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Proteinase-induced formation of focal tight junctions in HT 29 adenocarcinoma cells does not require extracellular calcium

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The human adenocarcinoma cell line HT 29 grows virtually without tight junctions, but the formation of focal tight junctions can be induced by brief treatment with proteinases. The freeze-fracture morphology of proteinase-induced tight junctions is not affected by treatment with EGTA or EDTA over a period of 30 min. The induction of tight junctions by trypsin or pronase can proceed in the presence of 3 mM EGTA or EDTA. Neither the formation nor the structure and complexity of the induced tight junctions is affected by the chelators. It follows that no extracellular divalent cations are required for the induced formation and the structural integrity of focal tight junctions in HT 29 cells.

Introduction

The zonula occludens (tight junction) represents the most lumenal part of the junctional complex and consists of focal contacts between the neighbouring membranes which appear as a meshwork of anastomosing strands in freeze-fracture electron micrographs. It is generally accepted that the zonula occludens controls the paracellular permeability of epithelia and endothelia [1].

The influence of Ca^{2+} depletion on the permeability of the tight junction seal has been investigated in epithelia and in epithelial-like cell sheets in culture such as confluent MDCK cells. It is well established that divalent metal chelators such as EGTA and EDTA lead to a marked and rapid breakdown of the electric resistance [2–8]. High levels of electric resistance can subsequently be restored by the reintroduction of Ca^{2+} into the culture medium [2–4]. This resealing process is inhibited by a monoclonal antibody against a uvomorulin-related protein [9]. It has also been documented that the paracellular permeability for ions [10], sucrose [10–12] and Phenol red [13] is increased in the absence of extracellular Ca^{2+}.

The Ca^{2+}-mediated modulation of electrical and osmotic phenomena are customarily described as 'opening' and 'resealing' of the tight junctions. However, it should be noted that the morphological correlate of these physiological phenomena is not well understood. Hirokawa [14] investigated mice liver cells by rapid freeze-fracture techniques after treatment with EDTA and suggested that the tight junctions split, thus exposing the true mem-

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Abbreviations: HBSS, Hanks' balanced salts solution; (−)HBSS, Ca^{2+}- and Mg^{2+}-free Hanks' balanced salts solution; PF, protoplasmic face; EF, exoplasmic face.

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brane surface of the tight junction area. This could imply a longitudinal splitting of the individual tight junctions' fibrils, with formation of two corresponding half fibrils in the neighbouring cell membrane. Other authors reported changes in the topological organization of the zonula occludens rather than actual splitting of fibrils. Thus, it was found that the zonula occludens showed discontinuities with formation of fascia occludens type strand arrangements. The breakdown of the electric resistance would be in line with either the longitudinal splitting of tight junction fibrils or with the topological reorganization of the strands [2,6–8,15–18].

Little is known about the role of Ca^{2+} in the de novo formation of tight junctions. Gonzalez-Mariscal et al. [8] studied the influence of Ca^{2+} on the formation of tight junctions in MDCK cells plated at high concentration after trypsinization. In the absence of Ca^{2+}, the cells did not develop electric resistance and the tight junctions consisted of single or double strands only. Following the addition of Ca^{2+}, the electric resistance reached a maximum value after 5 h, and the width and complexity of the zonula occludens increased in parallel. Ducibella et al. [19] found that early mouse embryos do not develop zonulae occludentes in the absence of extracellular Ca^{2+}, but the presence of some focal tight junctions was considered possible.

We have reported earlier on the rapid, massive assembly of tight junctions after proteinase treatment of the human adenocarcinoma cell line, HT 29 [20,21]. The induced tight junctions appear as anastomosing strands of the fascia occludens type but do not organize into zonulae occludentes. Since untreated HT 29 cells have virtually no tight junctions, the experimental system is very well suited for quantitative studies. This paper shows that the formation and the morphology of proteinase-induced focal tight junctions in this experimental system are not affected by the chelators, EGTA and EDTA.

