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Gap Junctions from the Lens: Purification and Characterization by Chemical Crosslinking Reagent

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SUMMARY: Quantitation of gap junction preparations from chick lens by transmission electron microscopy has indicated that 95.0% of the membrane bilayer material is in the form trilayer structures. The preparations were comprised of a major polypeptide component of 26K, as well as minor components of 49K, 46K and 22K-15K. Treatment with oxidizing agent resulted in the production of apparent homo-oligomeric complexes involving the 26K and 46K components. These results demonstrate that the 26K polypeptide is the major component of highly purified preparations of lens gap junctions. Furthermore, they demonstrate that this 26K component plus an additional 46K component are both involved in extensive nearest-neighbor interactions in the intact junctional complex.

INTRODUCTION

Gap junctions have been implicated as the structural entities involved in the direct passage of components between cells (1). As such, they may play a necessary role in intercellular communication in numerous cell types, (1-3). Analyses of enriched gap junction preparations by SDS-PAGE have indicated the presence of major polypeptide components of approximately 26K & 47K in the liver (4,5), 26K & 34K in the lens (6,7), and 31K, 33.5K & 38K in the heart (8). Definitive identification of these polypeptides as authentic gap junction components is still not firmly established, especially when consideration is made of the varying purity of the junction preparations under study.

X-ray analyses of preparations from the mouse liver have demonstrated an extensively ordered array of components that comprise the gap junction, and

ABBREVIATIONS

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetracetic acid; PMSF, phenylmethylsulfonylfluoride; Na-P buffer, 0.01 M sodium phosphate, pH 7.4; Cu-P, 5mM $CuSO_4$ + 15mM 1,10-phenanthroline; sample buffer, 1% (w/v) sucrose plus 4% (w/v) sodium dodecyl sulfate plus 0.063M Tris·HCl, pH 6.8.

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have suggested a hexameric form of the major gap junction polypeptide. In view of these results, additional evidence of a biochemical nature would be helpful in ascertaining the identity and organization of gap junction components. This report demonstrates that the 26K polypeptide is the major component of the lens gap junctions, and provides biochemical evidence suggesting extensive nearest-neighbor interactions of 26K with itself in the intact junctional complex.

MATERIALS & METHODS

Preparation of lens membrane: Decapsulated lenses from 1.0-1.5 year old white leghorn chickens were homogenized in ten times volume of 1 mM EDTA, 1 mM PMSF, 5 mM Tris·HCl, 7.9. After pelleting at 27,000 x g for 10 min, the homogenization and pelleting procedures were repeated five more times. The final pellet was resuspended in Na-P buffer to a final concentration of 5 mg/ml, then layered on a discontinuous gradient containing 0.5 ml of 45% (w/w) sucrose, 1.5 ml of 35% (w/w) sucrose and 1.5 ml of 30% (w/w) sucrose. After spinning for 1 hr at 125,000 x g, the membrane material at the 30%/35% interface was collected and washed twice with Na-P buffer. Protein was determined using the Coomassie Blue assay (9). From 15 g wet weight of lens, approximately 800 ug of purified lens membrane was obtained.

Preparation of purified gap junctions: Approximately 800 ug of lens membrane was resuspended with vigorous vortexing in 10.0 ml of Na-P buffer containing 0.1% (w/v) of Sarkosyl NL-97 detergent (ICN). Following incubation at room temperature for 20 min, the suspension was pelleted at 27,000 x g for 20 min. The pellet was resuspended in 5.0 ml of Na-P buffer and pelleted at 27,000 x g for 10 min. This washing procedure was repeated two more times. From 800 ug of lens membrane, approximately 50-100 ug of purified gap junction was obtained.

Electron microscopy: Samples of gap junctions were centrifuged in 500 µl microfuge tubes for 5 min in a Beckman Microfuge B. The resulting pellet was repeatedly resuspended and respun sequentially in solutions of glutaraldehyde, osmium, tannic acid, and uranyl acetate as previously described (10). The final pellet was dehydrated in ethyl alcohol and embedded in Spurr's low-viscosity embedding media. Thin sections of the embedded pellet were obtained with glass knives on a Reichart OM-2 ultramicrotome. These were mounted on 300 mesh grids and poststained with uranyl acetate and lead citrate. The grids were then viewed under a Phillips 201 electron microscope with an accelerating voltage of 60 KV. Pictures were taken of areas representative of each sample after viewing twenty to thirty different thin sections on each grid. Two to three pictures were taken of each sample at a magnification of 28,000 x. The resulting negatives were printed and enlarged on 8 x 10 inch photographic paper to give a final magnification of 83,000 x. All of the bilayers and trilayers were measured from these prints using a flexible wire whenever necessary. At least 20 bilayer or trilayer segments from each print were measured in this manner. The percentages of gap junctions were computed using the formula described in Table I.

