Comparative Analysis of the Major Polypeptides from Liver Gap Junctions and Lens Fiber Junctions

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ABSTRACT Gap junctions from rat liver and fiber junctions from bovine lens have similar septilaminar profiles when examined by thin-section electron microscopy and differ only slightly with respect to the packing of intramembrane particles in freeze-fracture images. These similarities have often led to lens fiber junctions being referred to as gap junctions. Junctions from both sources were isolated as enriched subcellular fractions and their major polypeptide components compared biochemically and immunochemically. The major liver gap junction polypeptide has an apparent molecular weight of 27,000, while a 25,000-dalton polypeptide is the major component of lens fiber junctions. The two polypeptides are not homologous when compared by partial peptide mapping in SDS. In addition, there is no detectable antigenic similarity between the two polypeptides by immunochemical criteria using antibodies to the 25,000-dalton lens fiber junction polypeptide. Thus, in spite of the ultrastructural similarities, the gap junction and the lens fiber junction are comprised of distinctly different polypeptides, suggesting that the lens fiber junction contains a unique gene product and potentially different physiological properties.

Gap junctions are plasma membrane specializations that are characterized by an apposition of the membranes of adjacent cells where the intercellular space narrows to a "gap" of 2–4 nm (1). Correlative studies have implicated this junction as the membrane structure associated with the pathway for the transmission of small molecules between cells (2, 3, 4). Gap junctions from a variety of tissues show many ultrastructural similarities, with minor variations (5, 6, 7). The possibility of structural and biochemical homology between gap junctions from different tissues is strongly supported by physiological studies, in which it has been found that most cells which are communication-competent and form gap junctions among themselves will form gap junctions and communicate with other communication-competent cell types in heterologous coculture (8, 9). Little direct information is available, however, which is relevant to the molecular basis for this apparent morphological and physiological homology.

The mammalian liver and lens contain unusually large quantities of cell junctions. The gap junction from mammalian liver is among the most extensively studied junctions and has been well characterized with regard to morphological (10–15), physiological (16), and biochemical (11, 17–19) properties. Although less well characterized, the vertebrate lens fiber cell junction also appears to function in a communication pathway (20, 21). Analysis of thin sections in the electron microscope demonstrates that the lens fiber junction has a very similar appearance to the gap junction seen in liver and other tissues (22–26), although differences between lens fiber junctions and gap junctions from other tissues are observed, especially by freeze-fracture analysis (25, 26). Indeed, the term "gap junction" has often been used to describe the lens fiber junction (23–28).

Since the liver and lens junctions can be isolated as subcellular fractions from their respective tissues, we have undertaken a direct comparison of the biochemical and immunological properties of the major polypeptides contained in these fractions. The major polypeptide associated with liver gap junctions is a polypeptide with an apparent molecular weight of 27,000 (18, 19). Lens fiber junction preparations, when analyzed on SDS polyacrylamide gels, have been reported to contain a predominant polypeptide of 26,000 daltons (26, 29–31).[1] The similarities in both junction morphology and the

[1] Although most investigators report that the major lens fiber junction polypeptide has an apparent molecular weight of 26,000, our analysis indicates a molecular weight of 25,000.

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molecular weights of the junctional proteins have led to the assumption that a biochemical homology exists between the two junctions. In fact, this possibility has provided a basis for studies of gap junction structure-function correlations by using the lens fiber junction as a model system (26, 32–35). It should be noted that while hepatocytes can readily form communicating junctions with heterologous cell types in culture (8, 36), suggesting molecular homology of gap junctions, there is no similar information available for lens fiber cells in coculture.

Here we use biochemical and immunological methods to compare the major proteins from rat liver gap junctions and bovine lens fiber junctions. A preliminary report of these observations has been published (37).