Materials and Methods

Cell culture

The human colon adenocarcinoma cell line HT 29 was isolated by Føgh and Trempe [22] and was obtained by courtesy of Prof. F. Doljanski, Jerusalem. Cells were grown in Dulbecco's modified Eagle's medium containing 0.37% glucose and 10% fetal calf serum at 37 °C in a humidified atmosphere containing 5% CO_2. They were transferred every 5 days by treatment with trypsin (0.05 mg/ml) in phosphate-buffered saline containing 0.53 mM EDTA. Experiments were performed on cultures grown for 4 days. Viability of cells subsequent to treatment with enzymes and/or chelators was monitored by Trypan blue exclusion and/or subculturing.

Electron microscopy

Cells were fixed in suspension by 1% glutaraldehyde in 0.1 M cacodylate buffer or in phosphate-buffered saline (pH 7.4) for 1 h at room temperature. They were then washed and suspended in 30% glycerol in 0.1 M cacodylate buffer or phosphate-buffered saline (pH 7.4) at 4 °C overnight. Samples were frozen by immersion in liquid propane. Freeze-fracturing was performed at −100 °C in a Balzers freeze-etching unit. Replicas were examined with a Jeol 100 CX electron microscope. When cells were still attached to the culture dishes after treatment, they were fixed in situ and subsequently scraped off with a rubber policeman. They were then processed for freeze-fracturing as described above.

Quantitation of tight junctions

Numerical evaluation [21]. A minimum of 50 freeze-fractured membranes were evaluated in each experiment. Each exposed contiguous membrane surface which could be unequivocally identified as part of the cell membrane was counted as one unit, irrespective of size. The fragments were monitored for the presence or absence of tight junctions. It was also documented whether the fracture plane changed from P-face to E-face within a tight junction area of a given cell. Continuity of ridges on PF and grooves on EF were considered as proof for a true tight junction as opposed to a (hypothetical) split tight junction.

Morphometric evaluation. Freeze-fracture electron micrographs of cell membranes with and without tight junctions were obtained at 3300-fold magnification. In a given replica, every cell membrane which could be unequivocally identified was

photographed to obtain a random sample. The negatives were analyzed with a semiautomatic Leitz image analysis system (total magnification, 22000). The tight junction and the membrane perimeter were drawn with the electric cursor yielding the combined length of tight junction strands and the exposed membrane area. The number of tight junction anastomoses per membrane was counted.

Results

We have shown that the proteinase-induced formation of tight junctions in cultured HT 29 cells can be monitored by counting membranes with and without tight junctions in freeze-fracture replicas [21]. HT 29 cells grown in culture are virtually devoid of tight junctions. More specifically, less than 2% of observed cell membranes exhibit tight junction strands or networks. As shown earlier, tight junctions form rapidly in these cells after brief treatment with various proteinases [20]. Treatment with trypsin at a concentration of 1.5 mg/ml in HBSS for 15 min at 37°C yields tight junctions on about 68% of membranes investigated with a high degree of reproducibility (Table I, Fig. 1).

In order to study the influence of chelators on the structural integrity of tight junctions in HT 29 cells, we induced maximum tight junction formation by treatment with trypsin. The treated cells were subsequently incubated in Ca^{2+}- and Mg^{2+}-free HBSS containing 3 mM EGTA or EDTA. Control cells were incubated in HBSS containing 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}. The fraction of cell membranes with tight junctions was monitored by freeze-fracture electron microscopy after periods of 15 and 30 min (Fig. 2, Table I). In buffer with Mg^{2+} and Ca^{2+}, the fraction of cell membranes with tight junctions showed a slow decrease over the period of 30 min, in agreement with earlier findings [23]. This decrease with time was less apparent in the presence of chelators.

Transitions of the fracture plane from PF to EF in the area covered by tight junctions occurred on 61–74% of membranes with tight junctions. No significant difference was found in experiments with divalent cations or chelators, respectively (Fig. 2, Table I). In each case where a PF/EF transition occurs, it is clear that we are dealing with the membranes of two closely apposed cells which were intimately connected by the tight junction structure. It follows that the fibrils exposed in the freeze-fractured membrane are not split tight junctions.

