Comparative Characterization of the 21-kD and 26-kD Gap Junction Proteins in Murine Liver and Cultured Hepatocytes

Otto Traub, Jutta Look, Rolf Dermietzel,* Franz Brümmer,‡ Dieter Hülser,‡ and Klaus Willecke

Institut für Genetik, Abteilung für Molekulargenetik, Universität Bonn, 5300 Bonn 1; *Institut für Anatomie, Universitätsklinikum, 4300 Essen 1; and ‡Biologisches Institut, Abteilung Biophysik, Universität Stuttgart, 7000 Stuttgart 80, Federal Republic of Germany

Abstract. Affinity-purified antibodies to mouse liver 26- and 21-kD gap junction proteins have been used to characterize gap junctions in liver and cultured hepatocytes. Both proteins are colocalized in the same gap junction plaques as shown by double immunofluorescence and immunoelectron microscopy. In the lobules of rat liver, the 21-kD immunoreactivity is detected as a gradient of fluorescent spots on apposing plasma membranes, the maximum being in the periportal zone and a faint reaction in the perivenous zone. In contrast, the 26-kD immunoreactivity is evenly distributed in fluorescent spots on apposing plasma membranes throughout the rat liver lobule. Immunoreactive sites with anti–21 kD shown by immunofluorescence are also present in exocrine pancreas, proximal tubules of the kidney, and the epithelium of small intestine. The 21-kD immunoreactivity was not found in thin sections of myocardium and adult brain cortex. Subsequent to partial rat hepatectomy, both the 26- and 21-kD proteins first decrease and after ∼2 d increase again. By comparison of the 26- and 21-kD immunoreactivity in cultured embryonic mouse hepatocytes, we found (*a*) the same pattern of immunoreactivity on apposing plasma membranes and colocalization within the same plaque, (*b*) a similar decrease after 1 d and subsequent increase after 3 d of both proteins, (*c*) cAMP-dependent in vitro phosphorylation of the 26-kD but not of the 21-kD protein, and (*d*) complete inhibition of intercellular transfer of Lucifer Yellow in all hepatocytes microinjected with anti–26 kD and, in most cases, partial inhibition of dye transfer after injection of anti–21 kD. Our results indicate that both the 26-kD and the 21-kD proteins are functional gap junction proteins.

GAP junctions are defined as cell-to-cell channels that are visible in the electron microscope as plaque structures and can be isolated by differential centrifugation due to their resistance towards nonionic detergents. Gap junction plaques consist of aggregated hexameric structural units which are interpreted to be built out of channel-forming proteins (reviewed in Bennett and Spray, 1985; Loewenstein, 1981, 1987). When purified gap junction plaques from mouse or rat liver are dissolved in SDS and separated by PAGE, two proteins of 26 kD (also described as 27 or 28 kD in different laboratories)[1] and of 21 kD are found in addition to protein bands of 45–50 kD which appear to consist of aggregated 26- and 21-kD proteins (Henderson et al., 1979; Hertzberg and Gilula, 1979; Finbow et al., 1980). A protein of 16 kD which does not appear to be related to the 26-kD protein was found as the main constituent when gap junctions were isolated from mouse liver after treatment with 1% Triton X-100 and trypsin (Finbow et al., 1983). The 21-kD protein had been considered to be a degradation product of 26 kD (Henderson et al., 1979) or an independent membrane protein (Traub et al., 1982). Affinity-purified 21-kD antibodies had not been available for further characterization at that time. Recently, it was discovered that the NH_2-terminal amino acid sequences of the 28- and 21-kD gap junction proteins from rat and mouse liver show ∼48% homology and that both proteins are probably located in the same gap junction plaques in liver sections (Nicholson et al., 1987). Here we report on a comparative analysis of both proteins using affinity-purified antibodies for immunoblot, immunoprecipitation, immunofluorescence, and immunoelectron microscopy. Since dye coupling through gap junctions is blocked in cultured hepatocytes after microinjection of affinity-purified anti–21 kD or anti–26 kD, we conclude that both proteins are functional components of gap junction channels in mouse liver.

Materials and Methods

Isolation of Embryonic Hepatocytes and Conditions of Cell Culture

Mouse hepatocytes were isolated from 18-d-old embryos (BALB/c) and cultured in serum-free MX83 medium as described (Traub et al., 1987). For immunoblots and immunoprecipitations the cells were plated at 4.6 × 10^6 cells per 60-mm dish and for microinjections at 1.7 × 10^6 cells per 35-mm

---

[1] During the International Conference on Gap Junctions in Asilomar in July 1987, it was suggested that the 26-kD gap junction protein should now be referred to as connexin32, whereby "connexin" designates this family of sequence-related gap junction proteins and "32" is an abbreviation of the theoretical molecular mass (32,007 Daltons) of the rat liver protein, deduced from the corresponding cDNA (Paul, 1986; Beyer et al., 1987).

© The Rockefeller University Press, 0021-9525/89/03/1039/13 $2.00
The Journal of Cell Biology, Volume 108, March 1989 1039–1051

dish. Cells grew to confluency within 40 h after attachment and continued to grow through a few more rounds of division.

Labeling of Gap Junction Protein with Radioisotopes

Pulse-chase experiments were carried out as described (Traub et al., 1987). For labeling, the hepatocytes were incubated with [^{35}S]methionine (100 μCi/ml, sp act 800 Ci/mmol, 30 TBq/mmol; Amersham International, Amersham, UK) in methionine-free MX83 medium for 1 h. In vitro labeling of isolated gap junction proteins with [^{32}P]ATP was performed using the catalytic subunit of cAMP-dependent protein kinase (Walter et al., 1979).

Affinity Purification of Rabbit 21-kD Antiserum

Rabbit antiserum to SDS-denatured mouse liver 21-kD gap junction protein was raised as described and shown not to cross-react with the 26-kD protein under immunoblot conditions (Traub et al., 1982). For preparation of the affinity column, 1 mg of purified mouse liver gap junction plaques was dissociated in 200 μl NaHCO$_3$ buffer (0.1 M), pH 8.3, containing 0.5 M NaCl and 2% SDS (wt/vol) and sonicated with a Branson Sonic Power Co. (Danbury, CT) sonifier (30 W; 5 × 5 s). Undissociated gap junction proteins were pelleted by centrifugation at 15,000 *g* for 20 min. The supernatant was diluted to a final SDS concentration of 0.2% and used for coupling to cyanobromide-activated Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the Pharmacia coupling protocol. The crude rabbit anti-21 kD serum was applied to this column, but first washed with 0.5 M NaCl to eliminate unspecific 21-kD antibodies. For some experiments these affinity-purified 21-kD antibodies were further affinity purified using electrophoretically separated mouse liver 21-kD protein and transferred onto nitrocellulose paper, according to the method of Olmsted (1981). For some microinjection experiments, the affinity-purified 21-kD antibodies were additionally purified by "negative adsorption" to 26-kD gap junction protein coupled to tresyl-activated Sepharose 4B (Pharmacia Fine Chemicals). Affinity-purified antibodies to 26 kD were prepared in an analogous manner.

Immunoprecipitation

For termination of the incorporation of [^{35}S]methionine, or [^{32}P]orthophosphate, the labeling medium was removed and the cell layer washed three times with PBS without calcium and magnesium ions. The cells were then treated for 5 s with RIPA buffer (10 mM sodium phosphate buffer, pH 7.2, 40 mM NaF, 2 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, and 1% Trasylol [Bayer AG, Leverkusen, FRG]). The mixture was slightly agitated to preserve intact nuclei. After centrifugation at 460 *g* (5 min, 4°C), the supernatant was aspirated and brought to a final concentration of 1.5% SDS. To facilitate solubilization of membrane proteins, the supernatants were agitated twice on a vortex mixer for 5 s. The solution was diluted to a final concentration of 0.16% SDS and agitated twice for 5 s on a vortex mixer. After centrifugation at 13,000 *g* (20 min, 4°C) the supernatants were aspirated, stored in aliquots at −70°C, or used directly for immunoprecipitation as described (Traub et al., 1987).

Quantification of Gap Junctional Proteins

The relative amounts of electrophoresed (SDS-PAGE) 26- and 21-kD gap junction proteins were evaluated by densitometry of SDS-PAGE (stained with Coomassie blue). The accuracy of the Coomassie blue method was checked in the following way. The 26- and 21-kD protein bands were electrically eluted from a nonstained gel. After measuring the protein amounts according to Lowry et al. (1951), aliquots of the eluted 26- and 21-kD proteins were subjected to SDS-PAGE using ratios of 1:1 and 2:1, respectively. Densitometric quantitation of this gel stained with Coomassie blue revealed the expected ratios of 1:1 and 2:1. Further the use of a modified Coomassie blue stain (Neuhoff et al., 1985) which binds to proteins relatively independently of their amino acid sequence confirmed our results.

