a narrow near-apical belt whereas the catenins and E-cadherin were found in addition in numerous sites along the lateral cell walls (Fig. 6, *a* and *b*, and Fig. 7, *a* and *b*). The zonular apical reaction of symplekin was strikingly different from the punctate immunostaining pattern of

desmoplakin along the lateral cell walls (Fig. 8 *a*). Observing double-label immunofluorescence stainings with laser scanning confocal microscopy it was clear that symplekin did not co-localize with the plaque proteins of both the adherens junctions and the desmosomes, but was located more apically (Fig. 8, *a* and *b*), together with the tight junction plaque protein ZO-1 (Fig. 8, *c-f*).



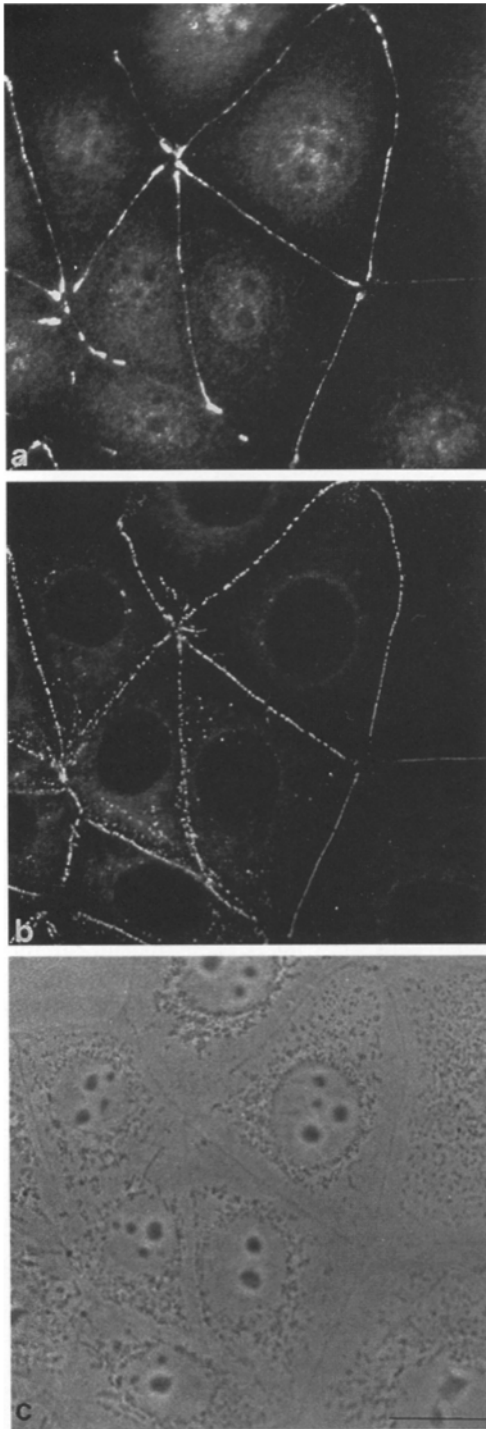
**Figure 10.** Single-(*a* and *b*) and double-(*c* and *d*) label immunofluorescence microscopy of symplekin (*a-c*) and protein ZO-1 (*d*) in monolayer cultures of human epithelial cells, showing the dual location of symplekin. (*a*) Caco-2 cell monolayers treated in the absence of detergent according to protocol 1 (see Materials and Methods). Under these conditions, optimized for minimal protein loss and redistribution, intense fluorescence is seen at cell-cell junctions and in the nucleoplasm (nucleoli are negative) whereas free cell edges are negative. Not also, in the center, a cell in mitosis (late anaphase), with transient dispersion of symplekin over the cytoplasm. (*b*) Caco-2 cell monolayers treated in the presence of detergent (5 min) according to protocol 2, showing intense junctional staining but greatly reduced nuclear staining. (*c* and *d*) Double-label immunostaining of symplekin (*c*) and protein ZO-1 (*d*) in PLC cell monolayers treated briefly with detergent (2 min) according to protocol 2. Note the identical staining pattern of the two proteins at the intercellular borders. With the shorter duration of exposure to detergent, symplekin is still detected in the nucleus (*c*) whereas protein ZO-1 reaction is very weak. Bars, 5  $\mu\text{m}$ .

