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Induction of Tight Junctions in Human Connexin 32 (hCx32)-Transfected Mouse Hepatocytes: Connexin 32 Interacts with Occludin

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Small gap junction plaques are associated with tight junction strands in some cell types including hepatocytes and it is thought that they may be closely related to tight junctions and the establishment of cell polarity. In order to examine roles of gap junctions in regulating expression and structure of tight junctions, we transfected human Cx32 cDNA into immortalized mouse hepatocytes (CHST8 cells) which lack endogenous Cx32 and Cx26. Immunocytochemistry revealed that endogenous integral tight junction protein occludin was strongly localized and was colocalized with Cx32 at cell borders in transfectants, whereas neither was detected in parental cells. In Northern blots, mRNAs encoding occludin and the other integral tight junction proteins, claudin-1 and -2, were induced in the transfectants compared to parental cells. In Western blots, occludin protein was increased in the transfectants compared to parental cells, and binding of occludin to Cx32 protein was demonstrated by immunoprecipitation. In freeze fracture of the transfectants, tight junction strands were more numerous and complex compared to parental cells, and small gap junction plaques appeared within induced tight junction strands. Nevertheless, no change in barrier function of tight junctions was observed. These results indicate that in hepatocytes, gap junction, and tight junction expression are closely coordinated, and that Cx32 may play a role in regulating occludin expression.

Key Words: **gap junction; ZO-1; claudin-1 and -2; CHST8 cells.**

Gap junction channels, composed of proteins termed connexins, mediate reciprocal exchange of ions and small molecules of less than 1,000 Da, including second messengers, such as cyclic AMP, IP_3 and Ca^{2+} between adjacent cells (1–3). Gap junctions in hepatocytes are composed of both connexin 26 (Cx26) and connexin 32 (Cx32) (4–6). Gap junctional intercellular communication is thought to play a crucial role in development, cell growth and cell differentiation (7–11). Moreover, as epithelial cells differentiate, tight junction strands establish cell polarity, and small gap junction plaques ($<0.05\ \mu m^2$) appear within the tight junction web in freeze fracture replicas (12–16). Recently, it has become established that Cx43 interacts with ZO-1, a 220 kDa peripheral membrane component of tight junctions and adherens junctions (17, 18) in Cx43 transfectants, in normal fibroblasts and in cardiac myocytes (19, 20). This interaction is direct, through binding of the extreme carboxyl terminus of Cx43 and the second PDZ domain of ZO-1 (19, 20).

Tight junctions, the most apical component of intercellular junctional complexes, separate the apical from the basolateral cell surface domains to establish cell polarity (performing the function of a fence); tight junctions also provide a barrier function, inhibiting solute and water flow through the paracellular space (21, 22). Tight junctions show a particular net-like meshwork of fibrils (23) and several peripheral or integral membrane proteins have been specifically localized to tight junctions (24, 25). Occludin is a 65 kDa integral membrane protein that has been shown to localize to tight junctional strands through immunogold labeling of freeze-fracture replicas, and occludin was until recently believed to be the protein responsible for both fence and barrier functions (26, 27). However, because targeted disruption of the occludin gene did not abolish tight junctions in epithelial cells (28), the search for new integral membrane proteins localized at tight junctions was renewed, resulting in the identification

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of claudin-1 and -2 proteins as new integral tight junction membrane proteins whose expression is even more closely correlated with the formation of tight junction strands (29, 30).

In this study, in order to examine roles of gap junctions in regulating expression, structure and function of tight junctions, we transfected human Cx32 cDNA into an immortalized mouse hepatocyte cell line (CHST8 cells) which lacked endogenous expression of hepatocyte gap junction proteins Cx26 and Cx32, and established two clonal cell lines which stably expressed Cx32 protein (Kojima *et al.*, submitted). When we examined changes in expression and function of tight junctions in the transfectants compared to that of parental cells, gap and tight junction expression were closely correlated, and Cx32 expression might play a role in regulating occludin expression.