MATERIALS AND METHODS

Gap junctions from rat liver were isolated as described previously (18). Lens fiber junctions were isolated essentially by the procedure of Goodenough (26), in which membrane pellets from homogenized bovine lenses were washed with buffer to remove crystallins and loosely associated material, then treated with urea to remove peripheral membrane proteins. As reported by Goodenough (26), the subsequent detergent extraction and sucrose gradient centrifugation which yield a junction enriched fraction do not produce a major change in protein composition as observed using SDS polyacrylamide gels. Partial peptide map analysis (below) indicated that the proteins present in the urea-washed membranes, including the 25,000-dalton junction polypeptide,[1] were indistinguishable from those isolated with the junction fraction. Because of the substantial loss of material (>90%) during subsequent detergent treatments, urea-washed membranes were used for most studies.

Junctional proteins were iodinated by the chloramine T technique (38) as described previously (18), except that samples were solubilized in buffer containing 2% SDS before iodination. They were subsequently chromatographed on a Sephadex G-25 column equilibrated with buffer containing 0.1% SDS to remove reactants and free ^{125}I. A similar protocol was used to radiolabel junctions with the reagent described by Bolton and Hunter (39).

Protein A was radioiodinated to a specific activity of 50 μCi/μg by the chloramine T technique in the absence of SDS. After termination of the reaction by the addition of sodium meta-bisulfite, hemoglobin was added to 5 mg/ml and chromatography was carried out in the presence of 5 mg/ml hemoglobin.

SDS PAGE was performed as described previously (18), except that the samples were not heated during preparation (19). Partial peptide map analysis in SDS was carried out by the procedure of Cleveland et al. (40) as modified by Bordier and Crettol-Jarvinen (41). Subsequent to electrophoresis on a 12.5% gel (42) in the first dimension, gel lanes were excised, placed at right angles on a second gel, and sealed into place with stacking gel buffer (42) containing 1% agarose. The upper reservoir buffer was then added and 2.0 ml protease-containing solution (stacking buffer with 10% glycerol and a trace of bromphenol blue) was carefully layered over the agarose. Partial proteolysis of each polypeptide in the original gel takes place during stacking in the second dimension. The resolving gel (42) for this dimension was modified to contain 15% acrylamide and 8 M urea. To improve resolution of low molecular weight fragments, the ratio of acrylamide to bisacrylamide in stock solutions was doubled (43). The gel used for the second dimension was large enough to accommodate two first-dimension gel lanes side by side, thereby permitting direct comparison of two different samples.

Antibodies to the lens fiber junction 25,000-dalton polypeptide were prepared in rabbits by injection of material purified by SDS PAGE (44). Injection of either gel bands or electroeluted material proved successful in eliciting a response. Double diffusion and two-dimensional crossed immunoelectrophoresis were performed as described by Chua and Blomberg (45). The antigen was localized in SDS polyacrylamide gels after electrophoresis by the procedure of Bigelis and Burridge (46) using ^{125}I-protein A. Protein A and Sephadex G-25 were obtained from Pharmacia Fine Chemicals (Div. of Pharmacia, Inc., Piscataway, N. J.); *Staphylococcus aureus* V8 protease from Miles Laboratories Inc. (Research Products Div., Elkhart, Ind.); and α-chymotrypsin (× 3 crystallized), pronase P, and mercuripapain (crystallized suspension) from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

The morphological appearance of junctions between rat hepatocytes and bovine lens fiber cells are similar, although some differences can be detected. In thin sections, both junctions have a characteristic septilaminar appearance due to the presence of two trilaminar plasma membranes and an intervening space or "gap" of 2–4 nm (Figs. 1 *a* and *c*). This intervening space is readily detectable in liver junctions, whereas it is frequently difficult to resolve in the fiber junctions. Both junctions display similar freeze-fracture characteristics (Figs. 1 *b* and *d*), with one notable exception; in the liver junction, the intramembrane particles are closely packed, while in the fiber junction plaques the particles are more loosely organized. Both junctions contain a high density of intramembrane particles on the inner membrane half (P face), with a complementary arrangement of pits or depressions on the outer membrane half (E face). The complementary pits are usually more difficult to detect in the fiber junction since the particles are not tightly packed.