TABLE I

INFLUENCE OF CHELATORS ON TRYPSIN-INDUCED TIGHT JUNCTIONS (TJ) IN HT 29 CELLS

Cells were washed for 5 min with HBSS. The formation of tight junctions was induced by treatment with a solution of trypsin (1.5 mg/ml) in HBSS for 15 min at 37°C. The cells were centrifuged (5 min, 300 × *g*) and washed with soybean trypsin inhibitor (500 μg per ml of (−)HBSS). They were then incubated in (−)HBSS containing the specified agents at 37°C for the time indicated. Cells were fixed and analyzed by freeze-fracture electron microscopy.

Agent	Time (min)	Membranes				
		total [a]	with TJ [b]	with TJ/FT [c]	% TJ [d]	%FT [e]
–	0	446	303	187	68	62
1.2 mM Ca^{2+} + 0.8 mM Mg^{2+}	15	67	38	28	57	74
	30	60	22	14	37	64
3 mM EGTA	15	60	40	28	67	70
	30	65	40	29	62	73
3 mM EDTA	15	63	42	27	67	64
	30	61	30	22	49	73

[a] Number of evaluated membranes.
[b] Number of membranes with tight junctions.
[c] Number of membranes with tight junctions showing transition from PF to EF.
[d] Fraction of membranes with tight junctions.
[e] Fraction of tight junctions indicating a transition from PF to EF.

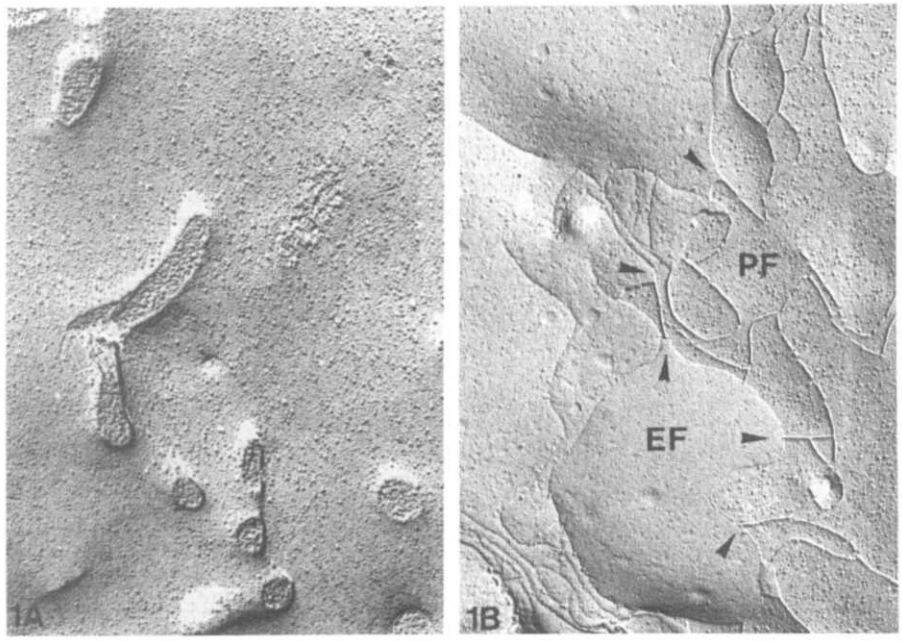

Fig. 1. Freeze-fractured membranes of HT29 cells treated for 15 min at 37 °C with (A) HBSS, (B) trypsin (1.5 mg/ml) in HBSS. EF, exoplasmatic face; PF, protoplasmatic face; wedges, PF/EF transitions; ×42000.