### Immunogold Electron Microscopic Localization

Using different immunogold labeling protocols, with or without the “silver enhancement” technique, we were able to localize symplekin specifically to the electron-dense cytoplasmic undercoat of the *zonula occludens* (Fig. 9), thus directly demonstrating symplekin as a tight junction plaque protein. In cryosections of human colon tissue, for example, immunogold particles decorated exclusively the plaques at either side of the tight junction, but were practically absent from the plaque structures of both the *zonula adhaerens* and the desmosomes (Fig. 9, *a-c*). Furthermore, the as-

sociation of symplekin with the plaque was underscored by its maintenance during retraction of the plaque from the membrane under slightly hypotonic conditions (Fig. 9 *c*). From the immunogold distribution pattern displayed in longitudinal sections (Fig. 9, *insert*) symplekin appeared to be situated in a juxtmembranous position at the interface between the plaque structure and the membrane proper.

Similar results were obtained in cultured cells. In electron micrographs of silver-enhanced immunogold localization of symplekin in PLC hepatocellular carcinoma cell monolayers, the concentration of gold particles in the tight



**Figure 11.** Double-label immunofluorescence localization of symplekin (a) and desmoplakin (b) in monolayers of MCF-7 breast carcinoma cells, treated according to protocol 2 (see Materials and Methods; C, phase contrast image of the same field). Symplekin staining is zonular, reflecting the organization of tight junction structures in this culture, in contrast to the distinct, desmosome-typical punctate staining pattern of desmoplakin. Note still detection of some symplekin in the nucleus, in contrast to the lack of nuclear staining of the plaque protein desmoplakin (b). Bar, 5  $\mu$ m.

junction plaque compositionally distinguished this structure from the otherwise morphologically indistinguishable subjacent plaque of the *zonula adhaerens* (Fig. 9 d).