MATERIALS AND METHODS

Cell culture. CHST8 is a clonal cell line derived from female C3H/HeJ mouse hepatocytes conditionally immortalized with the temperature-sensitive SV40 large T antigen gene as described previously (31). The cells were incubated with DMEM medium (GIBCO BRL, Gaithersburg, MD) with 4% fetal bovine serum (Sigma Co., St. Louis, MO), 20 mM Hepes (Sigma), 0.5 mg/L insulin (Sigma), 10^{-7} M dexamethasone (Sigma), 1 g/L galactose (Sigma), 30 mg/L proline (Sigma), 1 mM sodium pyruvate (Sigma) and antibiotics at 33°C.

cDNA construction and transfection A fragment containing nucleotides 1 to 1558 of human connexin 32 (Cx32) cDNA (32) was subcloned into the expression vector pREP9 (Invitrogen, Carlsbad, CA) at the *Kpn*I–*Bam*HI restriction site. CHST8 cells were transfected with 2 μg of Cx32 cDNA using the LIPOfectamine reagent (GIBCO BRL, Gaithersburg, MD). After 48 h, the cells were transferred to selection medium containing 2 μg/ml puromycin (Sigma).

Immunofluorescence microscopy. Immunocytochemistry with anti-Cx32 (Zymed, South San Francisco, CA), anti-occludin (Zymed), anti-ZO-1 (Zymed), anti-pancadherin (Sigma), anti-α catenin (Sigma) and anti-β catenin (Sigma) antibodies was performed as described previously (15, 33). The specimens were examined by an epifluorescence microscope and a laser-scanning confocal microscope (MRC 1024; Bio-Rad, Hercules, CA).

Furthermore, in order to examine intracellular trafficking of Cx32, occludin and ZO-1, the transfectants were treated with 10 μg/ml Brefeldin A (Sigma) for 24 h, and then immunostaining was performed for Cx32, occludin and ZO-1.

RNA isolation and Northern blot analysis. Total RNA was extracted from the cells using TRIzol reagent (GIBCO BRL). For Northern blots (15), 20 μg of total RNA was electrophoresed and blotted onto a nylon membrane (Hybond-N; Amersham Corp., Buckinghamshire, UK) and fixed by ultraviolet light. The membranes were hybridized at 68°C in Quick Hyb solution (Stratagene, La Jolla, CA) with ^{32}P-labeled cDNA probes for Cx32 (34), occludin, claudin-1 and -2. Occludin and claudin-1 and -2 cDNAs were amplified from mouse liver first strand DNA by PCR using primers corresponding to the previously reported sequences of mouse occludin and claudin-1 and -2 (29, 35). The membranes were washed at 60°C before exposure to film.

Western blot analysis. Western blots were performed as described previously (15, 33). The membranes was incubated with the primary antibodies (see Immunofluorescence Microscopy, above) and then were incubated with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The detection was carried out using an enhanced chemiluminescence (ECL) Western blotting system (Amersham Corp., Buckinghamshire, UK).

Immunoprecipitation. For immunoprecipitation, the dishes were washed twice with PBS and 300 μl of NP-40 lysis buffer (50 mM Tris-HCl, 1% NP-40, 0.25 mM Na-deoxycholate, 150 mM NaCl, 2 mM EGTA, 0.1 Na_3VO_4, 10 mM NaF and 2 mM PMSF) was added to 60 mm dishes (19). The cells were scraped and collected in Eppendorf tubes and then sonicated for 10 sec. Cell lysates were incubated with mM A-Sepharose Cl-4B (Pharmacia LKB Biotechnology Inc.) for 1 h at 4°C and then clarified by centrifugation at 15,000 $\times$ g for 10 min. The supernatants were incubated with anti-occludin antibody bound to protein-A-Sepharose Cl-4B overnight at 4°C. After incubation, immunoprecipitates were extensively washed with the same lysis buffer. Immunoprecipitates were subjected to Western blot analyses with anti-Cx32 antibody.

