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Biochemical and Genetic Investigations on Gap Junctions from Mammalian Cells*

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Abstract. Gap junction protein (26K) in mouse or rat liver has been studied using a rabbit antiserum directed against the sodium dodecylsulfate denatured 26K protein from mouse liver. The liver 26K protein has been localized in gap junction plaques of hepatic plasma membranes by immuno electron microscopy. Affinity purified anti-26K antiserum showed weak cross reactivity with mouse or bovine lens gap junction protein (MIP26). This result suggests some structural homology between the different gap junction proteins in liver and lens. After partial hepatectomy of young rats the liver 26K protein appears to be degraded and later resynthesized. A variant of established Chinese hamster fibroblastoid cells has been isolated and shown to be defective in metabolic cooperation via gap junctions.

Key words: Gap junction – Protein – Antisera – Immunoblot – Immuno electron microscopy – Liver – Metabolic cooperation

The cells of most mammalian tissues are electrically and ionically coupled, i.e., ions and metabolites of a molecular weight < 900 Daltons can diffuse directly from one cell into the next without being diluted into the surrounding medium (Loewenstein 1981). The membrane structures which most likely mediate this mutual exchange of small molecular weight compounds between cells are called gap junctions. They are composed of arrays of intramembranous particles representing hemichannels in the plasma membranes of apposing cells. The hemichannels in one membrane bind to the hemichannels in the apposing membrane and form connexons, i.e., structures of polygonal symmetry through which hydrophilic channels can be opened between neighboring cells. Unwin and Zampighi (1980) studied purified rat liver gap junctions by electron microscopy using Fourier and image reconstruction techniques. The authors

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concluded from their results that a connexon is a cylinder of six subunits tilted around its axis. Gap junction plaques (connexons) can be purified from liver plasma membranes by differential centrifugation in the presence of urea, Triton X-100 and Na-deoxycholate. Under these conditions, extrajunctional membranes are dissolved, whereas connexons adhere to each other. Some controversy exists concerning the molecular weight of the protein(s) which is (are) the structural component(s) of liver connexons. Henderson et al. (1979) described two major proteins of 26,000 Daltons (26K) and 21,000 Daltons (21K) apparent molecular weight as constituents of purified *mouse* liver gap junction plaques after polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS). The authors suggested the 21K protein to be a degradation product of the 26K protein. Finbow et al. (1980) characterized the 26K to 28K protein from purified gap junction plaques of *rat* liver. These preparations contained very little 21K protein compared to purified mouse liver plaques. Recently the N-terminal 52 amino acids of the rat liver 28K protein were sequenced (Nicholson et al. 1981).

Since gap junctions consist of structural proteins which presumably lack any enzyme activity, specific antibodies are an indispensable experimental tool for analysis of the biosynthesis, regulation of expression, and the ultrastructural localization of gap junction proteins. Furthermore, antibodies directed against the cell surface part of the gap junction proteins presumably block the "docking" of the two hemichannels and may thus specifically inhibit gap junction function. We therefore decided to prepare antisera against the 26K and 21K protein component from purified mouse liver gap junctions. Both proteins were eluted from SDS-polyacrylamide gels and used for immunization of rabbits and rats. In addition, antisera against urea/detergent purified gap junction plaques were prepared. We studied the specificity of our antisera with the immunoblot technique: the proteins were separated on SDS-polyacrylamide gels blotted onto nitrocellulose paper, and incubated with antisera and ^{125}I-protein A. To our surprise, no immunological cross reactivity between the 26K and the 21K protein could be demonstrated. Each antiserum recognized only the 26K or the 21K protein, respectively, even when tested with the proteins of total liver plasma membranes after SDS-polyacrylamide gel electrophoresis (Traub et al. 1982a). In the enzyme immunoassay, the anti-26K antiserum reacted much better with purified gap junction plaques than the anti-21K antiserum. The simplest explanation for our results is that the 26K and 21K components are independent of each other, the 26K protein being most likely the major constituent of gap junction plaques. This explanation was recently confirmed by an immuno electron microscopic study: Urea/detergent purified gap junction plaques or native liver plasma membranes were adsorbed to electron microscopic grids or thin-sectioned after incubation with rabbit anti-26K antiserum and protein A complexed with colloidal gold (5 μm) (Janßen-Timmen et al. 1982). We found that the anti-26K antiserum reacted with the cytoplasmic face of purified plaques 30-fold more frequently and with native plaques in plasma membranes 2-fold more frequently than preimmune serum did. The rabbit anti-26K antiserum appeared neither to recognize the former cell surface part of gap junction hemichannels nor did it react with the cytoplasmic face of hemichannel sheets

derived from split gap junction plaques. This is consistent with the notion that under the latter conditions the 26K protein has changed its conformation or has been protected against reaction with the anti-26K antibodies. Furthermore, the anti-26K antiserum did not recognize the 26K protein in extrajunctional areas of liver plasma membranes. Thus at present it appears that the anti-26K antibodies recognize only the cytoplasmic side of connexons in the gap junction plaques configuration, in addition to their reactivity with the SDS denatured 26K protein. This result may be of particular importance for studying the biosynthetic steps of gap junction plaque assembly.