Partial Hepatectomy

BDIX rats, 23–25 d old, were used for partial hepatectomy as described (Higgins and Anderson, 1931). In brief, the rats were partially hepatectomized under ether anesthesia. The median and left lobes were removed while the other lobes were minimally disturbed and left in situ. Sham-operated animals were treated in the same manner except that the liver was manipulated without any portion being removed. One control and two experimental animals were anesthetized again at 19, 26, 32, 42, 48, and 52 h after the operation. After flushing the liver with Ringer solution, the right lateral lobe was immediately removed, sliced, and the slices were frozen in liquid propane maintained at its freezing point. Cryostat sections of the regenerating livers were subjected to immunofluorescence as described below.

Immunoblot

For immunoblot analyses liver plasma membranes were isolated by sucrose gradient centrifugation (Traub et al., 1983). Purification of plasma membranes from cultured hepatocytes and conditions of immunoblot analyses have been described (Traub et al., 1987).

Microinjection

Lyophilized, affinity-purified antibodies (rabbit preimmune IgG, polyclonal rabbit anti-26 kD, polyclonal rabbit anti-21 kD, and rat monoclonal anti-26 kD [Janssen-Timmen et al., 1986]) were dissolved in PBS (without calcium and magnesium ions). Monoclonal mouse antidesmoplakin I and II (Progen, Heidelberg, FRG) was dialyzed against Ca^{2+}- and Mg^{2+}-free PBS, lyophilized, and resuspended in bidistilled water. In the first series of experiments, antibodies were used at a protein concentration of 0.2 mg/ml. Later, when new batches of antibodies were used, we found that a protein concentration of 1 mg/ml was necessary for inhibition of Lucifer Yellow coupling. Only these datas are documented in this paper. Lucifer Yellow CH (Fluka AG, Buchs, Switzerland) was used as a 4% solution in 1 M LiCl.

Antibodies were microinjected iontophoretically with a hyperpolarizing current (Iontophoresis Programmer model 160 from World Precision Instruments Inc., New Haven, CT).

During injection, the cell culture dishes were kept on a heated block at 37°C and were flushed with CO$_2$ gas. About eight injections of antibodies were carried out in one dish within 45 min. During this time interval the hepatocytes did not change their morphology and capability of dye coupling. 4 min after each injection of antibodies or preimmune immunoglobulin, Lucifer Yellow was injected into one or more adjacent cells. 2 min later the extent of dye coupling between each antibody-containing cell and its surrounding cells were recorded.

To estimate the average volume injected per cell, an aqueous solution of ^{125}I-labeled sheep anti-mouse IgG (100 μCi/ml or 1.8×10^5 cpm/μl; 17 μCi/μg; Amersham International) was microinjected by iontophoresis under the same conditions as used for the antibody injection experiments. A total number of 2,225 cells were microinjected and washed three times with PBS whereby the radioactivity in the wash solution decreased to background level. The cells on cover slips were washed four additional times and the remaining radioactivity was counted in a Gamma-scintillation counter. We calculated an average injected volume of 7×10^{-8} μl per cell. In parallel 3,800 injections with the same ^{125}I-labeled antibodies were made into a droplet of 0.9% NaCl. From the radioactivity in the droplet, a volume of 6×10^{-8} μl per injection was calculated.

Immunofluorescence

Immunolabeling of the 26- and the 21-kD proteins in different tissues was carried out on cryostat sections (6–8 μm) placed on round glass cover slips. The labeling procedure for both gap junction proteins followed the scheme as described (Dermietzel et al., 1984).

Immunoelectron Microscopy

Immunogold-labeling was performed on LR (London Resin)-White-embedded ultrathin sections. Fixation was done by vascular perfusion with 2% paraformaldehyde plus 0.1% glutaraldehyde in PBS (pH 7.4) for 5 min, followed by immersing dissected cubes of liver samples in 2% paraformaldehyde for 1 h. The tissue was then rinsed in PBS at 4°C overnight, dehydrated in a graded series of ethanol concentrations, and immersed in 100% LR-White at 4°C for 12 h. After reincubation of the samples in fresh LR-White, the samples were encapsulated in gelatine and resin, and polymerized by irradiation using a black light source (UV light, 282 nm) at 4°C. Suitable consistency of the blocks was obtained after 3–4 d of polymerization. Ultrathin sections were sliced by means of a Reichert Scientific Instruments (Buffalo, NY) Ultratome, mounted on Formvar/carbon-coated Cu electron microscopical grids, and further processed for immunolabeling. Labeling with the primary antibody was achieved by using either a 10-μl droplet of polyclonal, affinity-purified anti-26 kD or anti-21 kD (5 μg/ml) at room

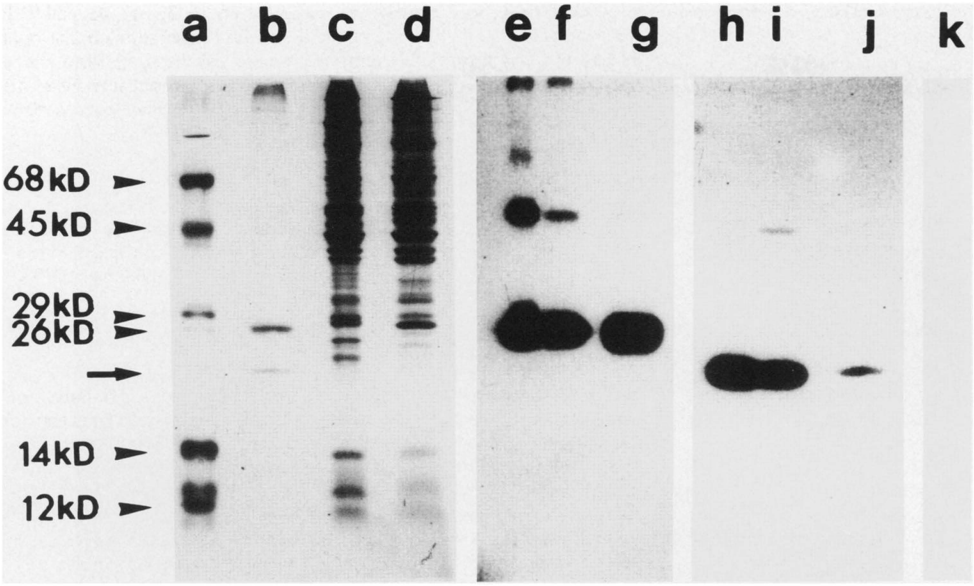

Figure 1. Immunochemical identification of 26- and 21-kD proteins from mouse liver gap junction plaques as well as mouse and rat liver plasma membranes. Purified gap junction proteins and total plasma membrane proteins were separated on SDS-polyacrylamide gels and analyzed after transfer onto nitrocellulose paper, incubation with affinity-purified anti-26 kD, affinity-purified anti-21 kD as well as IgG of preimmune serum, and reaction with ^{125}I-protein A according to standard immunoblot conditions. (lane *a*) Reference proteins: bovine serum albumin (68 kD), ovalbumin (45 kD), carbonic anhydrase (29 kD), chymotrypsinogen (26 kD), ribonuclease A (14 kD), and cytochrome *c* (12 kD) (Serva Fine Biochemicals Inc., Garden City Park, NY), stained with Coomassie brilliant blue; (lanes *b*, *e*, and *h*) mouse liver plaque proteins; (lanes *c*, *f*, and *i*) mouse liver plasma membrane proteins; (lanes *d*, *g*, and *j*) rat liver plasma membrane proteins; (lanes *a–d*) staining with Coomassie brilliant blue; (lanes *e–k*) autoradiographs after immunoblot with anti-26 kD (lanes *e–g*), anti-21 kD (lanes *h–j*), or IgG from preimmune serum (lane *k*). The arrow at the left indicates the position of the 21-kD protein.

temperature for 15 min. Affinity-purified goat anti-rabbit IgG coupled to colloidal gold (5–6 nm, Janssen Pharmaceutica, Beerse, Belgium) was used as a secondary marker. The goat anti-rabbit IgG-gold solution exhibited minimal background labeling at a dilution of 1:20. Staining of the sections was obtained with a solution of 5% uranylacetate in ethanol. Electron microscopy was performed using a Philips Electronic Instruments, Inc., model EM 400 (Mahwah, NJ) fitted with a goniometer cartridge.

Double immunolabeling was performed according to Bendayan and Stephens (1984). For this purpose the sections were mounted upon uncoated Cu grids, dried down, and then transferred into a droplet of one of the appropriate antibodies. After several washes the immunogold stain was applied by incubating the grid turned upside down onto a droplet of the diluted gold solution. We used either 5- or 15-nm gold beads, for each antigen. Labeling and transfer of the grids were done with great care in order to avoid spilling of the primary or secondary antibodies onto the unexposed side. After labeling of one of the gap junction antigens, the grids were flipped over onto another droplet of the appropriate gap junction antibody for a further immunolabeling procedure. Staining and handling for electron microscopy was done as described above. For quantitative evaluation of labeling density, electron micrographs were compared at the standard magnification of 82,000×. Counting and measurements of particle density and length of gap junctions, respectively, were carried out using a semiautomatic morphometric device (ASM-Leitz, Wetzlar, FRG).