### **Immunofluorescence Microscopy of Cultured Epithelial Cells**

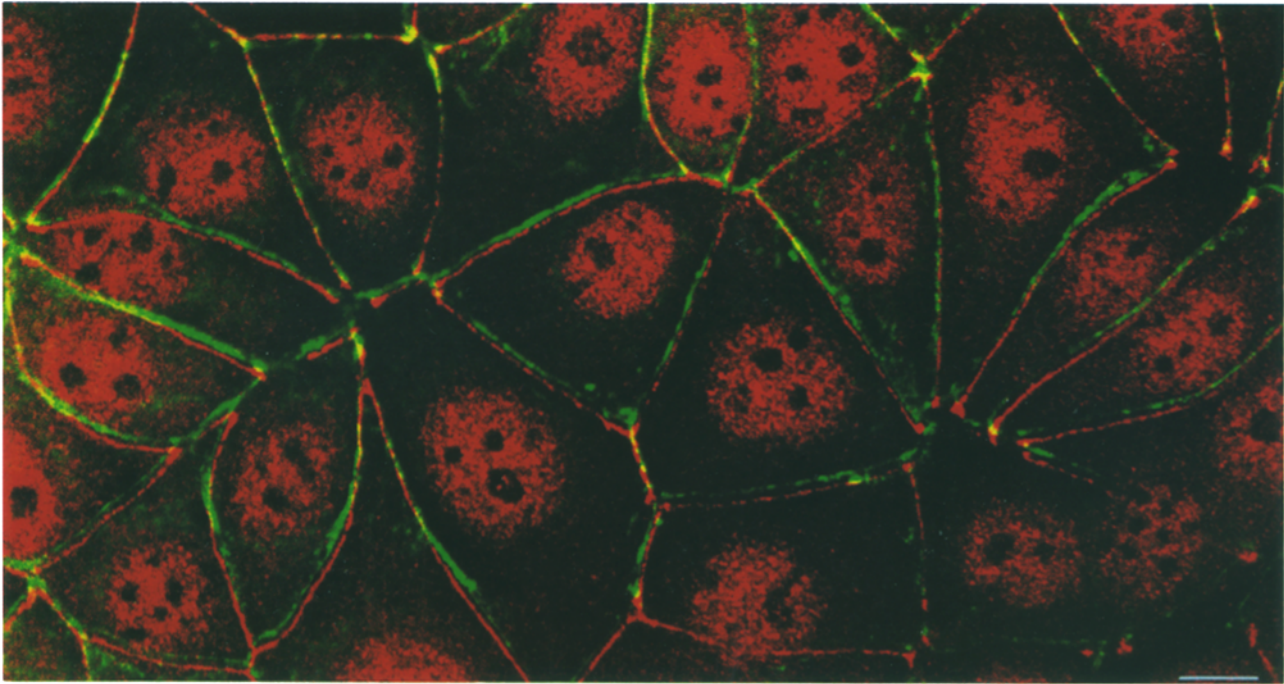
In colonies of cultured cells derived from polarized epithelia or adenocarcinomas, symplekin could be localized to a zonular region of cell-cell contact but was absent from the “free” cell surfaces not bordering on another cell (Figs. 10 and 11). Again, coimmunostaining of symplekin with ZO-1 was obvious (Fig. 10, c and d), whereas desmoplakin (Fig. 11) and the adherens junction markers (Fig. 12) displayed distinctly different staining patterns and positions. Laser scanning confocal microscopy (Fig. 12) allowed us to resolve directly the *zonula adhaerens*, seen by its E-cadherin reaction, from the more apically placed *zonula occludens*, also demonstrating that in monolayers of cultured cells the adherens junctions and the tight junction domains are spatially and compositionally distinct.

Surprisingly, we noticed, with mAb Symp-TJ-E150 as well as the guinea pig antibodies, an intense immunoreaction in the nucleoplasm, excluding the nucleoli, which varied according to the specific preparative conditions: while with extremely rapid fixation in methanol and after short wash and incubation times strong staining was only seen in the nucleoplasm (not shown), more extended incubations showed the simultaneous appearance of the nuclear and the junctional immunoreaction (Figs. 10, a and c, 11 a and 12), and the latter was usually enhanced when a detergent step was included. Increasing times of detergent treatment and washes resulted in a reduction of nucleoplasmic and thus a more prominent junctional immunofluorescence (Fig. 10 b). The significance of this selective nucleoplasmic reaction of symplekin was also evident from the redistribution of this protein during mitosis when it transiently spreads over the cytoplasm (an anaphase cell is shown in Fig. 10 a) but is effectively reaccumulated into the telophase daughter nuclei. The specificity of the strong nuclear immunostaining of symplekin was further demonstrated in double-labeling experiments, in comparison with, for example, protein ZO-1 (Fig. 10, c and d) and desmoplakin (Fig. 11, a–c).

In culture cell lines derived from non-polarized epithelia such as in human HaCaT keratinocytes and A-431 squamous carcinoma cells we noted a heterogeneous immunostaining reaction in that only some cells showed short regions of symplekin positivity at cell–cell borders, often in whiskers or punctate arrays (not shown). In these cells as well as in non-epithelial cells devoid of tight junctions but forming extensive adherens junctions, such as human endothelial HuVEC cells, calf lens-, rat RVF- and murine 3T3-cells or in cells that form no stable junctions at all, such as lymphoma and erythroleukemia cells, we only and consistently observed the nuclear reaction.