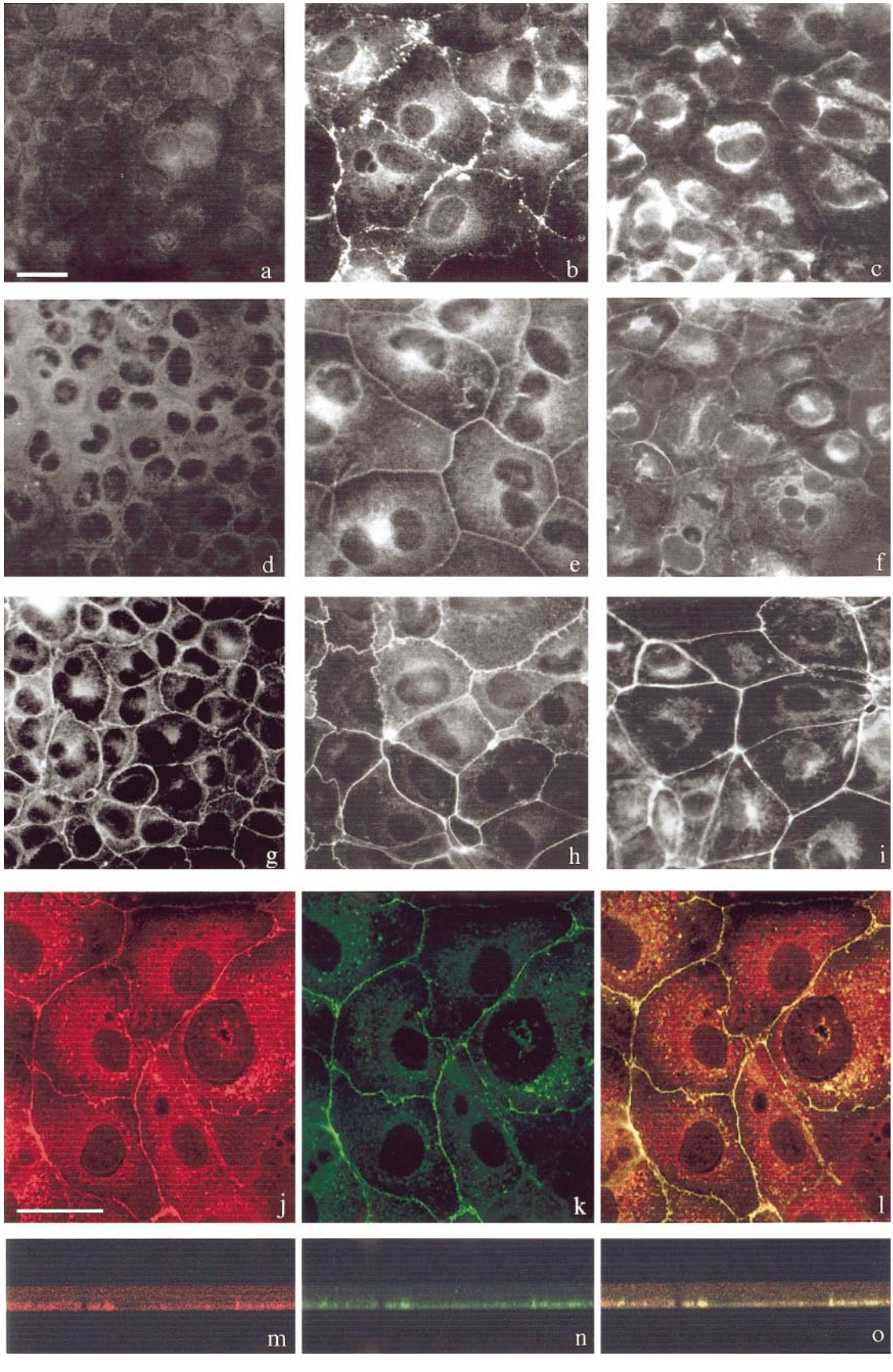
Freeze-fracture analysis. Freeze fracture was performed as described previously (15, 33, 36). The number of small gap junction plaques and tight junction strands in the replicas were measured on coded 20 prints of electron micrographs (60,000$\times$ magnification) and numbers of small gap junctions and tight junction strands were counted in the regions where tight junction strands were expressed at 200-nm intervals.

Measurement of transepithelial electrical resistance (TER). Cells were grown on 12-mm transwell filters (Costar Corp., Cambridge, MA) to confluence. Transepithelial resistance (TER) was measured directly in the wells. Current pulses (4-mA) were passed across the monolayer using a pair calomel electrodes via KCl salt bridges, and voltage was measured by a conventional voltmeter across the same cell monolayer using a pair of Ag/AgCl electrodes via KCl salt bridges (EVON, World Precision Instruments). TER was calculated from the measured voltage and normalized by the area of the monolayer. The background TER of blank transwell filters was subtracted from the TER of the cell monolayers.