Results

Immunochemical Identification of 26- and 21-kD Proteins

Affinity-purified antibodies to the 26- and 21-kD proteins were used for the characterization of gap junctions proteins of mouse and rat liver membranes. Purified gap junction plaques from mouse liver and whole plasma membrane proteins from mouse as well as rat liver were separated on SDS–polyacrylamide gels and analyzed according to standard immunoblot criteria (Fig. 1). Affinity-purified anti–26 kD or anti–21 kD specifically recognized the 26- or 21-kD protein components of mouse liver gap junction plaques, respectively, but did not show any cross-reactivity (Fig. 1, lanes *e* and *h*). The same specificity was found when the 26- and 21-kD proteins were analyzed in whole plasma membranes of mouse or rat liver (Fig. 1, lanes *f*, *g*, *i*, and *j*). In mouse liver gap junction plaques, the ratio of 26- to 21-kD protein was estimated to be 2:1 by means of densitometric evaluation of Coomassie blue–stained protein bands. This ratio was confirmed by different methods of quantitation as described under Materials and Methods. Immunoblot analysis of whole plasma membranes showed that the amount of the 21-kD protein of mouse liver exceeded that of rat liver significantly (Fig. 1, lanes *i* and *j*), whereas the amounts of the 26-kD proteins were comparable in both species (Fig. 1, lanes *f* and *g*).

Immunofluorescence in Rat Liver

Indirect immunofluorescence was performed on sections through various tissues. We have already reported on the colocalization of the 26- and the 21-kD gap junction proteins in mouse liver (Nicholson et al., 1987). The published results indicate an almost exact matching of immunofluorescent sites of 26- and 21-kD protein in mouse liver. Subsequently, we found evidence of a disproportionate distribution of both proteins in lobules of rat liver. To get an unambiguous local definition of both antigenic sites, we performed double immunolabeling using a monoclonal anti–26 kD raised in rats (Janssen-Timmen et al., 1986) and affinity-purified polyclonal 21-kD antibodies obtained from rabbits. By applying antisera from different species to rat liver sections, we were able to avoid cross-reaction of the secondary antibodies. Immunoreactive sites of the 26-kD protein were found to occur evenly distributed throughout the rat liver lobules (Fig. 2 *a*). However, anti–21 kD immunolabeling was most prominent in the peripheral zones of the lobules and decreased in its

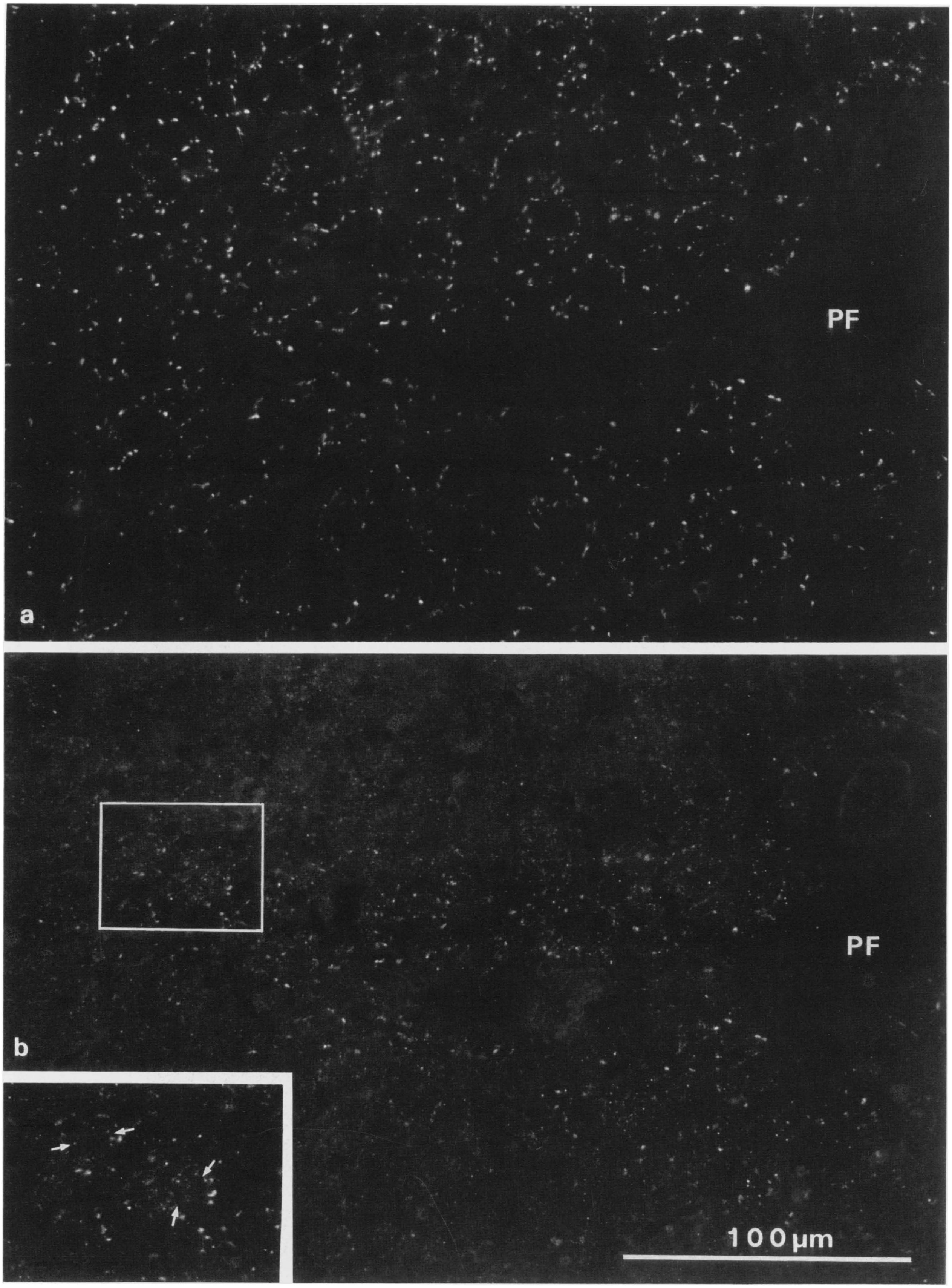

Figure 2. Double immunolabeling of the same rat liver cryostat section with monoclonal 26-kD antibodies and affinity-purified polyclonal 21-kD antibodies. There is a striking discrepancy in the distribution of the immunoreactive sites. Anti-26 kD stains uniformly within the rat liver lobules (*a*), whilst anti-21 kD (*b*) shows an increased concentration of immunoreactive sites around the periportal fields (*PF*). The inset shows the magnified area outlined in *b* depicting some intracytoplasmic staining (*arrows*) which was frequently found in rat liver after incubation with anti-21 kD.

staining intensity towards the central vein (Fig. 2 *b*). This pattern of 21-kD immunoreactivity was consistently found in adult as well as in young (3–4-wk-old) rats. At sites where anti–21 kD immunoreactivity was prevalent, an almost exact superimposition with the anti–26 kD immunolabeling was detected.

Immunofluorescence in Cultured Mouse Hepatocytes

Cultured embryonic mouse hepatocytes were screened for anti–21 kD and anti–26 kD double immunoreactivity. An intensive intracytoplasmic anti–21 kD reactivity was found within single cells between 12 and 24 h after plating (Fig. 3 *a*), whereas only a minimal anti–26 kD reactivity was seen (Fig. 3 *b*). At this time the plasma membranes appear to be free or relatively low in 21-kD immunolabeling (Fig. 3 *a*; cf. Fig. 7). The first well-defined regions of 21-kD immunoreactivity within plasma membranes were seen after 24 h of culture. Concurrently, there was significant increase in labeling with anti–26 kDa. The immunoreactive sites of both proteins within the plasma membrane were found to exactly superimpose on each other. Both proteins were extensively expressed on the membranes of cultured mouse hepatocytes after 72 h (Fig. 3, *c* and *d*). However, even at this later time of culturing, intracytoplasmic labeling was more evident for the 21-kD than for the 26-kD protein.

Immunofluorescence of Different Tissues

Table I summarizes the results of immunoreactivity found in various other organs. We emphasize those findings where major differences in immunolabeling of some other epithelial derivatives such as exocrine pancreas, epithelium of the small intestine, and kidney were detected. In addition, myocardium (Fig. 4, *c* and *d*) and adult brain cortex (not shown) were subjected to immunolabeling. While the latter two tissues were negative when 21-kD antibodies were applied (the same result has been documented for 26-kD immunoreactivity; cf. Dermietzel et al., 1984) all the other tissues of epithelial origin so far studied reacted positively. In the exocrine pancreas a strong fluorescent signal was obtained for 21-kD immunoreactivity exceeding that of the anti–26 kD reaction. The endocrine part of the pancreas showed no immunoreaction. Strong labeling occurred in the epithelium of kidney tubules, although confined only to the proximal part (Fig. 4, *a* and *b*). The 26-kD immunolabeling in kidney was less intense but occurred preferentially within the proximal epithelial domains. The epithelium of the small intestine revealed a faint 21-kD immunoreactivity along the lateral portion of the plasma membranes (Table I).