### **Immunofluorescence Microscopy of Nuclear Symplekin**

The detection of symplekin in the nucleus of a broad range of cultured cell types prompted us to examine whether symplekin could also be detected in the nuclei of solid tissues. As we had originally noted the high extractability of



**Figure 12.** Double-label immunofluorescence comparison of symplekin (red) with E-cadherin (green) in a monolayer culture of MCF-7 cells, treated according to protocol 1, as viewed by laser-scanning confocal microscopy. Symplekin and E-cadherin do not colocalize, but shadow one another, resulting in the distinction of the *zonula occludens* (red contour line) from the *zonula adherens* (green line), which is clearly subjacent to the *zonula occludens*. Again, using a protocol designed to minimize losses of soluble proteins and redistributions, an intense and specific nuclear reaction of symplekin is observed. Bar, 10  $\mu\text{m}$ .

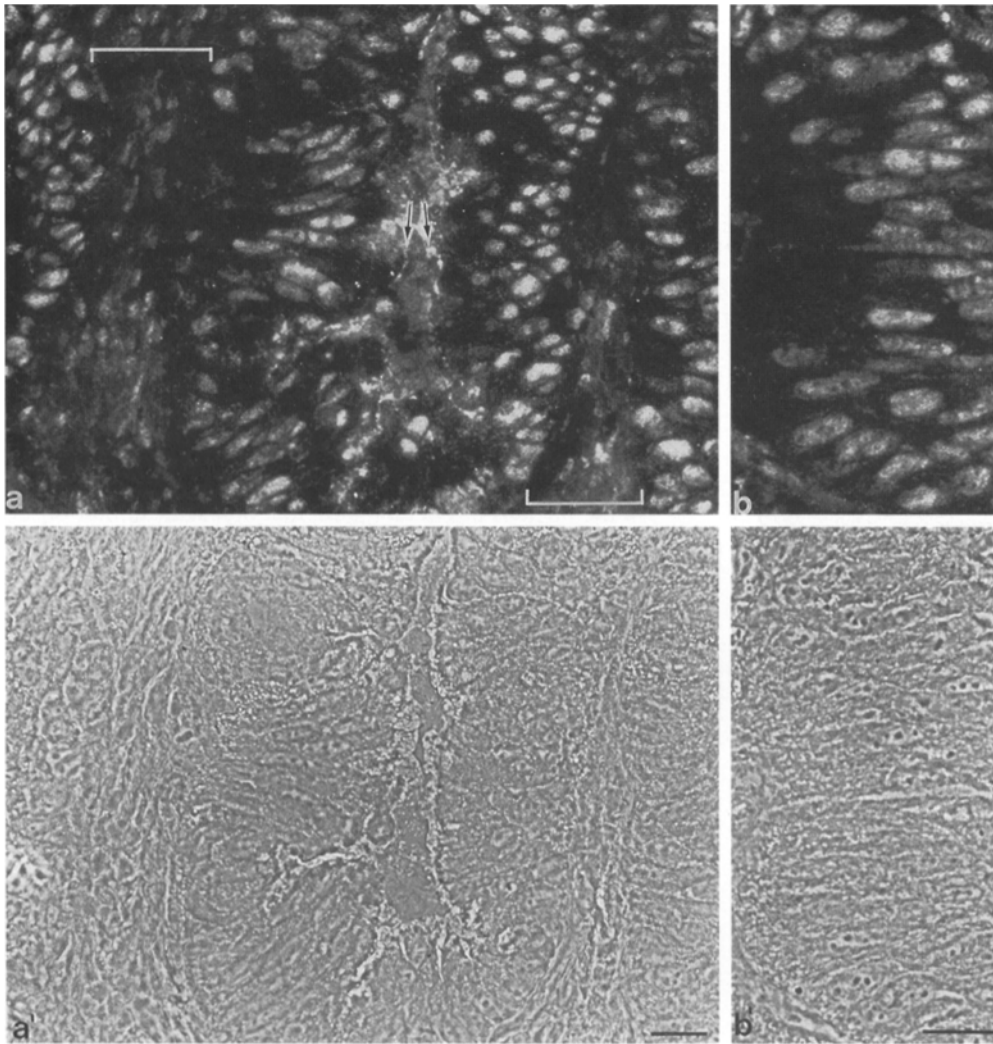
soluble nuclear proteins to be a specific problem with thawed sections of frozen tissue, we used several alternative fixation protocols for immunofluorescence localization (for details see Materials and Methods; a more comprehensive report of these results is in preparation): (a) Fixation in acetone for 10 min at  $-20^{\circ}\text{C}$ ; (b) fixation in Carnoy solution (methanol/chloroform/acetic acid) for 20 min followed by an ethanol wash for 10 min; and (c) formaldehyde fixation for 15 min. Furthermore, for each of these fixation protocols other variables were also tested such as treatments with detergent, Triton TX-100, and various antibody incubation times. By using several marker antibodies as controls it became clear that different antigens require different procedures for optimal nuclear retention and accurate detection in situ.