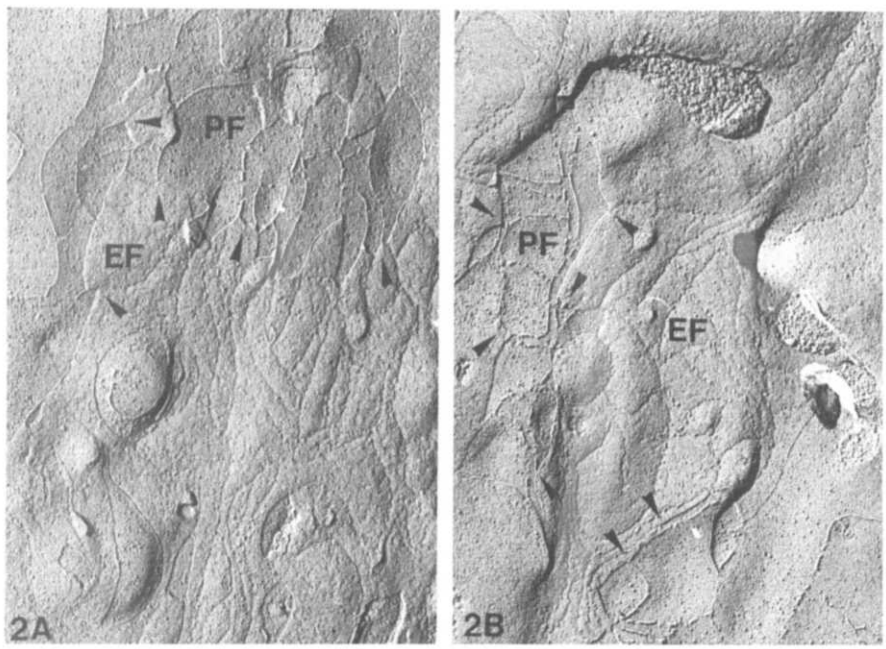

Fig. 2. Freeze-fractured membranes of HT29 cells pretreated with trypsin (1.5 mg/ml) for 15 min at 37 °C and postincubated for 30 min at 37 °C with (A) 3 mM EGTA, (B) 3 mM EDTA; ×42000.

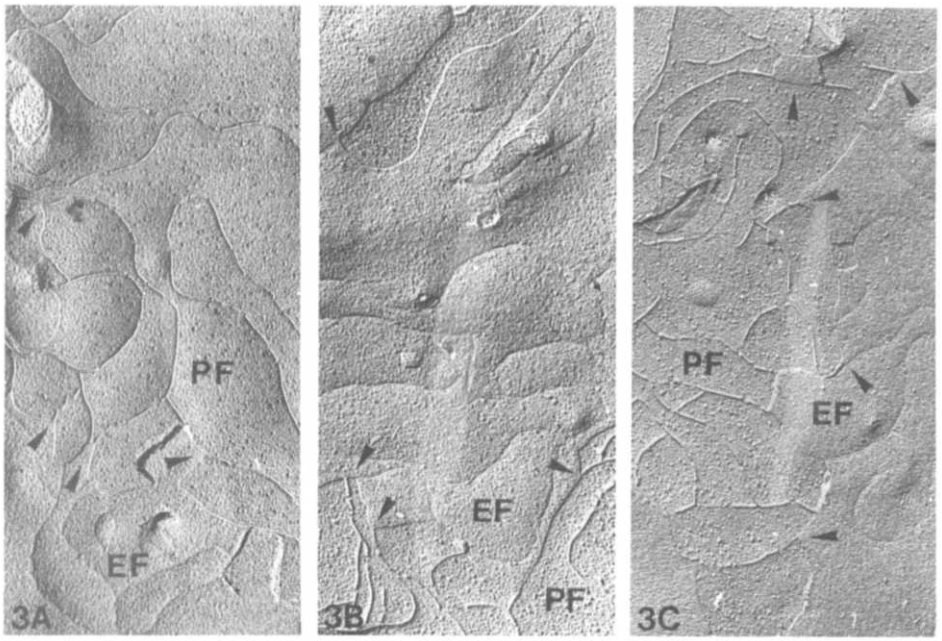

Fig. 3. Freeze-fractured membranes of HT29 cells treated with trypsin (1.5 mg/ml) for 30 min at 37 °C in the presence of (A) 1.2 mM Ca^{2+} + 0.8 mM Mg^{2+}, (B) 3 mM EGTA, (C) 3 mM EDTA; wedges, PF/EF transitions; ×42000.