Immunoelectron Microscopy

Ultrathin sections of mouse liver were subjected to immunogold labeling. As has been reported (Dermietzel et al., 1984) the use of 26-kD antibodies resulted in intense immunogold labeling at the gap junction membrane domains (cf. Fig. 5 *a* for comparison). Significant decoration with immunogold particles was also obtained when the sections were incubated with 21-kD antibodies (Fig. 5 *b*). The intensity of the 21-kD immunoreactivity was less, however, than that of the 26-kD immunoreactivity. The ratio of immunogold labeling of 26- to 21-kD protein within the junctional domains was ∼2:1 when the 5-nm gold particles were used. The beads were counted on a total length of at least 22 μm of gap junction plaques on each specimen. These results corroborate our recent fluorescence microscopical finding of a colocalization of the 26- and the 21-kD protein (Nicholson et al., 1987). Fig. 5 *c* shows a gap junction decorated with immunogold beads of two different sizes. The small 5-nm particles label the 26-kD protein while the larger 15-nm ones mark the 21-kD protein. The average labeling index (gold particles/μm) was 42 (anti–26 kD) to 8 (anti–21 kD) when the above protocol was used. When the reverse order of particle size (15-nm beads for anti–26 kD, and 5-nm beads for anti–21 kD) was used, the labeling index for the 26-kD protein decreased six times while the labeling intensity of the 21-kD protein increased by a factor of three. An unequivocal determination of the ratio of 26- to 21-kD protein by immunoreaction cannot be given by this method because the large-sized particles are particularly prone to being washed away from the sections during the several rinsings which follow the immunoincubation.

Furthermore, we observed that the gold decoration of both antigens occurred in clusters rather than in an equal distribution. Only in rare cases were an exact superimposition of the small- and large-sized particles within the same gap junctional area obtained.

Half-life Time of the 21-kD Protein in Cultured Hepatocytes

We estimated the half-life time of the 21-kD protein in cultured mouse hepatocytes by pulse incorporation of [^{35}S]methionine, chase with unlabeled methionine, immunoprecipitation, and densitometric evaluation of the 21-kD band on autoradiographs. A half-life time of ∼1.3–2 h was found in different experiments one of which is shown in Fig. 6. In another experiment, the half-life time of the 26- and 21-kD proteins were determined simultaneously by using a mixture of affinity-purified anti–26 kD and anti–21 kD for immunoprecipitation. In this experiment the autoradiograph showed a similar exponential decrease of 26- and 21-kD proteins. Previously, a half-life time of ∼3 h has been reported for the 26-kD protein under similar conditions (Traub et al., 1987). Fallon and Goodenough (1981) as well as Yancey et al. (1981) had reported somewhat longer half-life times of gap junction proteins in liver tissue.

Changes in the Amounts of 21- and 26-kD Proteins in Cultured Mouse Embryonic Hepatocytes

Fig. 7 presents an immunoblot analysis of the amount of 21- and 26-kD proteins in plasma membrane proteins of mouse embryonic hepatocytes at five different time points of culture. The amounts of the two proteins decreased and increased similarly. These results are concomitant with the immunofluorescence in the membranes (cf. Fig. 3) indicating that the appearance of both proteins in hepatic plasma membranes is similarly regulated. Immunoblot as well as immunofluorescence analyses were carried out with the same affinity-purified 26- and 21-kD antibodies. Previously, we have shown (Heynkes et al., 1986) that mRNA of the 26-kD protein decreased and increased in cultured mouse embryonic hepatocytes. The mRNAs for albumin, α-fetal protein, and tyrosine amino transferase are stable under these experimental conditions (Paul, D., unpublished observations).

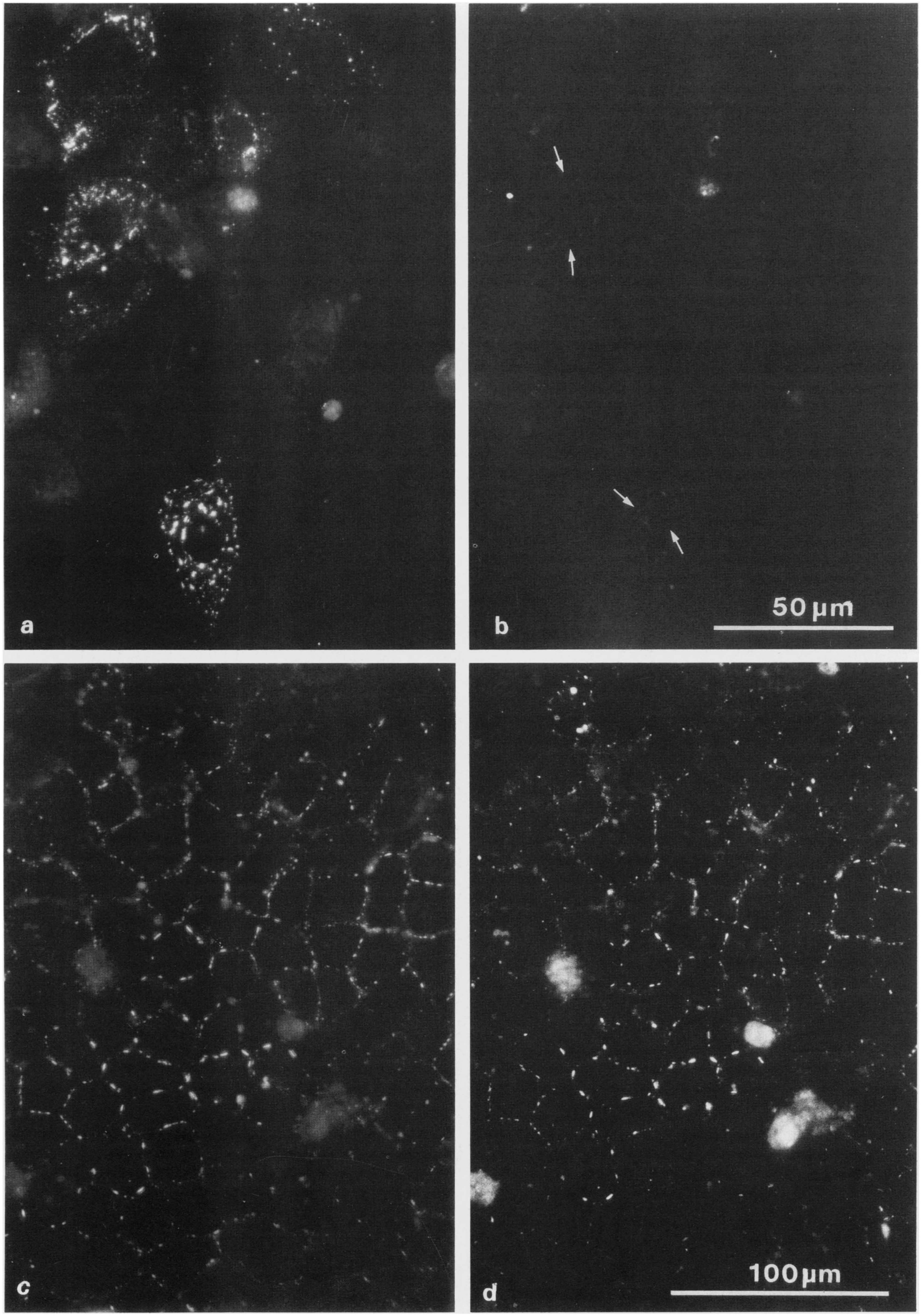
a
b
50 µm
c
d
100 µm

Table I. Immunofluorescence of Tissues Screened with Affinity-purified 21-kD and 26-kD Antibodies

Tissue	Species	Immunoreactivity Anti–21 kD	 Anti–26 kD*
Liver	Mouse	+	+
	Rat	±	+
Pancreas	Mouse	+	
(exocrine part)	Rat		+
Kidney	Mouse	±	
	Rat		±
Small intestine	Mouse	+	
	Rat		+
Myocardium	Mouse	−	
	Rat	−	−
Brain cortex	Mouse	−	
(adult)	Rat	−	−

Positive immunoreactivity is represented by +, no immunoreactivity is represented by −. The results of ± are described and discussed in detail in the text.
* For comparison, data taken from Dermietzel et al. (1984) are included.

Amounts of 26- and 21-kD Proteins in Plasma Membranes of Regenerating Rat Liver after Partial Hepatectomy

Fig. 8 summarizes the changes in the amount of 26- and 21-kD proteins analyzed by quantitative immunoblot during rat liver regeneration using affinity-purified anti–21 kD and anti–26 kD. Both the 26- and the 21-kD proteins decreased and increased after partial hepatectomy but the amount of the 21-kD protein at the minimum was ∼35% of its initial value compared to 15% of the 26-kD protein. The decrease of the 21-kD protein had not been noticed in previous experiments (Traub et al., 1983) when crude anti–21 kD serum was used in analyzing this protein after partial hepatectomy. We can now conclude that the 26- and 21-kD gap junction proteins are similarly regulated in their expression after partial hepatectomy.