Figs. 13 and 14 present some results obtained with near-optimal protocols, showing intense nuclear immunofluorescence of epithelial and non-epithelial (submucosal) cells in sections through human colon carcinoma (Fig. 13, *a-b'*) and esophagus (Fig. 14, *a-b'*) after Carnoy fixation. In contrast to nuclear localization this protocol yielded only poor reactions at the tight junctions of the *zonula occludens* (arrows in Fig. 13 *a*). It is also notable that in stratified epithelia such as esophagus (Fig. 14), tongue mucosa, and epidermis (not shown), which lack a *zonula occludens*, the nuclei are the only site of symplekin immunoreaction, and that this nuclear reaction is seen in the proliferative compartment of the basal cell layer as well as in the non-proliferative compartment of the upper strata. Other protocols, including those using formaldehyde or involving a short treatment with Triton X-100, revealed a relatively in-

tense tight junction reaction but a weaker nuclear staining. The non-epithelial cells showing strong nuclear immunostaining in tissue sections included fibroblasts, endothelial cells, and lymphocytes, and the latter reaction was particularly impressive in lymph node sections (not shown).

### Discussion

In this study we have identified and characterized symplekin, a novel and unique human protein with a polypeptide molecular mass of 126.5 kD, which is a specific component of the *zonula occludens* plaque associated with the tight junctions of polar epithelia and Sertoli cells of testis but not of vascular endothelia. Both by its aa sequence and by its intracellular distribution, symplekin is notably different from other tight junction-associated proteins as its junctional localization is clearly more specific for tight junctions than protein ZO-1, so far the most widely used "tight junction marker," as it does not occur at other kinds of junctions such as between vascular endothelial cells in vivo and in vitro, between the foot processes of kidney podocytes, between fibroblasts or muscle cells, or in the intercalated disks of myocardium (for protein ZO-1 see Stevenson et al., 1986; Schnabel et al., 1990; Howarth et al., 1992, 1994; Itoh et al., 1993; Miragall et al., 1994). Similarly, it has been reported that in uncoupled canine kidney epithelial cells of line MDCK protein ZO-1 localizes in aggregates deep in the cytoplasm where it can be associated with the E-cadherin/ $\alpha$ - and  $\beta$ -catenin/plakoglobin complex, and that it is dispersed over the cytoplasm in Moloney sarcoma virus-transfected MDCK cells (Rajasekaran et al., 1996).



**Figure 13.** Immunohistochemistry showing nuclear immunofluorescence staining with mAb Sym-TJ-E150 (*a; a'*, phase contrast optics of the same field) and polyclonal guinea pig antibodies (*b; b'*, phase contrast) on cryostat sections through frozen human colon carcinoma reacted according to protocol B of Materials and Methods, including Carnoy fixation and 30-min incubation with primary antibodies. Note intense nuclear immunostaining in the epithelium-derived cells as well as in non-epithelial (stromal) cells (stroma marked by brackets). Arrows point to the cell apex regions showing only weak residual *zonula occludens* immunostaining under these conditions (compare with Fig. 7). Bars, 5  $\mu$ m.