Data analysis. Scanning-densitometry was performed using a Macintosh computer and an EPSON GT5000 scanner. Signals were quantified by the NIH Image 1.52 Densimetric Analysis Program (Wayne Rasband, NIH, Bethesda, MD). Each set of results shown is representative of three separate experiments.

RESULTS AND DISCUSSION

It was recently reported that transfection of mouse L cells with mouse occludin cDNA induced both tight junction-like strands and interspersed gap junction plaques (30). Transfection with an N-terminally truncated occludin construct was also reported to induce small gap junction plaques that were associated with P-face tight junction strands (37). In the present study, we transfected human Cx32 cDNA into immortalized mouse hepatocytes (CHST8 cells) which lack endogenous Cx32 and Cx26 and selected both moderately and highly expressing clones, Tr 1 and Tr 2, respectively. Immunocytochemistry identified Cx32- and occludin-immunoreactive products in Tr 2, which were localized both in perinuclear regions and at cell borders (Figs. 1b and 1e), while immunoreactive products were not detected in parental cells (Figs. 1a and 1d). Staining for another tight junction associated protein, ZO-1, detected strong immunoreactivity both at the cell borders and within the cytoplasm in both parental cells and Tr 2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(Figs. 1g and 1h). Double immunostaining of Tr 2 using confocal laser microscopy demonstrated that Cx32 immunoreactivity was in part colocalized with occludin immunoreactivity at cell borders and within the cytoplasm in the same optical section (Figs. 1j, 1k, 1l, 1m, 1n, and 1o). As a further test for coordinated regulation of Cx32 and occludin, Tr 2 cells were treated with the ER-Golgi blocker Brefeldin A, which has been shown to disrupt delivery of Cx32 to the surface membrane (14, 38). In Tr 2 following 24 h treatment with Brefeldin A, both Cx32- and occludin-immunoreactive products were reduced at cell borders and increased in perinuclear regions of the cytoplasm (Figs. 1c and 1f). However, distribution of ZO-1 immunoreactivity at the cell borders was unaffected by Brefeldin A treatment (Fig. 1i). These findings suggest a close association between Cx32 and occludin expression.

In order to determine whether Cx32 transfection altered the expression of mRNAs encoding tight junction associated proteins, Northern blot analyses for Cx32, occludin, and claudin-1 and -2 were performed on parental and transfected cells and mouse liver. As illustrated in Fig. 2, Cx32 mRNA was strongly expressed in Tr 1, Tr 2 and in mouse liver, whereas it was absent in parental CHST8 cells. Expression of occludin mRNA was markedly increased in both Tr 1 and Tr 2 compared to parental cells and mouse liver. Expression of claudin-1 mRNA, which was readily detected in mouse liver, was not demonstrable in either Tr 1 or parental cells but was faintly observed in Tr 2. Expression of claudin-2 mRNA was increased in Tr 1 and Tr 2 compared to parental cells and mouse liver, with expression being higher in Tr 1 than in Tr 2.

Western blots were performed on parental cells and the transfectants in order to compare levels of expression of Cx32 and the tight junction associated proteins occludin and ZO-1. As shown in Fig. 3a, expression of Cx32 protein was strongly observed in Tr 1 and Tr 2, but not in parental cells. Expression of occludin protein in Tr 1 and Tr 2 was markedly increased compared to that of parental cells, with levels of both Cx32 and occludin proteins being higher in Tr 2 than Tr 1. Expression of ZO-1 protein was at similar levels among parental cells and both transfectants.

Recently, it has become established that Cx43 interacts with ZO-1 in Cx43 transfectants, in normal fibroblasts and in cardiac myocytes (19, 20). This interaction is direct, through binding of the extreme carboxyl terminus of Cx43 and the second PDZ domain of ZO-1 (19, 20). In order to examine whether a direct interaction existed between Cx32 and occludin, we performed immunoprecipitation experiments using NP-40 lysis buffer (19). As illustrated in Fig. 3b, immunoprecipitation using anti-occludin antibody led to the identification of Cx32 in Western blots of the immunoprecipitates obtained from Tr 2 but not in either parental cells or Tr 1.