Phosphorylation of Gap Junction Proteins

In contrast to the 26-kD protein, the 21-kD protein was not phosphorylated by in vitro labeling of mouse liver gap junction plaques using cAMP-dependent protein kinase (Fig. 9 *b*). After metabolic labeling of mouse hepatocytes, more ^{32}P-label was detected after immunoprecipitation in the 26-kD protein (Traub et al., 1987) than in the 21-kD protein taking into account the apparent mass ratio of both proteins. Thus the two gap junction proteins may be differently modified by postttranslational phosphorylation.

Antibodies to the 21-kD or the 26-kD Proteins Inhibit Dye Transfer after Microinjection into Hepatocytes

72–96 h after start of the hepatocyte cultures, maximal coupling between the cells was observed after microinjection of Lucifer Yellow. At this time affinity-purified 21-kD antibodies were injected into hepatocytes and 4 min later the fluorescent dye Lucifer Yellow was injected into one or more adjacent cells. Fig. 10 illustrates that the cells microinjected with anti–21 kD as well as anti–26 kD (Fig. 10, *asterisks*) were inhibited for transfer of Lucifer Yellow. Dye transfer was inhibited in almost all of the cells injected with anti–21 kD (94% on the average with negatively adsorbed anti–21 kD) and in all cells injected with anti–26 kD (100%) (Table II). When control cells were injected with IgG from preimmune serum or with antidesmoplakin I and II no inhibition of dye transfer was detected (Table II). The latter experiment indicates that inhibition of dye transfer is not an unspecific effect of antibody binding to membrane proteins on the cytoplasmic face of the cell membrane. Furthermore, the monoclonal anti–26 kD (12/1-C5) did not interfere with dye transfer (Table II) although it has been shown by indirect immunofluorescence to bind specifically to gap junction plaques (Janssen-Timmen et al., 1986). The expected pattern of immunoreactivity on peripheral membranes of embryonic mouse hepatocytes was obtained by analysis of indirect immunofluorescence using antidesmoplakin I and II (data not shown).

Microinjection of sheep anti-mouse IgG (^{125}I-labeled) showed that $\sim 6 \times 10^{-8}$ μl of antibody solution per cell were injected by iontophoresis (see Materials and Methods). This is about the same volume as determined for pressure injections (Stacey and Allfrey, 1976). Since we used polyclonal antibodies for microinjection, we do not know the fraction of antibodies which causes inhibition of dye transfer.

Discussion

The results presented in this publication confirm and extend the notion that there are (at least) two gap junction proteins in liver, the 26- and 21-kD protein. Both proteins are copurified with liver gap junction plaques, but the ratio of the 26- to 21-kD protein (i.e., intensity of protein bands stained with Coomassie blue on gels) was ∼10:1 in isolated rat liver plaques (Nicholson et al., 1987) and ∼2:1 in isolated mouse liver plaques, respectively. We have shown that both proteins are colocalized in the same plaques in sections of mouse liver (Nicholson et al., 1987) and in cultured mouse embryonic hepatocytes (this paper). The latter conclusion is based on immunofluorescence analysis and immunoelectron microscopy. Surprisingly, we detected a gradient of 21-kD immunoreactivity in sections of rat liver. The strongest reaction was found in the periportal zone of the liver lobule; i.e., in the proximity of the terminal afferent vessels. Relatively little 21-kD immunoreactivity was observed near the central vein of the rat liver lobule. In contrast, the 26-kD immunoreactivity was equally distributed on apposing plasma membranes in mouse as well as rat liver as discrete fluorescent spots that have been shown to represent areas of gap junction plaques (Dermietzel et al., 1987). The unequal distribution of the 21-kD immunoreactivity in rat liver argues against the possi-

Figure 3. Double immunofluorescence of 26- and 21-kD proteins in cultured hepatocytes. *a* and *b* are taken from hepatocytes 12 h after plating. At this time there is a strong intracytoplasmic anti–21 kD immunoreactivity in some hepatocytes (*a*), whereas anti–26 kD immunolabeling is barely visible (*b*, *arrows*). 72 h after plating both proteins occur equally expressed within the plasma membranes (anti–21 kD, *c*; anti–26 kD, *d*).

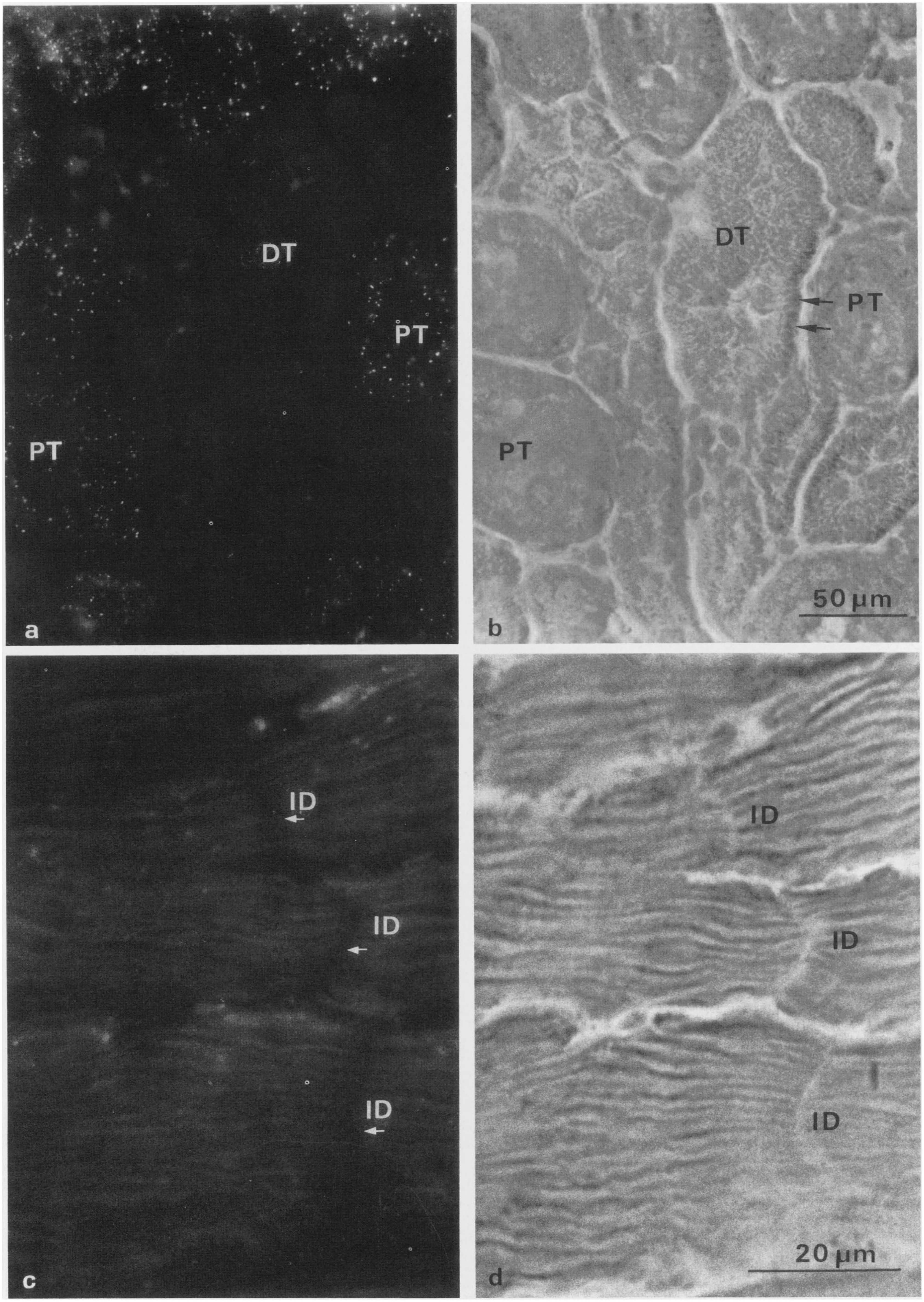

Figure 4. Anti–21 kD labeling in epithelial cells of the proximal tubules of kidney (*PT*). Distal parts of the tubules (*DT*) do not display any staining (*a*). Phase-contrast micrograph (*b*) elucidates *a* with regard to the respective parts of the tubules. Distal parts of the tubules are made prominent by their more pronounced basal striation (*b*, *arrows*). Immunofluorescence (*c*) and phase-contrast micrograph (*d*) of rat heart muscle. Three intercalated discs (*ID*) are visible. No anti–21 kD immunoreactivity is seen.