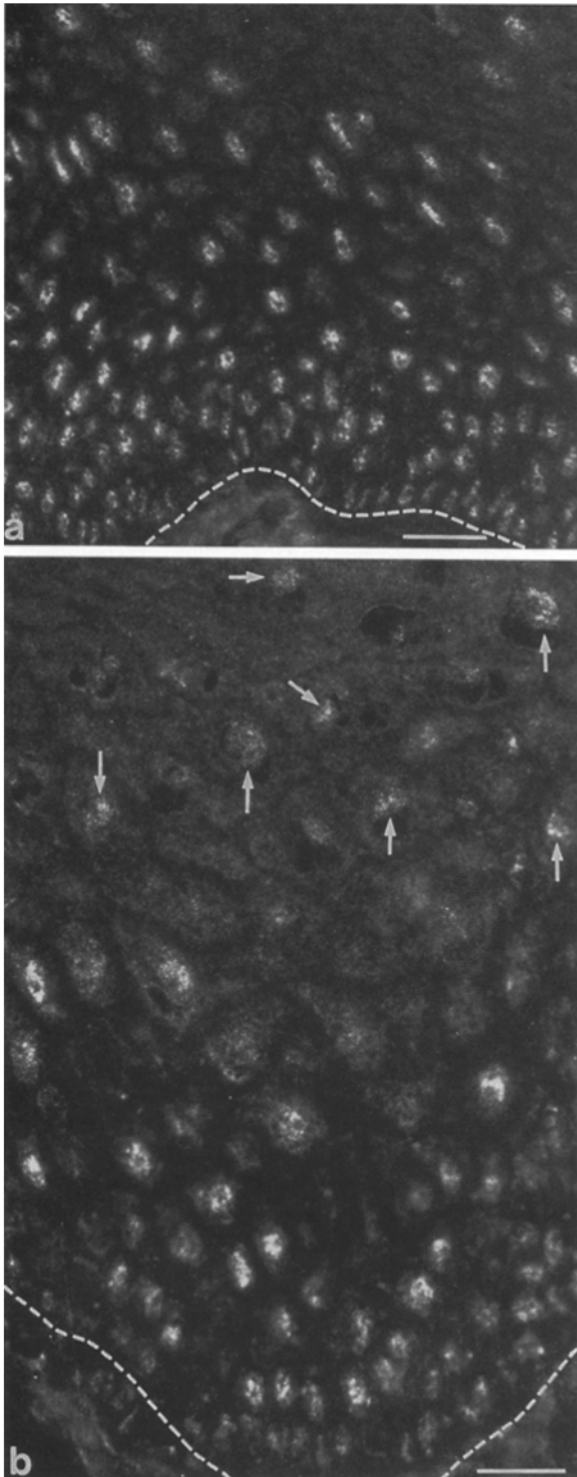
We are currently studying the mode of association of symplekin with the forming *zonula occludens* to test the possibility of transient complexes with components of the *zonula adherens* ensemble, as recently reported by Rajasekaran et al. (1996).

Our immunohistochemical results with actual carcinomas also show that in differentiated colon carcinomas and adenocarcinomas symplekin is assembled in a tight junction-associated plaque structure in a near-apical position, similar to its location in normal polarized epithelia. We therefore propose to use symplekin as a differentiation marker in the differential diagnosis of tumors, notably for different types of carcinomas and certain "epithelioid" tumors.

Remarkably, we detected symplekin and its encoding mRNA not only in cells that are connected by tight junctions or have the developmental potential to form a *zonula occludens* but also in a wide range of other cells and tissues devoid of tight junctions or even of stable cell-cell junctions in general. Such symplekin-producing, non-tight junction-forming cells do not only include cultured cells derived from stratified squamous epithelia or squamous cell carcinomas (e.g., lines HaCaT and A-431) but also non-epithelial cells such as various endothelial cultures,

lens-forming cells and fibroblasts, and totally uncoupled cells such as lymphocytes, lymphoma cells and erythroleukemia cells. While in some of these cells, immunolocalization revealed symplekin in occasional small dot-, whisker- or ring-like structures at the cell surface or in the cytoplasm (immunoelectron microscopic identification of these sparse structures is underway), all of the cultured cells and tissues examined have shown intense symplekin immunoreaction in the nuclear interior. In fact, under the specific appropriate immunolocalization conditions, i.e., preventing extraction of diffusible proteins, very strong symplekin labeling has consistently been seen throughout the entire nucleoplasm, and this is also the case in the tight junction-coupled polar epithelial cells. Consequently, in cells devoid of tight junctions, the nucleus is the only demonstrable location of accumulated symplekin.