A comparison of the SDS polyacrylamide gel profiles of isolated liver and lens junction shows that the major polypeptides associated with the two preparations migrate with distinctly different M_r's. We have previously reported that the major liver junction polypeptide migrates with an apparent molecular weight of 27,000 when analyzed on a 10–15% gradient gel (18). On a 12.5% gel (Fig. 2, lane *a*) this polypeptide migrates with an apparent molecular weight of 26,000. In addition to this major polypeptide, several additional polypeptides migrating somewhat faster can be detected and are present in variable quantities from preparation to preparation. When analyzed by either gel system, the major lens fiber junction polypeptide migrated consistently faster than the liver junction polypeptide, with an apparent molecular weight of 25,000 (Fig. 2, lane *b*). The lens preparation also contains components of molecular weight 34,000 and 18,000. The lower molecular weight species is thought to be α-crystallin (24). The 34,000-dalton band obtained is variable among different lens junction preparations and has been reported to be the major junctional protein (47), a finding not in agreement with the work of others (26, 39–31), or our own observations.

Several types of analysis were used to compare the 25,000-dalton lens fiber junction polypeptide and the 27,000-dalton liver gap junction polypeptide. One set of experiments was a comparison of the polypeptides by partial peptide mapping in SDS. This procedure is particularly useful for membrane proteins which are relatively insoluble and more difficult to map by conventional techniques. After the separation of polypeptides in a SDS polyacrylamide gel in the first dimension, the gel lanes were excised, placed at right angles on top of a second SDS polyacrylamide gel, overlayered with a protease-containing solution, and then electrophoresed in the second dimension. The fragments generated by partial proteolysis of each gel band were resolved in the second dimension gel.

Figs. 3–6 are autoradiographs of experiments in which fragments generated from radioiodinated liver gap junctions and lens fiber junctions were mapped using four different proteases. The protease used in Fig. 3 was *S. aureus* V8 protease. Several fragments were generated from the 27,000-dalton liver gap junction polypeptide (Fig. 3 *a*). In contrast, the 25,000-dalton lens fiber junction polypeptide was only slightly digested (Fig. 3 *b*), as indicated by the presence of a minor, slower migrating spot, corresponding to the native polypeptide. Treatment with higher levels of *S. aureus* V8 protease did not lead to further degradation of this polypeptide (not shown), a result consistent with the report of Horwitz and Wong (48), in which fragments were detected by staining rather than by autoradiography. This proteolytic fragment of the 25,000-dalton lens fiber junction polypeptide does not have the same molecular weight as any of the fragments derived from the liver gap junction polypep-