Another question of gap junction research which can be tackled by the use of specific antisera concerns the relationship between gap junction proteins from different mammalian tissues. As a first example we have investigated the reactivity of the gap junction protein (MIP26) from bovine or mouse lens fiber cells with rabbit anti-liver 26K antiserum (Willecke et al. 1982a). In order to exclude unspecific reactions we used anti-26K antibodies which had been affinity-purified according to Olmsted (1981). These affinity-purified antibodies reacted weakly with MIP26 from lens fiber tissue when tested with the immunoblot procedure. In a control experiment affinity-purified rabbit anti-vinculin antibodies as well as affinity-purified rabbit anti-collagen type I antibodies did not react with MIP26, thus excluding a general binding affinity of MIP26 for rabbit IgG molecules. We conclude from our results that liver 26K protein and lens fiber MIP26 protein are widely different proteins and therefore must be coded for by different genes. The weak cross reactivity shown by the use of anti-26K antiserum tentatively suggests, however, that there may be regions of homology between the liver and lens gap junction proteins which may not be detected by analysis of proteolytic degradation peptides (Hertzberg et al. 1982; Hertzberg and Gilula 1982). Rabbit anti-bovine lens MIP26 antiserum did not react with the liver 26K protein (Hertzberg et al. 1982; Willecke et al. 1982a). Further studies are needed to demonstrate whether or not the concept of a multigene family for gap junction proteins with tissue specific expression can be substantiated. Probably only the comparison of the complete amino acid sequences of liver and lens fiber gap junction protein will permit an unequivocal decision as to what extent these proteins are homologous to each other.

An alternative approach to the use of antibodies for studying the structure and function of gap junctions is the characterization of cell mutants defective in gap junction function, i.e., metabolic cooperation. We have recently completed the characterization of a Chinese hamster fibroblastoid cell line which we selected in several consecutive cycles for being defective in metabolic cooperation (i.e., mec^-) (Willecke et al. 1982b). By use of the survival cloning assay (Slack et al. 1978), fluorescent dye injection, electrical as well as autoradiographical studies, and electron microscopic localization, this cell variant appeared to be defective in cell-cell coupling. The genetic defect could be partially complemented in Chinese hamster × mouse somatic cell hybrids by the presence of mouse chromosome 16 (Willecke et al.1982b). These selected and other spontaneous mec^- cell variants (Hooper and Subak-Sharpe 1980) may be very useful for functional reconstitution of gap junctions, for example by fusion with purified gap junction proteins integrated in liposomes. Furthermore, these

variants may help to dissect experimentally the contribution of different cellular proteins (for example cell-cell adhesion proteins etc.) to the phenotype of metabolic cooperation.

Finally we have recently shown that the rabbit anti-26K antiserum can be used to quantitate the 26K protein in regenerating livers after partial hepatectomy or cholestasis due to bile duct ligation and recanalization. (Traub et al. 1982b). It had been shown previously (Yee and Revel 1978, Yancey et al. 1979; Meyer et al. 1981; Metz and Bressler 1979; Metz 1982) that rat liver gap junction plaques dissappeared completely after partial hepatectomy or cholestasis, respectively, and reappeared later in regenerating liver. These morphometric results could be explained by dispersal of gap junction protein subunits in liver plasma membranes. Alternatively the gap junction protein may be degraded and resynthesized under these pathophysiological conditions. Using the quantitative immunoblot of liver 26K protein with anti-26K antiserum, we demonstrated that the 26K protein disappeared from hepatic plasma membranes to a large extent after partial hepatectomy or cholestasis, respectively (Traub et al. 1982b). At these times, no corresponding increase of the amount of the 26K protein was found in crude lysosomal membranes isolated from the same livers. We conclude that the liver 26K protein must be degraded and later resynthesized under these conditions. The molecular mechanisms underlying the degradation of the 26K protein after partial hepatectomy or cholestasis may be quite different from each other. The biosynthesis of the liver 26K protein may be dependent on the G_1 phase of the cell cycle of hepatocytes. Alternatively the biosynthesis or the "docking" of the gap junction hemichannels on apposing cellular membranes may be inhibited by the increased concentration of bile acids after cholestasis. Other explanations are equally conceivable. The use of anti-26K antibodies should allow clarification of these hypothetical mechanisms. Interestingly, no change in the concentration of the 21K protein was noticed in regenerating liver under conditions where the amount of the 26K protein decreased and increased again. This result confirms our previous conclusion (Traub et al. 1982a) that the 26K and the 21K protein are independent of each other. Possibly the 21K protein is a contaminant of purified mouse liver gap junction plaques.

The prospects after 2 years of research with anti-liver gap junction antisera are encouraging. It appears that many of the problems concerning the biosynthesis, structure, and function of gap junctions can be tackled and possibly solved in the future with these anti-gap junction antibodies. Probably different kinds of anti-gap junction antibodies which recognize the cell surface part of the 26K molecule are required for inhibition of gap junction function.

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