The finding of a junctional plaque protein in the cell nucleus, while surprising at first sight, is not without precedence. For example, the occurrence of protein ZO-1 in the nuclei of certain cells has recently been demonstrated by immunolocalization and fractionation experiments (Gottardi et al., 1995) although, as shown in the present study, nuclear protein ZO-1 is much less apparent and less stably bound in the nucleus. When exogenous plakoglobin, the



**Figure 14.** Nuclear symplekin immunostaining on cryostat sections through frozen human esophageal tissue, using mAb Sym-TJ-E150 after Carnoy fixation (protocol B) and detergent wash (0.2% Triton X-100, protocol 2 of Materials and Methods). Note exclusively nuclear localization in cells of the basal as well as suprabasal strata (*a*, survey picture; *b*, higher magnification). The dashed line denotes the level of the basal lamina, the arrows in *b* point to the finely granular-appearing reaction in the nuclei of the upper cell layers. Bars, 5  $\mu$ m.

plaque protein common to adhering junctions of both the desmosomal and the *zonula adhaerens* type, was introduced into *Xenopus laevis* embryos it was found in both junctions and nuclei, and the same was noted for the adherens junction plaque protein,  $\beta$ -catenin (Karnovsky and Klymkowsky, 1995; Funayama et al., 1994). Moreover, the desmosomal plaque proteins, plakophilin 1 and plakophilin 2, have been shown to be proteins of a dual location, as they occur in the nucleoplasm of a large diversity of cells and tissues as well as in desmosomal plaques of various epithelial and non-epithelial cells and tissues (Schmidt et al., 1994; Heid et al., 1994; Mertens et al., 1996). Plakophilin has even been identified in the manually isolated nuclei (germinal vesicles) of amphibian oocytes (Franke, H.W.W., unpublished results). Although possible interactive nuclear partners of any of these plaque proteins have not yet been identified, their dual residence at cell junctions and in the nucleus has been taken, together with similar observations for the  $\beta$ -catenin-related *Drosophila* protein encoded by the *Armadillo* gene, as an indication for a role not only in junction structure formation and cell-cell coupling but also as nuclear signaling molecules in complex signal transduction pathways (for reviews see, e.g., Peifer, 1995; Takeichi, 1995).

The perplexing finding that the tight junction-associated plaque protein symplekin, and also other junctional plaque proteins, also occur as “housekeeping” proteins in the nucleus of a very wide range of different cell types forces one to reconsider the views of the topogenesis, evolution and the functions of these proteins. Apparently, they are not synthesized in relation to the appearance of certain types of cell–cell junctions in the course of specific pathways of cell differentiation. They rather seem to represent generally and genuinely nuclear proteins which in certain cell differentiations can be recruited to contribute to the assembly of specific kinds of junctional plaques: plakophilins 1 and 2 to desmosomal plaques,  $\beta$ -catenin to adherens junction plaques, plakoglobin to both, and symplekin to the plaque of tight junctions. Clearly, our observations of nuclear immunostaining of symplekin in non-proliferative states such as in densely grown cell cultures or in upper strata of squamous epithelia indicate that, in contrast to what has been reported for protein ZO-1 (Gottardi et al., 1995), symplekin does not appear in the nucleus only in response to replication and cell proliferation. Obviously, it will be important to determine the possible modifications and/or complex of partners that are involved in the regulation of the specific topogenic assembly of symplekin.

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