Since we found no detectable effect of chelators on the structural integrity of tight junctions, we decided to analyze the question whether tight junctions can be formed de novo in the presence of chelators. In order to ascertain the complete removal of extracellular divalent cations, the cells were washed with Ca^{2+}- and Mg^{2+}-free buffer and incubated with the chelators for 30 min. Trypsin was added subsequently. The formation of tight junctions was monitored after 15 and 30 min. Controls were treated in HBSS containing 1.2 mM Ca^{2+} and 0.8 mM Mg^{2+}. In each experiment, abundant tight junctions were observed on 57–70% of the exposed cell membranes (Fig. 3, Table II). The occurrence of PF/EF transitions was again carefully monitored on each cell membrane with tight junctions. The values ranged from 65 to 73%. Again, no significant difference was

TABLE II

INDUCTION OF TIGHT JUNCTIONS IN HT 29 CELLS BY TRYPSIN IN THE PRESENCE OF CHELATORS

Cells were washed at 37 °C for 5 min with (−)HBSS and incubated for 30 min in (−)HBSS containing 3 mM of the agent indicated. Trypsin in (−)HBSS was added to a final concentration of 1.5 mg/ml, and the cultures were incubated at 37 °C for the time indicated. Cells were fixed and analyzed by freeze-fracture electron microscopy.

Agent	Time (min)	Membranes				
		total [a]	with TJ [b]	with TJ/FT [d]	% TJ [c]	%FT [e]
1.2 mM Ca^{2+} + 0.8 mM Mg^{2+}	15	92	63	41	68	65
	30	65	44	32	68	73
3 mM EGTA	15	111	76	52	68	68
	30	53	38	29	72	76
3 mM EDTA	15	61	41	28	67	68
	30	64	44	34	69	77

[a–e] See Table I footnotes.

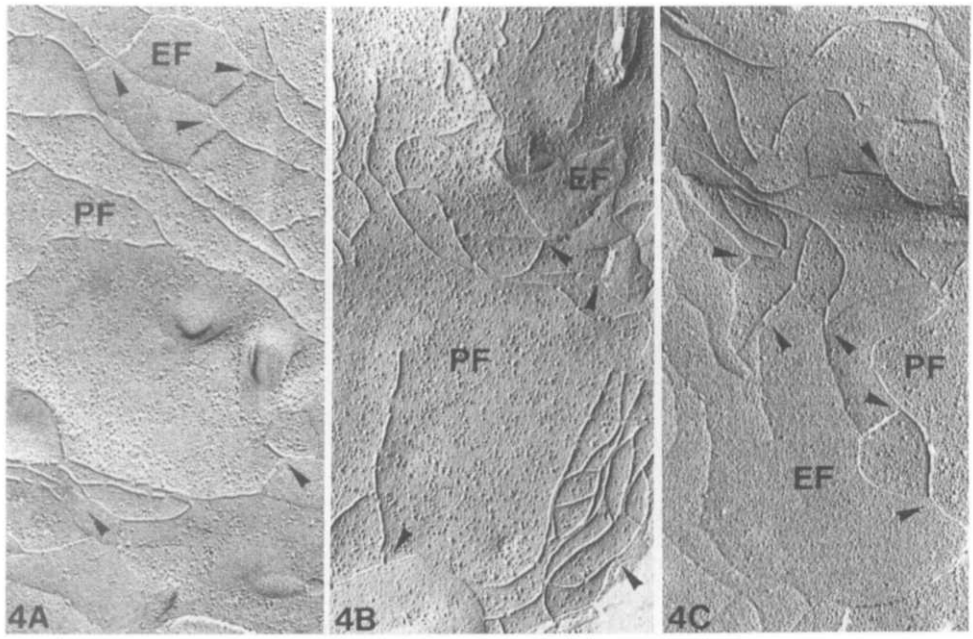

Fig. 4. Freeze-fractured membranes of HT29 cells treated with pronase (10 μg/ml) for 30 min at 37°C in the presence of (A) 1.2 mM Ca^{2+} + 0.8 mM Mg^{2+}, (B) 3 mM EDTA, (C) 3 mM EGTA; wedges, PF/EF transitions; ×47000.

found in the presence or absence of divalent cations. The high frequency of PF/EF transitions identifies the ridges and grooves observed in the replicas as actual tight junctions, i.e., intimate contacts between the membranes of two adjacent cells. No evidence whatsoever for the occurrence of "split" tight junctions could be obtained.