Crosslinking of gap junctions: A suspension of purified gap junctions was adjusted to a concentration of 1 mg/ml using Na-P buffer. To this suspension

Table 1

Purity of Gap Junction Preparations

Preparation	Section	Length trilayers/ Length of bilayers	Percent*	Average of Each Preparation
1	A	679/6	99.6	99.5
	B	646/9	99.3	
2	A	433/6	99.3	
	B	439/26	97.1	96.5
	C	647/98	93.0	
3	A	567/34	97.1	
	B	491/125	88.7	92.1
	C	264/55	90.6	
4	A	370/15	98.0	
	B	386/84	90.2	91.8
	C	208/61	87.2	
			Average	95.0

Table 1: Representative sections from 4 different preparations were quantitated as described in Materials and Methods.

* Precent = $\frac{\text{Length of trilayers} \times 2}{\text{Length of trilayers} \times 2 + \text{Length of bilayers}} \times 100$

was added a stock solution of Cu-P (11) to achieve a final concentration of 0.17 mM $CuSO_4$ and 0.5 mM 1,10-phenantholine. Following incubation at room temperature for 20 min, the crosslinking reaction was terminated by addition of an equal volume of solution containing sample buffer plus 50 mM N-ethyl-maleimide.

Gel Electrophoresis: Approximately 100 ug of protein was dissolved in sample buffer containing 2% (v/v) of 2-mercaptoethanol, then resolved on 10% polyacrylamide gels according to Laemmli (12). Human erythrocyte membrane proteins were used as molecular weight markers (13). For diagonal electrophoresis, 100 ug of protein was dissolved in sample buffer without 2-mercaptoethanol and resolved using 6% polyacrylamide gels in both dimensions as described by Takemoto et al. (14). After destaining, components with a molecular weight of 46,000 or 26,000 in the second dimension were scanned using a Joyce-Loebl recording microdensitometer (Model MK III C). The molecular weights in the second dimension were determined by mixing the gap junction preparations with a small amount (less than 1 ug) of radioiodinated bovine serum albumin and human erthrocyte membrane proteins. Following staining and destaining of the second dimension gels, they were dried and the location of the molecular weight markers were determined by autoradiography.

RESULTS

Figure 1 illustrates the morphology of lens membrane treated with 0.1% Sarkosyl detergent. Present are the trilayer structures characteristic of gap