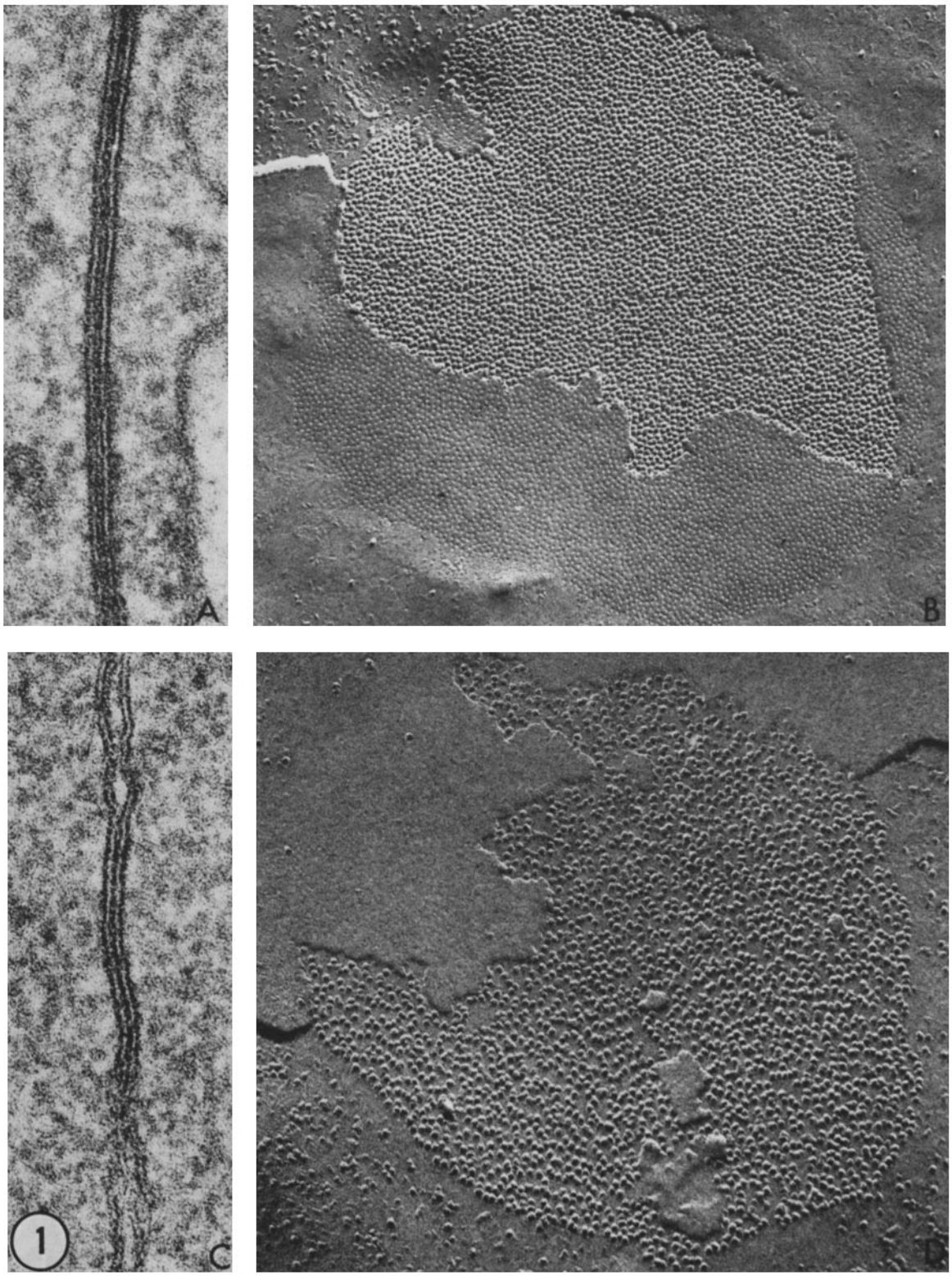

FIGURE 1 Ultrastructural features of the liver gap junction and the lens fiber junction. (*a*) Thin section of a liver gap junction. (*b*) Freeze-fractured liver gap junction containing closely packed intramembrane particles. (*c*) Thin section of a lens fiber junction. (*d*) Freeze-fractured lens fiber junction with a loose arrangement of intramembrne particles. *a* and *c*, × 200,000; and *b* and *d*, × 100,000.

tide. In a similar experiment in which chymotrypsin was the protease (Fig. 4), six fragments were generated from the liver 27,000-dalton gap junction polypeptide (Fig. 4*a*), while the four fragments generated from the lens fiber junction 25,000-dalton polypeptide (Fig. 4*b*) migrated with both distinctly different mobility (apparent molecular weight) and different intensities (reflecting different iodotyrosine content). Our observation of four chymotryptic fragments from the lens polypeptide is also consistent with the results of Horwitz and Wong (48). Analysis with papain (Fig. 5) indicated not only different

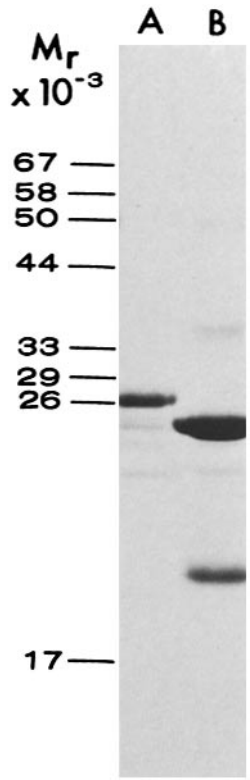

FIGURE 2 SDS polyacrylamide gel analysis of rat liver gap junctions and lens fiber junctions. Samples were prepared and analyzed on a 12.5% gel. Lane *A* contained rat liver gap junctions and lane *B* lens fiber urea-washed membranes. The molecular weight standards used were: bovine serum albumin (67,000), catalase (58,000), rabbit IgG (heavy chain) (50,000), ovalbumin (44,000), urate oxidase (33,000), carbonic anhydrase (29,000), chymotrypsinogen (26,000), and myoglobin (17,000).