Experiments with low concentrations of pronase were very similar to the results reported above, i.e., tight junctions were abundantly induced both in the presence and absence of extracellular divalent cations (Fig. 4, Table III). Once again, the PF/EF transitions were observed at the same frequency as noted above.

The numerical evaluation of tight junctions by counting membranes with and without tight junctions has been shown to be highly reproducible by earlier studies and in the present series of experiments. However, this method would not detect minor changes of the quantity and complexity of tight junction strands per membrane area. We have therefore performed morphometric measurements of tight junctions induced by trypsin in the presence of Ca^{2+} and Mg^{2+} or in the presence of chelators. The total length of tight junction strands and the total membrane area were determined in samples of 22 to 100 freeze-fractured cell membranes photographed at random. Each cell membrane which could be unequivocally identified by topological criteria was photographed, irrespective of the presence of tight junctions. The combined tight junction strand length, the number of anastomoses and the total membrane area were de-

TABLE III

INDUCTION OF TIGHT JUNCTIONS IN HT 29 CELLS BY PRONASE IN THE PRESENCE OF EDTA OR EGTA

Cells were washed for 5 min at 37°C with (−)HBSS and incubated for 30 min in (−)HBSS containing the specified agent. Pronase in (−)HBSS was added to a final concentration of 10 μg/ml and the cultures were incubated at 37°C for 30 min. Cells were fixed and analyzed by freeze-fracture electron microscopy.

Agent	Membranes				
	total [a]	with TJ [b]	with TJ/FT [c]	% TJ [d]	% FT [e]
1.2 mM Ca^{2+} + 0.8 mM Mg^{2+}	71	47	34	66	72
3 mM EGTA	63	43	31	68	72
3 mM EDTA	60	41	30	68	73

[a–e] See Table I footnotes.

TABLE IV

MORPHOMETRIC ANALYSIS OF TRYPSIN-INDUCED TIGHT JUNCTIONS

HT 29 cells were treated as described in Table II. Tight junctions were analyzed quantitatively by morphometric measurement of tight junction length, number of anastomoses and membrane area.

Inducing agent	N [a]	L [b] (μm)	X [c]	A [d] (μm^2)	L/A [e] (μm^{-1})	X/A [f] (μm^{-2})
None	100	2.2	1	2340	0.001	0.0004
Trypsin/Ca^{2+} + Mg^{2+}	27	1057	1154	1050	1	1.1
Trypsin/EGTA	29	652	714	690	0.95	1.0
Trypsin/EDTA	22	720	903	740	0.97	1.2

[a] Number of membranes investigated by morphometry.
[b] Combined length of all tight junction fibrils measured.
[c] Number of anastomoses of all tight junctions measured.
[d] Area of all membranes measured.
[e] Length of fibrils (in μm) per μm^2 of membrane area.
[f] Number of anastomoses per μm^2 of membrane area.

termined (Table IV). The total length of tight junction strands ranged from 0.95–1.0 μm per μm^2 of membrane area in experiments performed either in HBSS (with Ca^{2+} and Mg^{2+}) or in (−)HBSS supplemented with one of the chelators. The number of anastomoses was also very similar in each of the experiments (1.0–1.2 per μm^2 of membrane area). Both experimental values (strand length and anastomoses) were very low in cells not treated with proteinase. This result confirms that the complexity of tight junctions induced by proteinases is not affected by chelators and thus does not require divalent ions.

In a control experiment, we have confirmed that the chelators used did not induce tight junctions per se. HT 29 cells were incubated with EDTA or EGTA in buffer without Ca^{2+} and Mg^{2+} for periods of up to 60 min. The cells were then fixed and analyzed by freeze-fracture electron microscopy. The fraction of membranes with tight junctions was always below 5% (data not shown).