In order to examine formation of gap and tight junction structures after Cx32 transfection, we performed freeze fracture analyses on the adluminal plasma membranes. In parental cells, small gap junction plaques were not detectable and only a few tight junction strands were observed on the luminal surfaces containing many microvilli (Fig. 4a). In Tr 2 cells, some small gap junction plaques were observed within much more elaborate, web-like tight junction strands (arrow, Fig. 4b), with the frequency of their occurrence being about 20% in regions of the tight junction strands (not illustrated). Numbers of tight junction strands are quantitatively compared in Fig. 4c, which shows that such strands were markedly more numerous in Tr 2 that in parental cells. These studies suggest a close correlation between gap and tight junction formation, as is demonstrated by the coordinated expression of gap and tight junction proteins.

In order to evaluate whether the more numerous and elaborate tight junction strands in Tr 2 resulted in enhanced barrier function of tight junctions, transepithelial resistance (TER) was measured at different times over a 24 h period after induction of cell-cell contact. TER in all cells was found to be very low (10–40 ohms per cm^2) and no significant differences were detected among TERs measured from the different cell types (Fig. 4d). Although previous studies have established a positive correlation between tight junction strand number and function, it has been difficult to extrapolate these generalizations to all cell types (39). It has been reported that phosphorylation of occludin protein is correlated with barrier function of tight junction (40), and it is conceivable that the lack of measurable TER in the present study might be due to lack of occludin phosphorylation (see Fig. 3a). Alternatively, even though we demonstrate an increased expression of claudin-2 (Fig. 2), it remains possible that additional yet to be discovered molecules necessary to induce tight junction function are missing from the transfectants.

FIG. 1. Immunocytochemistry for Cx32 (a, b, c, j, l, m, o), occludin (d, e, f, k, l, n, o), and ZO-1 (g, h, i) in parental CHST8 cells (a, d, g) and in Tr 2 (b, c, e, f, h, i, j, k, l, m, n, o). Cx32- and occludin-immunoreactive products were observed at cell borders in Tr 2, while in parental cells, these immunoreactive products were not detected (a, b, d, e). ZO-1 immunoreactivity was strongly observed at cell borders both in parental cells and in Tr 2 (g, h). Following Brefeldin A treatment, Cx32- and occludin-immunoreactive products were reduced on cell borders and increased around nuclei in the cytoplasm, whereas ZO-1 staining was not appreciably affected (c, f, i). By confocal laser microscopy after double staining with anti-Cx32 (red) and anti-occludin (green) antibodies (XY-sections: j, k, l; Z-sections: m, n, o), Cx32- and occludin-immunoreactive products in Tr 2 were colocalized in the same optical sections at cell borders (yellow). Bars: 10 μm.