partial peptide maps of the two junction polypeptides, but also a significant difference in susceptibility to digestion by this protease. The autoradiograph of the liver gap junctions (Fig. 5 *a*) required overexposure to visualize the fragments generated from the 27,000-dalton liver gap junction polypeptide and resulted in the appearance of large, overlapping spots of undigested polypeptides as a smear. Proteolysis with pronase (Fig. 6) yielded different fragments from the liver gap junction and lens fiber junction polypeptides, further demonstrating their lack of common proteolytic cleavage sites. It must be noted that in Figs. 4–6 one does observe, occasionally, spots migrating with the same apparent molecular weight for both the liver and

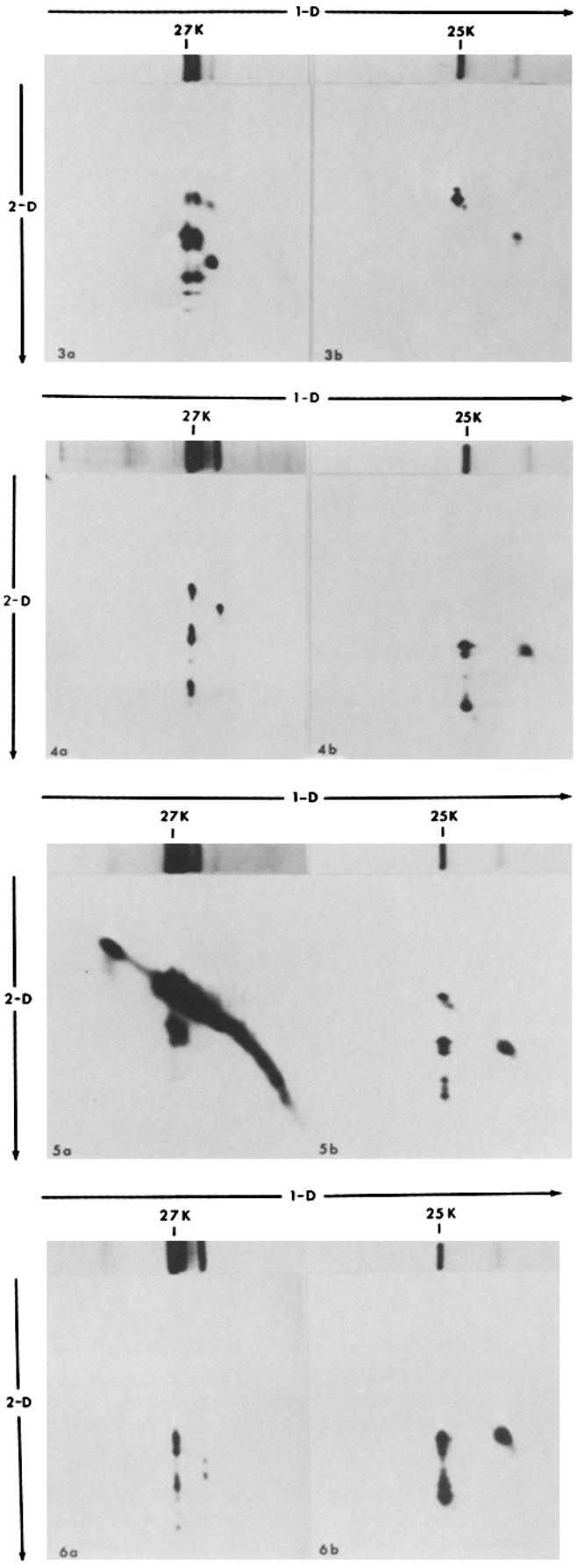

FIGURE 3–6 Partial peptide map analysis of radioiodinated rat liver gap junctions and lens fiber junctions. Junctions, radioiodinated by the chloramine T technique, were first electrophoresed on a 12.5% gel. Autoradiographs of samples run in parallel to those used for the two-dimensional analysis are shown at the top of the figures. Figs. 3 *a*, 4 *a*, 5 *a*, and 6 *a* are of rat liver gap junction samples, with the position of the 27,000-dalton polypeptide indicated. Figs. 3 *b*, 4 *b*, 5 *b*, and 6 *b* are of the lens fiber junction samples with the position of the 25,000-dalton polypeptide indicated. Directions of electrophoresis in the first dimension and second dimension (proteolysis and mapping) are indicated by arrows. 2.0 ml of protease-containing solution was used in all experiments. The proteases used were: Fig. 3, *S. aureus* V8 protease (7.5 μg/ml); Fig. 4, chymotrypsin (75 μg/ml); and Fig. 5, papain (1 μg/ml). Subsequent to electrophoresis, gels were fixed in 35% MeOH/7% HOAc, dried, and autoradiographed.

lens junction polypeptides. However, the majority of the peptide fragments generated by this technique have different mobilities for the two polypeptides, suggesting, at the least, little or no homology between the two polypeptides.

To avoid misinterpretation of the data due to limitations inherent in this method of peptide mapping, several of the experimental variables were subjected to further analysis. The extent of proteolysis was varied by altering the concentration of protease in the second dimension. In no case were fragments observed which suggested similarities between the liver and lens junction polypeptides. These experiments eliminate the possibility that the observed differences in peptide maps