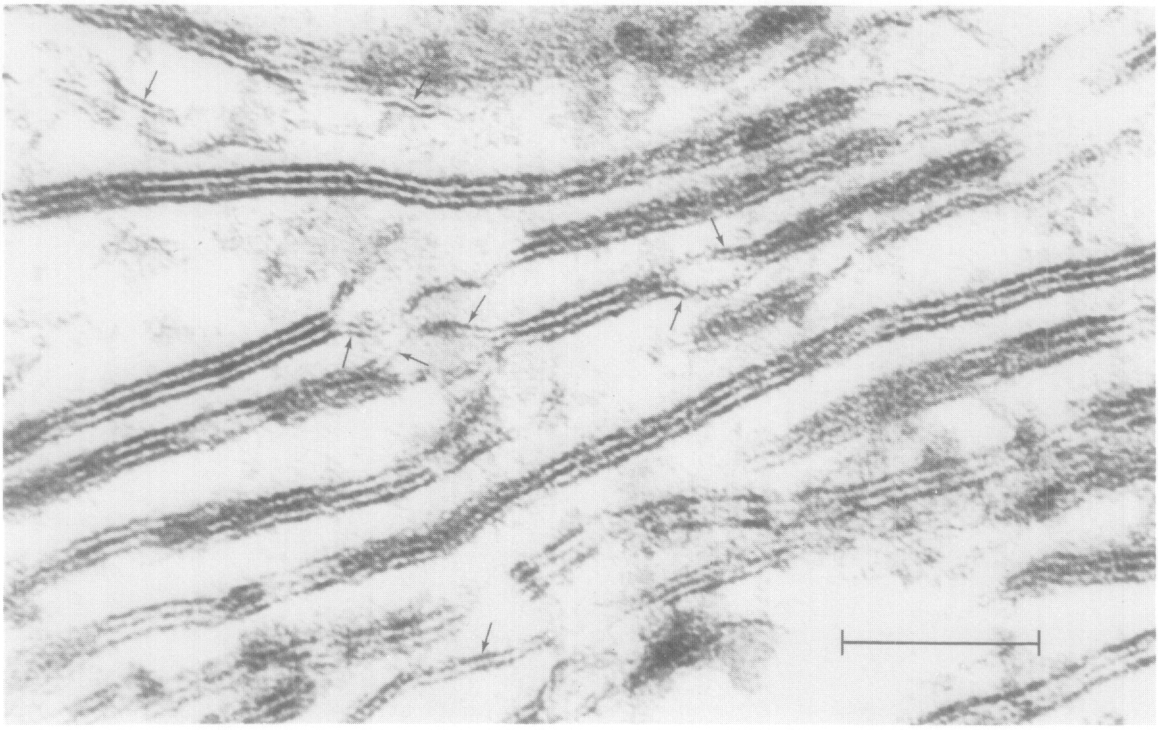

Figure 1: Transmission electron microscopy of purified gap junctions. See Materials and Methods for details of sample preparation. Magnification was 400,000 x. The bar represents 0.1 micron. Arrows designate examples of bilayer structures.

junctions from the lens and liver (7,15). This trilayer material was present as segments with widths of approximately 150 Angstroms and varying lengths of approximately 2,000 - 6,000 Angstroms. Occasionally, bilayer material was also observed (see arrows, Figure 1).

In order to obtain a more accurate estimate of the percentage of trilayer structures, 2-3 representative sections from 4 different gap junction preparations were assessed for the percentage of trilayer material in total membrane. The results of this quantitation are shown in Table 1. Each of these preparations contained over 90% of the visualized membrane in the form of trilayer structures.

The polypeptide composition of a purified gap junction preparation is shown in Figure 2. In agreement with previous reports (16,17), the whole lens

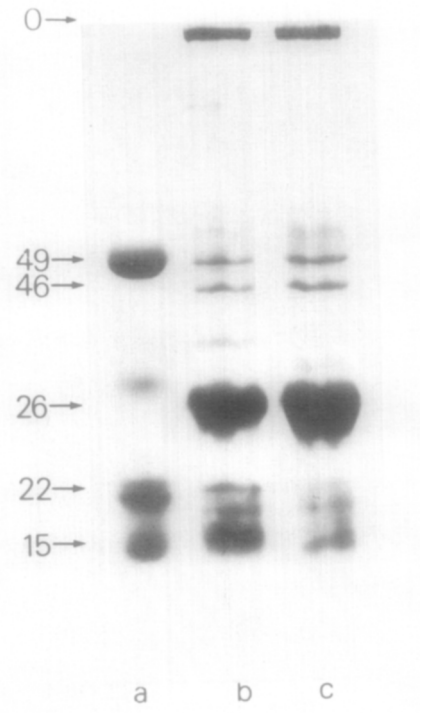

Figure 2: SDS-PAGE of lens preparations during purification of gap junctions. Approximately 100 ug of protein was resolved using 10% polyacrylamide (12), followed by staining with Coomassie Brilliant Blue R. Molecular weights ($x10^3$) are designated by arrows. a, lens homogenate; b, purified lens membrane; c, purified gap junctions.

contains a major component of approximately 49K (delta crystallin), as well as lower molecular weight beta and alpha crystallins of approximately 15K-22K (Figure 2a). The purified lens membrane contains a major component of 26K, plus significant amounts of beta and alpha crystallins of 15K-22K (Figure 2b). In this same preparation are present two components (49K & 46K) which are highly related in amino acid sequence to the major 26K component (Takemoto and Hansen, in press). These same 49K, 46K and 26K components, along with reduced amounts of crystallin components, are present in purified gap junctions (Figure 2c).