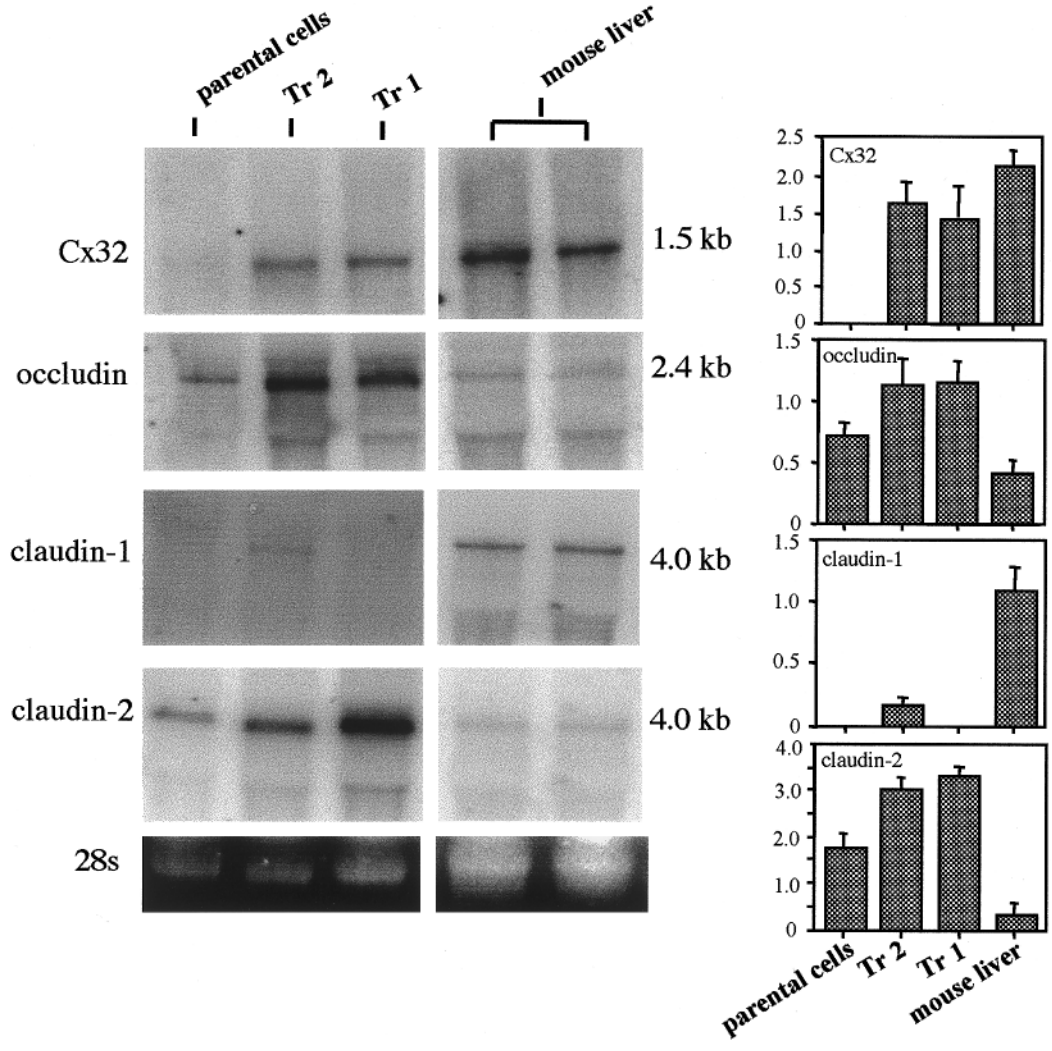

FIG. 2. Northern blot analyses for Cx32, occludin, and claudin-1 and -2 in parental cells and in transfectants. Brackets above bars in graphs represent SEM from three separate experiments. All mRNAs were detected in mouse liver tissue, providing a positive control. Expression of Cx32 mRNA was strongly observed in Tr 1 and Tr 2, whereas it was absent in parental CHST8 cells. Expression of occludin mRNA in Tr 1 and Tr 2 was markedly higher than that of parental cells. Expression of claudin-1 mRNA was slightly observed in Tr 2 but was not detected in Tr 1 or in parental cells. Expression of claudin-2 mRNA was increased in Tr 1 and Tr 2 compared to parental cells.

Cadherins and catenins are cell adhesion molecules and, as such, they are generally found near tight and gap junctions (41). Although we performed immunocytochemistry and Western blot analyses of pancadherin, and α and β catenin on parental cells and the transfectants, no significant changes were observed (not illustrated). Changes in the integral tight junction proteins occludin and claudins-1 and-2 were specific in the transfectants, as no changes were seen in localization or expression of ZO-1 or the adherens junction proteins cadherin or α or β catenin.

In the present study, in Cx32 transfectants, induction of tight junction strands and the integral tight junction proteins occludin, claudin-1 and -2 were observed, and small gap junction plaques appeared within the induced tight junction strands. We recently examined changes of occludin expression by transfection of the human Cx32 gene in a clonal cell line derived from Cx32 deficient mouse hepatocytes. Induction of occludin protein was also observed and was very similar to those seen in the experiments reported here (not illustrated). Furthermore, the induced endogenous occludin protein in the transfectants was found to bind to the exogenously expressed Cx32 protein. Our results indicate that gap junction and tight junction expression are closely correlated in hepatocytes, and we speculate that through this association gap junction expression may play a crucial role in the establishment of cell polarity via regulation of tight junction proteins. This finding