were a kinetics artifact. Also, since the data presented in Figs. 3–6 relied upon the presence of iodotyrosine for visualization, complementary experiments were carried out in which junctions were radiolabeled using the reagent of Bolton and Hunter (39) which results, predominantly, in acylation of ϵ-amino groups of lysine. Only rarely were spots observed which were labeled by one procedure, but not the other, and the results consistently confirmed those presented above using junctions labeled by the chloramine technique (not shown). These experiments rule out differences in maps as being due to single amino acid substitutions for tyrosine.

The mapping technique also permits examination of minor variations in the electrophoretic profile of the liver gap junction preparations. For example, the liver gap junctions used in Fig. 3 *a* contained a prominent doublet, observed in the first dimension autoradiograph (top). The map indicated that these two polypeptides are virtually identical, suggesting that some proteolysis of the material took place during preparation. However, a third, lower molecular weight polypeptide present in the liver gap junction sample is relatively resistant to proteolytic digestion, and the spots generated from it (Figs. 3 *a* and 6 *a*) are not adequate to define its relationship to the 27,000-dalton liver gap junction polypeptide.

Antibodies to the lens fiber junction 25,000-dalton polypeptide were obtained by immunizing rabbits. This antiserum (44) was used to determine the antigenic similarity of the lens fiber junction and liver gap junction polypeptides. Immunodiffusion (Fig. 7) demonstrated that only one component of lens fiber membranes, the 25,000-dalton fiber junction polypeptide, reacted with the antiserum. No cross-reaction was observed with either liver gap junctions or plasma membranes.

This antiserum was also used for indirect gel staining with radioiodinated protein A to detect antibody-binding bands in SDS gels (46). Fig. 8 *A* is the Coomassie Brillant Blue (CBB)-stained profile of an SDS gel containing plasma membranes (lane *d*) or gap junctions (lane *c*) from rat liver and buffer-washed (lane *a*) or urea-washed (lane *b*) calf lens membranes. The autoradiograph of this gel, Fig. 8 *B*, demonstrates that the antibodies raised against the 25,000-dalton lens fiber junction polypeptide are specific for this polypeptide. No binding of these antibodies to any other polypeptide in lens membranes, rat liver plasma membranes, or rat liver gap junctions was detected.