Discussion

The human colon adenocarcinoma cell line, HT 29, is an attractive model for the study of tight junction assembly. The presence of virtually no tight junctions in untreated cells and their massive and rapid formation after proteinase treatment provides a large dynamic range for quantitative evaluation. The numerical evaluation introduced earlier is rapid and can be used to obtain a large number of data points in complex experimental arrangements. The excellent reproducibility of this approach has been documented in earlier studies [21] and is again confirmed by the present results.

It has been shown that HT 29 cells can grow as epithelial-like cells with well-developed brush-borders and polarized distribution of membrane proteins under appropriate culture conditions (replacement of glucose by galactose or inosine) [24–26]. Thin section electron microscopy indicates the presence of tight junctions in these epithelial-like cells. The expression of this epithelial-like cell type requires a period of many days in culture. For the purpose of the present investigation it is advantageous that tight junctions do not form spontaneously in HT 29 cells growing rapidly in medium with glucose.

We have used this experimental system to study the influence of the divalent metal chelators, EGTA and EDTA, on the formation and on the structural integrity of tight junctions. Our initial observation indicates that tight junctions in HT 29 are stable and can actually form de novo in the absence of any divalent metal ions. It was then important to analyze whether the ridges and grooves observed in the freeze fracture images of cell membranes were true tight junctions or "split" tight junctions, i.e., hemifibrils in separate membranes, since Hirokawa [14] had suggested that liver tight junctions can split under the influence of chelators. This question could be addressed by

a careful study of topological criteria in the freeze-fracture replica. In adjacent cell membranes, the fracture plane can change from the membrane of one cell to the membrane of the neighbouring cell. The observation of ridges on the P-face of one membrane and grooves in register with these ridges on the E-face of the adjacent membrane is unequivocal proof for the presence of a true tight junction between two adjacent cells. This criterion has been applied quantitatively and shows that the occurrence of PF/EF transitions is not affected by chelators. We conclude that the structures observed in this study are true tight junctions throughout, and that no longitudinal splitting of tight junction fibrils occurred in our experiments.

In light of these findings, it follows that the structural integrity and the formation of tight junction fibrils does not require extracellular divalent ions in our experimental system. A structure which can actually form de novo in the absence of divalent metal ions is apparently not dependent on divalent ions for its stability. This could imply that focal tight junctions of HT 29 cells are different from tight junctions in other epithelial systems where Ca^{2+} appeared to be necessary for zonula occludens stability [2–8,17,18]. The alternative may be that the effects of chelators on cell sheets and epithelia, which have been documented many times by measurements of electric resistance and osmotic permeability, are not caused by actual splitting of the tight junction strands, but by a modulation of other cell properties which may ultimately and indirectly lead to a deterioration of the zonula occludens. It has been shown that the intermediate junction opens up rapidly in the absence of Ca^{2+} [27]. This could lead to the retraction of adjacent cell membranes and to the loss of tight junction integrity by mechanical shear. In line with this hypothesis, it has been suggested that the absence of Ca^{2+} may influence the tight junction pattern indirectly through the involvement of cytoskeletal elements [8,28]. Other Ca^{2+}-sensitive subsystems of the cell might also indirectly contribute to changes of electric and osmotic properties. In addition, it is also conceivable that Ca^{2+} ions modulate the leakiness of the tight junction seal at the molecular level, which would not be detected in an ultrastructural study.

Few studies have been performed to analyze the role of cations for tight junction formation. Mouse blastocysts kept at a low concentration of Ca^{2+} developed focal tight junctions but no zonular tight junctions [19]. Cultured MDCK cells developed single- or double-stranded tight junctions in the absence of Ca^{2+}; however, the full expression of the zonula occludens required extracellular Ca^{2+} and developed over a period of several hours upon addition of the ion [8]. In contrast to these relatively slow assembly processes, the data reported in this paper show that the fast tight junction assembly in proteinase-treated HT 29 cells is not sensitive to chelators and thus requires no extracellular Ca^{2+}. However, it should be emphasized again that the organization of the tight junction fibrils into zonula occludens does not occur in HT 29 cells under our experimental conditions. Thus HT 29 cells are a model of tight junction strand (fascia occludens) formation and not zonula occludens formation.

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