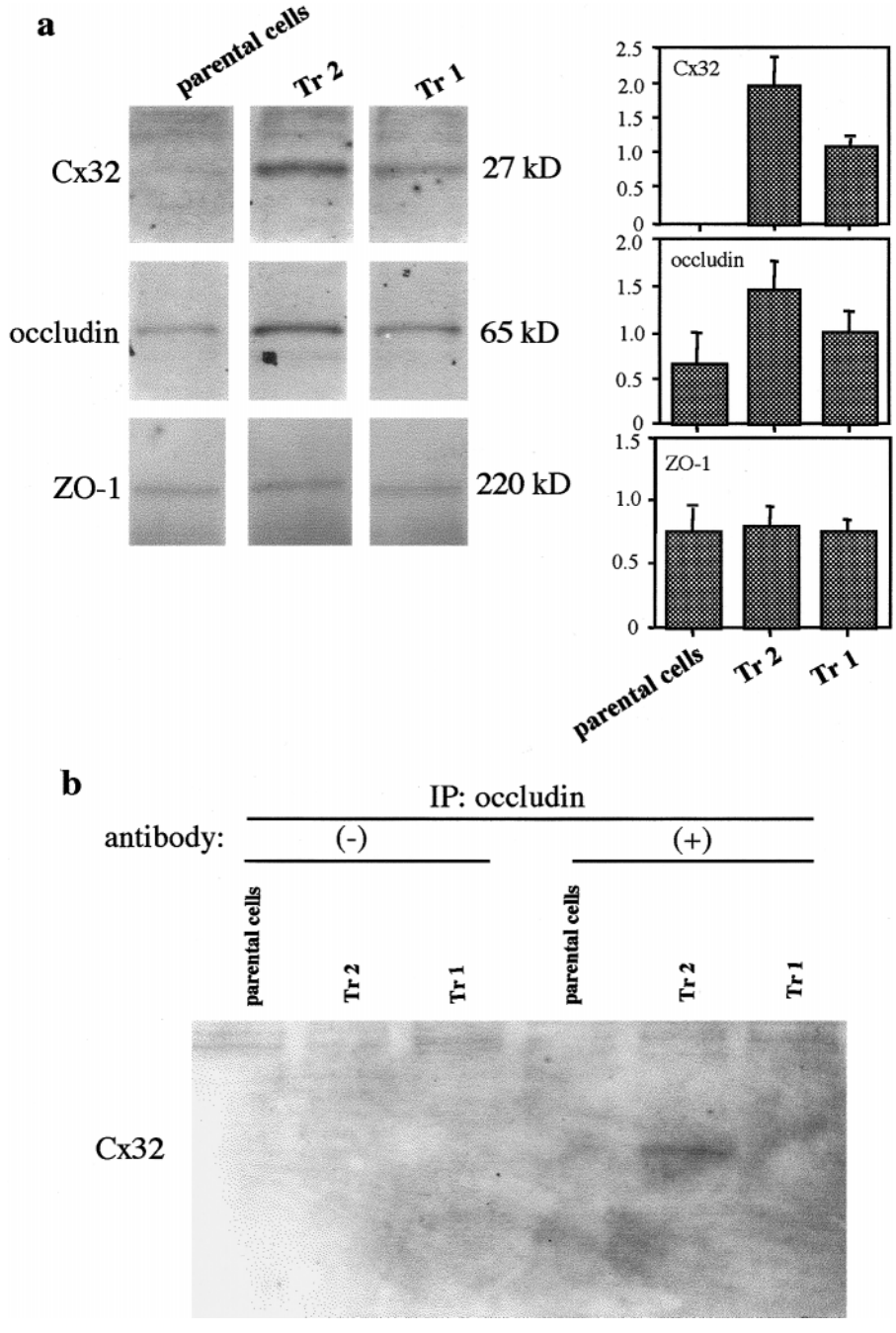

FIG. 3. (a) Western blot analyses for Cx32, occludin and ZO-1 in parental cells and in transfectants. Brackets above bars in graphs represent SEM from three separate experiments. Expression of Cx32 protein was stronger for Tr 2 compared to Tr 1, whereas it was not detected in parental cells. Expression of occludin protein was markedly higher in Tr 2 compared to either Tr 1 or parental cells. No difference in ZO-1 protein expression was observed in transfectants compared to parental cells. (b) Immunoprecipitation for occludin in parental cells and in transfectants. Immunoprecipitation using anti-occludin antibody led to the identification of Cx32 in Western blots of the immunoprecipitates obtained from Tr 2 but not in either parental cells or Tr 1.