DISCUSSION

This study provides some direct evidence that rat liver gap junctions and bovine lens fiber junctions differ with respect to their biochemical, immunochemical, and some morphological properties, in spite of the apparent general morphological similarities. Specifically, we have observed: that (*a*) the two junctions are similar when examined by thin-section electron

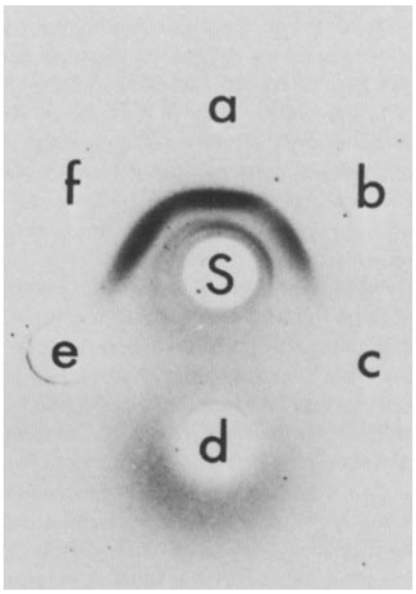

FIGURE 7 Comparative analysis of liver and lens fiber plasma membranes and junctions using antiserum to the lens fiber junction 25,000-dalton polypeptide. Immunodiffusion in the presence of Lubrol PX was carried out as described in Materials and Methods. 25 μl of antiserum was placed in the center well (S). The peripheral wells contained: (a) 7.5 μg urea-washed lens membranes; (*b*) 12.5 μg buffer washed lens membranes; (*c*) 12.5 μg purified rat liver gap junctions; (*d*) 150 μg rat liver plasma membranes; (*e*) buffer control; and (*f*) 3 μg purified, electroeluted 25,000-dalton lens fiber junction polypeptide. After incubation for 48 h at room temperature, the precipitin lines were visualized for photography by staining with CBB.

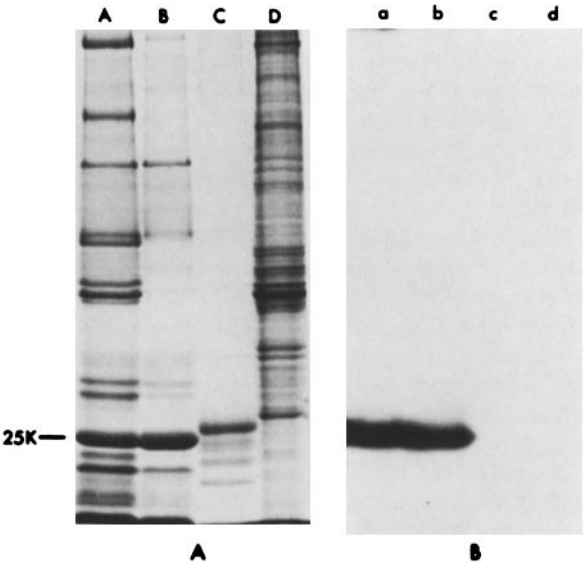

FIGURE 8 Identification of antigens in an SDS gel with antibodies to the fiber junction 25,000-dalton polypeptide. Samples were run on a 10% gel. After electrophoresis and sequential incubation with antibodies and ^{125}I-protein A, the gel was stained with CBB, dried, and autoradiographed. Panel *A* is the CBB stained profile and panel *B* is the autoradiograph. Lanes *A* and *a* contained buffer washed lens fiber membranes; lanes *B* and *b* urea washed lens fiber membranes; lanes *C* and *c* rat liver gap junctions; and lanes *D* and *d* rat liver plasma membranes. The autoradiograph demonstrates specific binding to only the 25,000-dalton lens fiber polypeptide. The position of this polypeptide is indicated as *25K*.

microscopy, but differ in freeze-fracture images with regard to the packing of intramembrane particles, as has been observed by others (25, 26); that (*b*) little or no detectable homology exists in the partial peptide maps of the lens 25,000-dalton and liver 27,000-dalton polypeptides; and that (*c*) there is no immunological cross-reactivity of antibodies to the fiber junction 25,000-dalton polypeptide with any polypeptide in rat liver junctions or plasma membranes or with any other polypeptide present in lens fiber cell membranes.