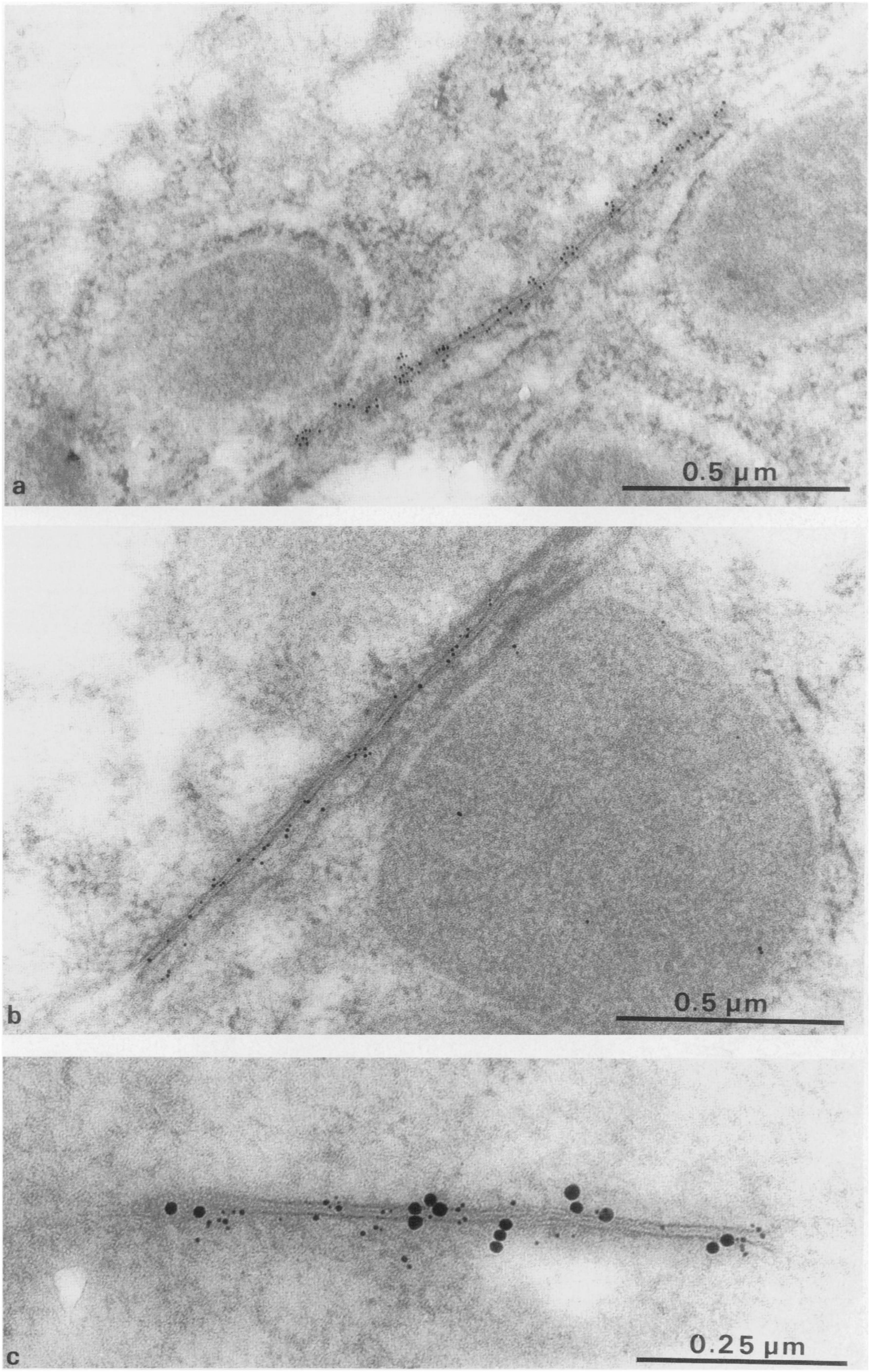

Figure 5. Immunogold labeling of mouse liver ultrathin sections. While *a* gives an approximate impression of the intensity of anti-26 kD labeling, *b* shows anti-21 kD reactivity. The clustered distribution of gold labeling along the gap junction domain is evident. The anti-21 kD staining (*b*) is weaker with some gold beads in the background. (*c*) Double immunogold labeling using 5-nm beads for anti-26 kD and 15-nm beads for anti-21 kD. Note that the larger sized particles occur in clusters.

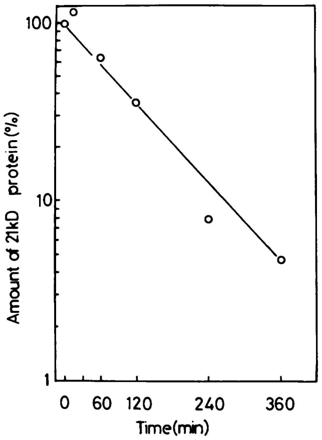

Figure 6. Half-life time of the 21-kD protein in cultured mouse hepatocytes. Confluent mouse embryonic hepatocytes (after 3 d in culture) were metabolically labeled with [^{35}S]methionine for 60 min and subsequently incubated with nonlabeled methionine for the indicated times. Affinity-purified anti-21 kD was used for immunoprecipitation. The 21-kD bands on autoradiographs were compared by densitometric evaluation.

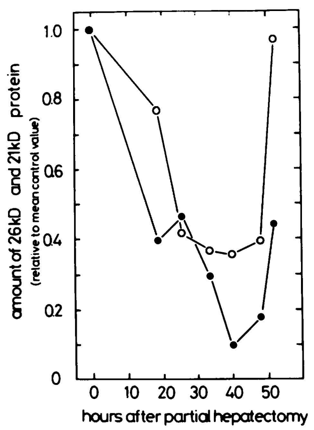

Figure 8. Amounts of 26- and 21-kD proteins in plasma membranes of regenerating rat liver after partial hepatectomy. Plasma membranes were prepared from regenerating rat livers at indicated times, subjected to immunoblot analysis, autoradiography, and densitometric evaluation as described in Fig. 7. The amounts of 26- and 21-kD protein after partial hepatectomy are expressed relative to the mean control values (sham-operated animals). (○) 21-kD protein; (●) 26-kD protein.

ble in vivo cross-reactivity of affinity-purified 21-kD antibodies with the 26-kD protein. The 21-kD antibodies have been shown not to cross-react with the 26-kD protein under SDS-denaturing conditions of immunoblot. The preferential localization of the 21-kD protein in plaques of the periportal zone of rat liver places this protein in the same category as enzymes for gluconeogenesis, oxidative energy metabolism, and amino acid use which have also been preferentially found in the periportal zone (Jungermann, 1986). In kidney, the 21-kD immunoreactivity was only found in the proximal tubules similar to the 26-kD immunoreactivity. The 21-kD immunoreactivity in kidney epithelium, however, was about three times stronger than the 26-kD immunoreactivity. This implies that the ratio of the two proteins in kidney is reversed as compared to that in mouse liver parenchyma. In rat myocardium Hertzberg and Skibbens (1984) had described positive immunoreaction using anti-27 kD whereas Paul (1985), similar to our results (Dermietzel et al., 1984), did not find specific immunofluorescence in this tissue. Possibly different antibodies to the liver 26-kD protein (connexin32) exhibit different cross-reactivity with the 43-kD gap junction protein (connexin43) (cf. Beyer et al., 1987), expressed in myocardium.

Recent data (Nicholson et al., 1987) indicate that 20 NH_2-

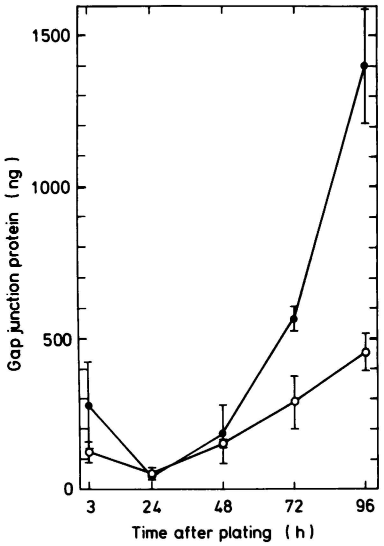

Figure 7. Amounts of the 21- and 26-kD proteins at different time points of mouse embryonic hepatocytes in culture. Plasma membranes were prepared from hepatocytes harvested at the indicated times and subjected to immunoblot analysis as well as autoradiography as described in Materials and Methods. Autoradiographs were evaluated by densitometry. Each point of the curves represents the mean of six (21 kD) and four (26 kD) measurements taken from three or two independent experiments, respectively. Gap junction protein (ng) refers to the total amount of protein in isolated mouse liver plaques. Four different standard amounts of gap junction plaques from mouse liver were electrophoresed on each gel as a reference. (○) 21-kD protein; (●) 26-kD protein.