Previous X-ray diffraction analyses of gap junctions from the mouse liver have suggested extensive protein-protein interactions of the major gap junction components. To ascertain the presence of nearest-neighbor interactions in the

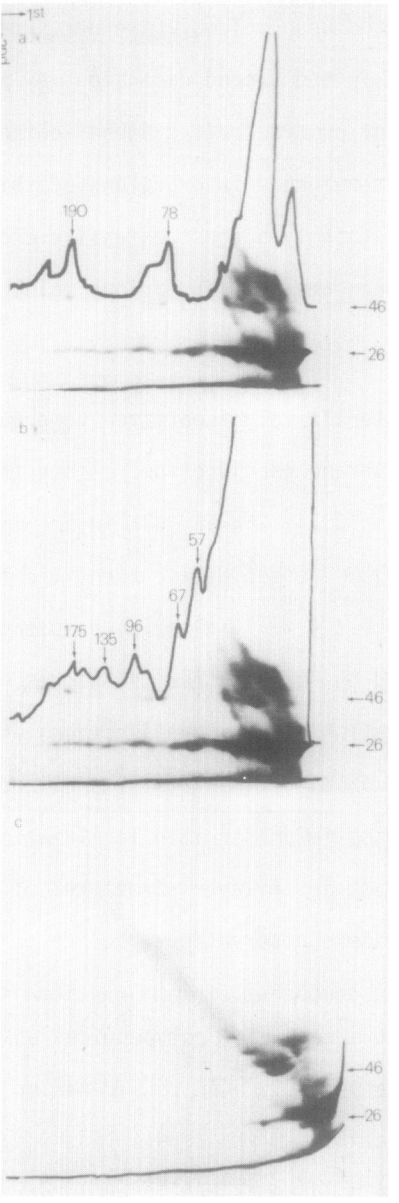

Figure 3: Two-dimensional, diagonal electrophoresis of gap junction preparations with and without treatment with the Cu-P crosslinking reagent. a, treatment of gap junctions with Cu-P, scan of components with a second dimension molecular weight of 46,000; b, treatment of gap junctions with Cu-P, scan of components with a second dimension molecular weight of 26,000; c, gap junctions without Cu-P treatment.

lens gap junction, purified preparations were treated with the Cu-P oxidizing reagent used by others (11,18,19) to induce intermolecular disulfide crosslinking. Figure 3a demonstrates the presence of extensive crosslinking involving the

26K and 46K components. Based upon molecular weight, the component of 78K in the first dimension and 46K in the second dimension may be a dimer of the 46K polypeptide. The presence of numerous off-diagonal components of the 26K polypeptide indicate nearest-neighbor interactions of this polypeptide in gap junctions (Figure 3b), and strongly suggest extensive crosslinking of 26K into apparent homo-oligomeric complexes of various molecular weights.

DISCUSSION

Previous reports have tentatively identified numerous polypeptides as components of the plasma membrane gap junction. Among these have been 26K and 34K polypeptides in the lens (6,7), 26K and 47K in the liver (4,5), and 31K, 33.5K & 38K in the heart (8). The definitive identification of all or some of these components would be greatly facilitated by preparation of gap junctions whose purity was ascertained by quantitative techniques. In this regard, this report describes the preparation of lens gap junctions with trilayer structures that comprise an average of 95.0% of the total visualized membrane. Recently, Bok and Horwitz (20) have used immunoferritin localization to demonstrate that the 26K polypeptide is present in trilayer structures of the bovine fiber lens membrane. Taken together, these observations provide definitive evidence that 26K is the major polypeptide component of gap junctions from the lens membrane.

The identification of the remaining polypeptides as components of the gap junction is still unclear, especially when consideration is made of the presence of significant amounts of beta and alpha crystallins in purified gap junctions from the chick. Since these components are found in very large excess in the initial lens homogenate, they could possibily originate from adsorption to the bilayer during homogenation. Alternatively, small amounts of soluble crystallins could be trapped in vesicles during the preparation procedure.

In contrast, the 49K and 46K components are not related to delta-crystallin and are highly enriched in membrane and gap junction preparations (Takemoto and Hansen, in press). Together with its sequence homology to 26K, the 46K component is involved in extensive nearest-neightbor interactions as assessed

by treatment with Cu-P crosslinking reagent. The nature of these protein-protein interactions must be very close, since Cu-P catalyzes the oxidation of neighboring sulhydryl groups. Furthermore, these interactions were not due to protein lateral diffusion, since Cu-P treatment at 1-2 degrees produced the same oligomers (results not shown). It is rather more likely that these crosslinked oligomers represent stable interactions of protein, perhaps in homo-oligomeric structures such as the "connexons" hypothesized to exist in gap junctions from the mouse liver (21).

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