The antibodies used in this study were also used in the examination of lens and liver membranes by other techniques. By indirect immunofluorescence, the antibodies to the lens 25,000-dalton fiber junction polypeptide specifically labeled the fiber cell membranes (44) in a manner virtually identical to that reported by Broekhuyse et al. (49), but showed no binding to liver tissue upon similar analysis. Similarly, we have been able to demonstrate antibody binding to the lens fiber junction polypeptide by two-dimensional crossed immunoelectrophoresis or by indirect immunoprecipitation with goat anti-rabbit immunoglobulin or *S. aureus* A as the precipitating agent (M. Friedlander, E. Morales, E. L. Hertzberg, and N. B. Gilula, manuscript in preparation). Again, no interaction with any liver plasma membrane or gap junction polypeptide was detected by these techniques. Because these are precipitating antibodies, as demonstrated by double diffusion (Fig. 7), binding must be to at least two determinants of the fiber junction polypeptide. The possibility that other domains of these polypeptides are homologous cannot be ruled out based upon these criteria since it is possible that the antisera used does not contain antibodies to all domains of the lens fiber junction polypeptide.

Based upon the close agreement of the biochemical and immunological data, we conclude that the major polypeptide present in lens fiber junctions is distinct from that in liver gap junctions. These results do not rule out the possibility that, upon the availability of different antisera, or sequence analysis, some degree of homology might be detected (50).

Our inability to detect homology is striking in light of the morphological similarities described here and by others (22–28) for these two types of junctions and the similar physiological role that they are thought to provide in their respective tissues (20, 21, 26, 32–35). Although many explanations might account for these observations, it is interesting to raise the following possibilities: (*a*) during the course of evolution, two entirely different proteins have evolved that may form similar structures with comparable physiological properties; and (*b*) the lens fiber junction, which has not been as well characterized as that from liver, may not be of the conventional communicating type. Although there is ample evidence for electrical coupling between lens fiber cells (20), only recently has the more detailed characterization of the permeability properties of this low resistance pathway been undertaken (21). If these junctions are not of the conventional communicating type, other roles might include providing mechanical stability during the change in lens shape during visual accommodation (20) or in the maintenance of the narrow intercellular space necessary for the efficient transmission of light (22).

One aspect of the studies on cell-cell communication has been the attempt to correlate a loss of communication (uncoupling) with a corresponding alteration of junction morphology (26, 32–35). In one study, involving a crayfish electrotonic synapse, a loss of coupling correlated with a tighter and more regular packing of junction particles as demonstrated by freeze-fracture analysis (51). Attempts have been made to extend these observations to other systems, including lens, by examining only the ultrastructural properties (26, 32–35). Our results indicate that extreme caution should be exercised in attempting to extrapolate from studies of communication regulation in different systems since different proteins and different regulatory mechanisms might exist.

This study does not contribute directly to the general issue of molecular homology of communicating junctions. However, the data demonstrate that the fiber junction represents a unique gene product different from the conventional gap junction polypeptide. Biochemical criteria similar to those described here have been used to demonstrate that molecular homology does exist between the major gap junction protein of rat liver, mouse liver, bovine liver, and rat ovary (E. L. Hertzberg and N. B. Gilula, unpublished observations). This biochemical homology correlates well with the observation that cells from these different tissues can readily establish cell-cell communication in heterologous cell cultures (8, 9, 52). At present, similar studies have not been carried out using lens fiber cells in cocultures with hepatocytes or other communicating cell types. One prediction from this study is that the lens fiber cells would fail to establish gap junctional communication with the other cell types. Another prediction is that an antibody to the liver gap junction polypeptide, when available, will not cross-react with the lens fiber junction polypeptide. Such results, when taken with the results here, would suggest that the lens fiber junction is a unique junction with properties and functions that are appropriate for the lens.

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