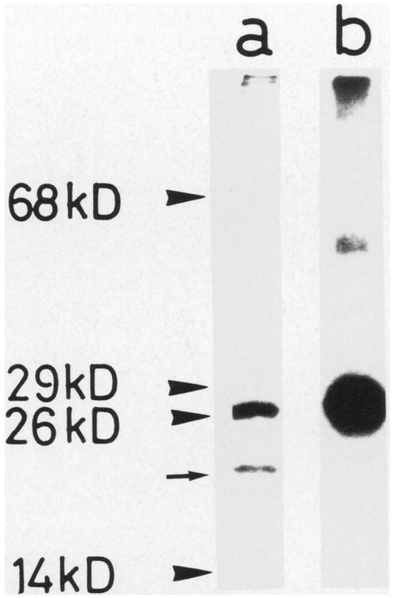

Figure 9. Phosphorylation of gap junction proteins in vitro. Isolated mouse liver gap junction plaques were incubated with cAMP-dependent protein kinase and [^{32}P]ATP. The labeled proteins were separated by SDS-PAGE and autoradiographed. (lane *a*) Staining with Coomassie brilliant blue; (lane *b*) autoradiograph. Molecular mass standards shown on left were bovine serum albumin (68 kD), Carbonic anhydrase (29 kD), Chymotrypsinogen (26 kD), and ribonuclease A (14 kD). The arrow indicates the position of the 21-kD protein.

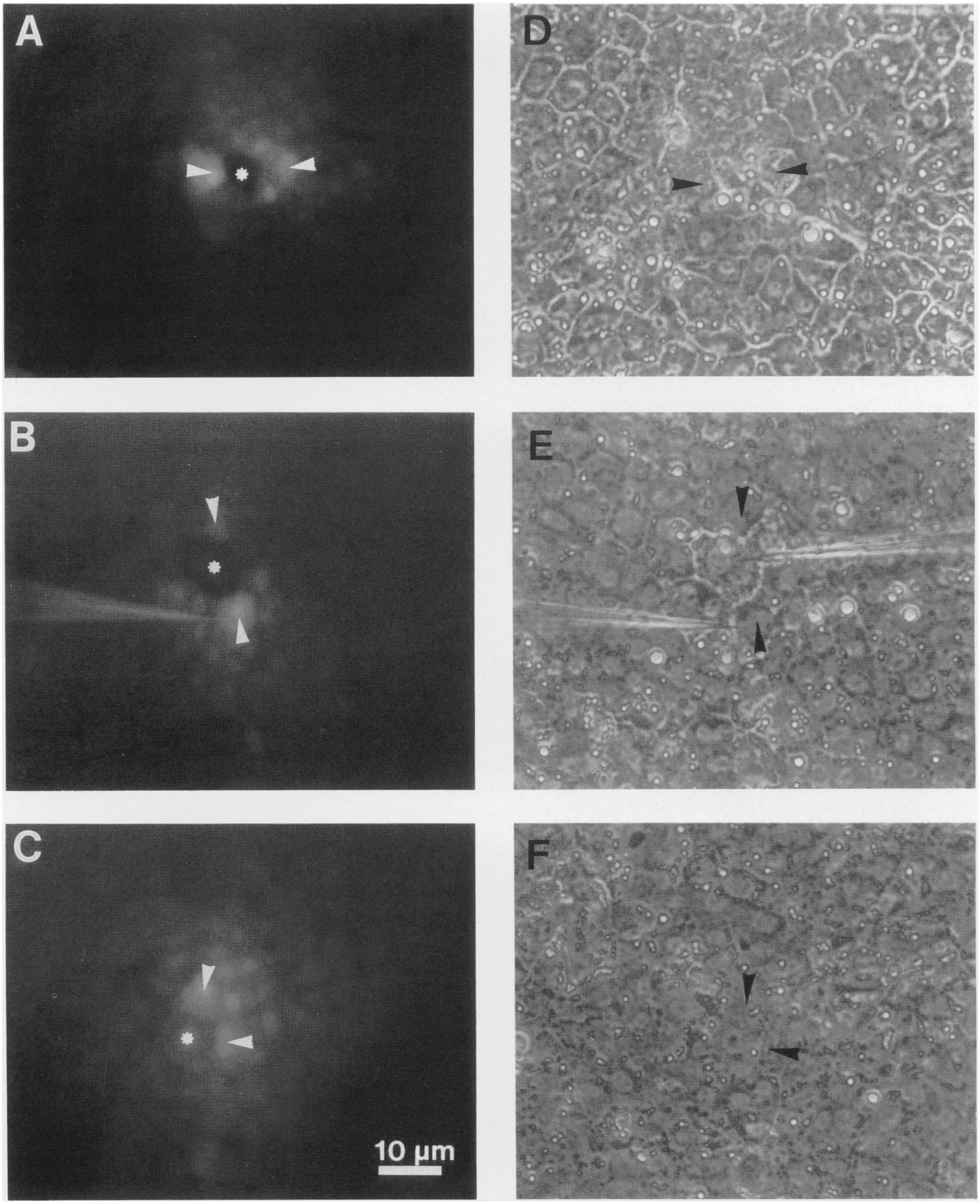

Figure 10. Inhibition of dye transfer after microinjection of antibodies. (*A* and *D*) Anti-21 kD; (*B* and *E*) anti-26 kD; (*C* and *F*) IgG from preimmune serum. Antibodies and IgG were microinjected at a concentration of 1 mg/ml 4 min before injection of two adjacent cells (indicated by arrowheads) with Lucifer Yellow. Antibodies or preimmune IgG-containing cells are marked by asterisks. Micrographs taken in fluorescent light are shown in *A*, *B*, and *C*; the corresponding phase-contrast micrographs are *D*, *E*, and *F*, respectively.

terminal amino acids of the rat liver 26- and 21-kD proteins show 48% homology. This infers that both proteins may have a similar structure. This conclusion is confirmed and extended by Nicholson and Zhang (1988) who concluded from comparison of cDNA sequences that the 26- and 21-kD proteins are coded for by genes of a multigene family ("connexin" genes). The rat liver 26-kD protein has been functionally reconstituted in lipid bilayers (Young et al., 1987) and

Table II. Inhibition of Intercellular Transfer of Lucifer Yellow After Microinjection of Antibodies

Antibodies	Concentration	Total number of injections	Number of injections showing Inhibition	Number of injections showing No effect	% Inhibition*
	mg/ml				
Rabbit preimmune IgG	1.0	10	0	10	0
Monoclonal antidesmoplakin I and II	1.0	12	0	12	0
Monoclonal anti-26 kD (12/1-C5)	1.0	8	0	8	0
Polyclonal anti-26 kD	1.0	10	10	0	100
Polyclonal anti-21 kD (negatively adsorbed)‡	1.0	16	15§	1	94

* Relative to total number of injections.

‡ Anti-21 kD was first affinity purified on a column of gap junction plaque protein and then negatively adsorbed on a 26-kD protein column (see Materials and Methods).

§ 9 out of 15 cells injected with antibodies showed a weak Lucifer Yellow fluorescence in the nucleus.

injection of its purified mRNA into *Xenopus* oocytes led to an increased electrical conductivity between contacting oocytes (Dahl et al., 1987). The results of these reconstitution experiments together with the resistance of the 26-kD protein to urea as well as to nonionic detergents and the protection of the structure after protease treatment strongly suggest that the 26-kD protein is part of the gap junction structure and most likely has a transmembrane orientation (Zimmer et al., 1987). No reconstitution or topology experiments have been reported concerning the 21-kD protein.

Our data confirm the close relationship of the 21-kD and the 26-kD proteins. Electron microscopical double immunolabeling demonstrates for the first time colocalization of two gap junction antigens within the same junctional domain at the ultrastructural level. The distribution pattern of either the small or the large-sized particles which represents one of the particular antigens appears to imply a patchy occurrence of both antigenic determinants within the same gap junctional plaque. At present we do not know whether this is due to selective removal of the larger immunobeads, selective proteolysis, or due to the occurrence of clustered homomeric channels.

We determined a half-life time of ~2 h for the 21-kD protein in primary mouse hepatocytes, similar as for the 26-kD protein for which a half-life time of ~3 h had been previously found (Traub et al., 1987). The relatively short half-life times of the two proteins are consistent with the notion of a common turnover of the two gap junction components. The results of our microinjection experiments in cultured mouse hepatocytes suggest that the 21-kD protein is a functional gap junction protein. Polyclonal anti-26 kD as well as polyclonal anti-21 kD inhibited dye coupling in almost all cells injected. The large extent of inhibition at the single cell level with either antibody preparation could hint to the existence of heteromeric channels. However, the notion of a minor population of homomeric channels which may not be influenced by one of the two different antibodies and which may allow weak dye transfer is also consistent with our results. We cannot exclude that the closure of channels due to antibodies bound may affect the conformation and permeability of neighboring channels. After injection of polyclonal anti-21 kD affinity purified on a column of plaque protein and subsequently negatively adsorbed by filtration through a column of 26-kD protein to remove antibodies which may cross-react with the 26-kD protein, we did not detect any dye transfer in 6 out of 15 cells. In 9 out of 15 microinjected cells, however, weak transfer of Lucifer Yellow was observed. This may indicate that homomeric 26-kD channels were left open in these cells. The interference with gap junctional dye coupling was specific since monoclonal antidesmoplakin I and II, monoclonal anti-26 kD (12/1-C5), and IgG from preimmune serum had no significant effect. Thus, the 21-kD protein appears to be a functional component of gap junctional channels.