supports previous studies in which gap junctions have been associated with other components of intercellular junctional components. More importantly, however, the present demonstration of a direct linkage between occludin and Cx32 and previous studies showing high affinity interactions between ZO-1 and Cx43 (19, 20) indicate that connexins may form selective association with specific components of adhesive or occluding junctions. Through binding of these proteins to cytoskeletal, adhesion and signaling proteins, we propose that different connexins may promote the aggregation of connexin-specific

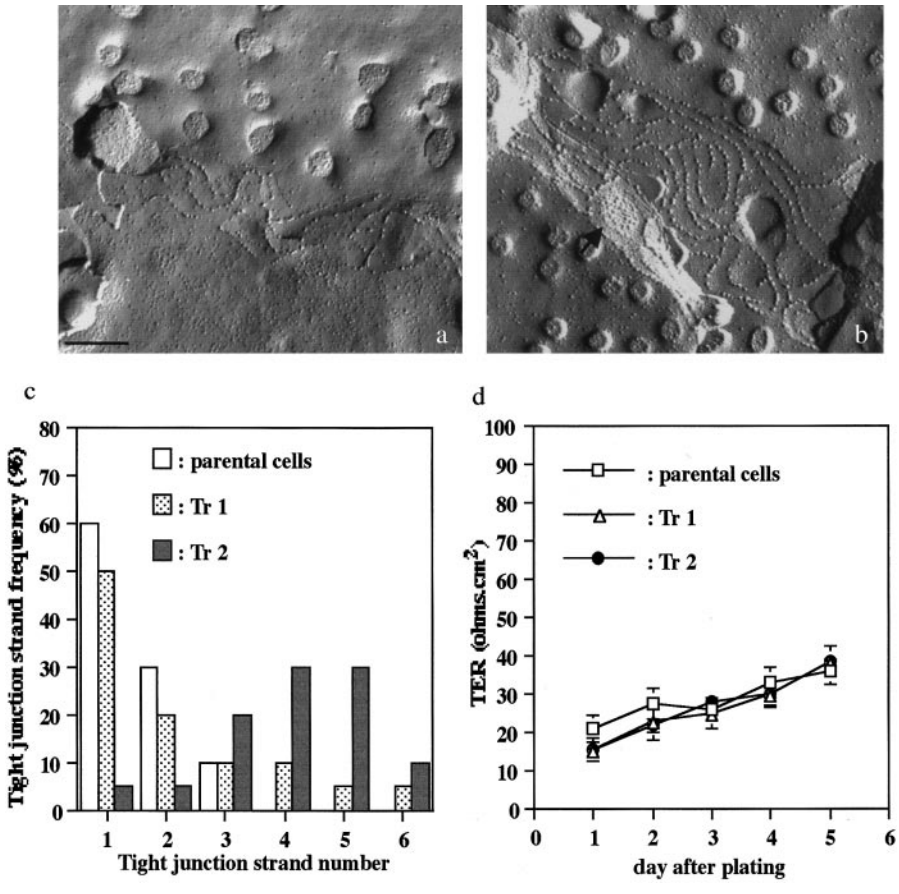

FIG. 4. Freeze-fracture replicas from parental cells (a) and from Tr 2 (b). Small gap junction plaques ($<0.05\ \mu m^2$) were observed in Tr 2 (arrow) but not in parental cells. In Tr 2, many tight junction strands were observed which formed well-developed networks, whereas in parental cells, the strands were fewer and much looser in arrangement. Bar: 100 nm. (c) The number of tight junction strands in the replicas from parental cells and transfectants were measured on coded 20 prints of electron micrographs (60,000× magnification) and were counted on the regions expressing tight junction strands at 200-nm intervals. The number of tight junction strands was markedly higher in Tr 2 compared to parental cells. (d) TER measurement in parental cells and in transfectants as a function of time in culture. TER in all cells was at a very low level (10–40 ohms per cm^2) and no significant differences were obtained comparing TERs measured from different cell types.

scaffolds at junctional regions, providing not only intercellular signaling but also sites where intracellular signaling is transduced (42, 43).

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