We report here that the 21-kD protein was not phosphorylated by cAMP-dependent protein kinase under in vitro conditions. The 26-kD protein, however, was phosphorylated by cAMP-dependent kinase in vitro and in vivo (Saez et al., 1986; Traub et al., 1987), a posttranslational modification that has been suggested to increase gap junctional conductance (Saez et al., 1986). Possibly those gap junction channels which are built up with 21-kD subunits may not respond to the same regulatory signals as homomeric 26-kD channels. Our data obtained after partial hepatectomy or with cultured hepatocytes suggest that the amount of the 21-kD protein does not decrease to the same extent in proliferating cells as that of the 26-kD protein. On the other hand, the relatively short half-life times found for both proteins in cultured hepatocytes indicate common regulatory properties. Furthermore, preliminary results show that the incorporation of radioactivity from [^{3}H]palmitic and [^{3}H]myristic acid is similar for the 26- and 21-kD proteins and is sensitive to treatment with hydroxylamine. This suggests that long chain fatty acid residues are covalently bound to both proteins via thioester linkage (Traub et al., 1988; Willecke et al., 1988). It will be of interest to clarify the regulatory properties of the 21- and 26-kD protein subunits for the structure and function of hepatic gap junction channels.

We thank Ms. L. Breuer, P. Altenhoff, B. Kunz, and D. Schünke for technical assistance; and P. Bühle for help with microinjection experiments. We are grateful to Dr. D. Paul (University of Hannover, Hannover, FRG) for making available to us the MX83 culture medium for hepatocytes developed in his laboratory.

This work was supported by the Deutsche Forschungsgemeinschaft (Wi 270/14 and De 292-2-1), and by grants from the Dr. Mildred Scheel Stiftung and the Fonds der Chemischen Industrie to K. Willecke.

Received for publication 22 August 1988 and in revised form 17 October 1988.

References

Bendayan, M., and H. Stephens. 1984. Double labelling cytochemistry applying the protein A-gold technique. *In* Immunolabelling for Electron Microscopy. J. H. Polak and I. M. Vandell, editors. Elsevier, Amsterdam. 143–154.

Bennett, M. V. L., and D. C. Spray. 1985. Gap Junctions. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 122 pp.

Beyer, E. C., D. Paul, and D. A. Goodenough. 1987. Connexin43: a protein from rat heart homologous to a gap junction protein from liver. *J. Cell Biol.* 105:2621–2629.

Dahl, G., T. Miller, D. Paul, R. Voellmy, and R. Werner. 1987. Expression of functional cell-cell channels from cloned rat liver gap junction complementary DNA. *Science (Wash. DC).* 236:1290–1293.

Dermietzel, R., A. Leibstein, U. Frixen, O. Traub, and K. Willecke. 1984. Gap junctions in several tissues share antigenic determinants with liver gap junctions. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:2261–2270.

Dermietzel, R., B. S. Yancey, U. Janssen-Timmen, O. Traub, K. Willecke, and J.-P. Revel. 1987. Simultaneous light and electron microscopic observation of immunolabelled liver 27 kD gap junction protein on ultra-thin cryosections. *J. Histochem. Cytochem.* 35:387–392.

Fallon, R. F., and D. A. Goodenough. 1981. Five-hour half-life of mouse liver gap-junction protein. *J. Cell Biol.* 90:521–526.

Finbow, M. E., J. Shuttleworth, A. E. Hamilton, and J. D. Pitts. 1983. Analysis of vertebrate gap junction protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1479–1486.

Finbow, M. E., B. S. Yancey, R. Johnson, and J.-P. Revel. 1980. Independent lines of evidence suggesting a major gap junctional protein with a molecular weight of 26,000 Daltons. *Proc. Natl. Acad. Sci. USA.* 77:970–974.

Henderson, D., H. Eibl, and K. Weber. 1979. Structure and biochemistry of mouse hepatic gap junctions. *J. Mol. Biol.* 132:193–218.

Hertzberg, E. L., and N. B. Gilula. 1979. Isolation and characterization of gap junctions from rat liver. *J. Biol. Chem.* 254:2138–2147.

Hertzberg, E. L., and R. V. Skibbens. 1984. A protein homologous to the 27,000 Dalton liver gap junction protein is present in a wide variety of species and tissues. *Cell.* 39:61–69.

Heynkes, R., G. Kozjek, O. Traub, and K. Willecke. 1986. Identification of rat liver cDNA and mRNA coding for the 28 kDa gap junction protein. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 202:56–60.

Higgins, G. M., and R. M. Anderson. 1931. Experimental pathology of the liver. *Arch. Pathol.* 12:186–202.

Janssen-Timmen, U., O. Traub, R. Dermietzel, H. M. Rabes, and K. Willecke. 1986. Reduced number of gap junctions in rat hepartocarcinomas detected by monoclonal antibody. *Carcinogenesis (Lond.).* 7:1475–1482.

Jungermann, K. 1986. Zonal signal heterogeneity and induction of hepatocyte heterogeneity. *In* Regulation of Hepatic Metabolism. R. G. Thurman, C. F. Kaufman, and K. Jungermann, editors. Plenum Press, New York. 445–469.

Loewenstein, W. R. 1981. Junctional intercellular communication: the cell to cell membrane channel. *Physiol. Rev.* 61:829–913.

Loewenstein, W. R. 1987. The cell-to-cell channel of gap junction. *Cell.* 48:725–726.

Lowry, O., N. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* 193:266–275.

Neuhoff, V., R. Stamm, and H. Eibl. 1985. Clear background and highly sensitive protein staining with Coomassie Blue dyes in polyacrylamide gels: a systematic analysis. *Electrophoresis.* 6:427–448.

Nicholson, B. J., and J. T. Zhang. 1988. Multiple protein components in a single gap junction: cloning of a second hepatic gap junction protein (MW 21,000). *Mod. Cell Biol.* 7:207–218.

Nicholson, B. J., R. Dermietzel, D. Teplow, O. Traub, K. Willecke, and J.-P. Revel. 1987. Two homologous protein components of hepatic gap junctions. *Nature (Lond.).* 329:732–734.

Olmsted, J. B. 1983. Affinity purification of antibodies from diazotized paper blots of heterogeneous protein samples. *J. Biol. Chem.* 256:11955–11957.

Paul, D. 1985. Antibody against liver gap junction 27 kDa protein is tissue specific and cross-reacts with a 54 kDa protein. *In* Gap Junctions, M. V. L. Bennett and D. C. Spray, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 107–122.

Paul, D. 1986. Molecular cloning of cDNA for rat liver gap junction protein. *J. Cell Biol.* 103:123–134.

Saez, J. C., D. C. Spray, A. C. Nairn, E. L. Hertzberg, P. Greengard, and M. L. V. Bennett. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27 kDa principal gap junction polypeptide. *Proc. Natl. Acad. Sci. USA.* 83:2473–2477.

Stacey, D. W., and V. G. Allfrey. 1976. Microinjection studies of duck globin messenger RNA in human and avian cells. *Cell.* 9:725–732.

Traub, O., P. M. Drüge, and K. Willecke. 1983. Degradation and resynthesis of gap junction protein in plasma membranes of regenerating liver after partial hepatectomy or cholestasis. *Proc. Natl. Acad. Sci. USA.* 80:255–259.

Traub, O., U. Janssen-Timmen, P. M. Drüge, R. Dermietzel, and K. Willecke. 1982. Immunological properties of gap junction protein from mouse liver. *J. Cell. Biochem.* 19:27–44.

Traub, O., J. Look, D. Paul, and K. Willecke. 1987. Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. *Eur. J. Cell Biol.* 43:48–54.

Traub, O., J. Look, R. Stutenkemper, and K. Willecke. 1988. Posttranslational modifications of the two 26 kDa and 21 kDa protein subunits in hepatic gap junctions. *Eur. J. Cell Biol.* 46(Suppl.):22–75.

Walter, U., M. R. C. Costa, X. O. Breakefield, and P. Greengard. 1979. Presence of free cyclic AMP receptor protein and regulation of its level by cyclic AMP in neuroblastoma-glioma hybrid cells. *Proc. Natl. Acad. Sci. USA.* 76:3251–3255.

Willecke, K., O. Traub, J. Look, R. Stutenkemper, and R. Dermietzel. 1988. Different protein components contribute to the structure and function of hepatic gap junctions. *Mod. Cell Biology.* 7:41–52.

Yancey, S. B., B. J. Nicholson, and J.-P. Revel. 1981. The dynamic state of liver gap junctions. *J. Supramol. Struct. Cell. Biochem.* 16:221–232.

Young, J. D., Z. A. Cohn, and N. B. Gilula. 1987. Functional assembly of gap junctional conductance in lipid bilayers: demonstration that the major 27 kDa protein forms the junctional channel. *Cell.* 48:733–743.

Zimmer, D. B., C. R. Green, W. H. Evans, and N. B. Gilula. 1987. Topological analysis of the major protein in isolated intact rat liver gap junctions and gap junction-derived single membrane-structures. *J. Biol. Chem.* 262:7751–7763.