The EMBO Journal vol.3 no.10 pp.2261 – 2270, 1984

Gap junctions in several tissues share antigenic determinants with liver gap junctions

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Communicated by K. Willecke

Using affinity-purified antibodies against mouse liver gap junction protein (26 K), discrete fluorescent spots were seen by indirect immunofluorescence labelling on apposed membranes of contiguous cells in several mouse and rat tissues: pancreas (exocrine part), kidney, small intestine (epithelium and circular smooth muscle), Fallopian tube, endometrium, and myometrium of delivering rats. No reaction was seen on sections of myocardium, ovaries and lens. Specific labelling of gap junction plaques was demonstrated by immunoelectron microscopy on ultrathin frozen sections through liver and the exocrine part of pancreas after treatment with gold protein A. Weak immunoreactivity was found on the endocrine part of the pancreas (i.e., Langerhans islets) after glibenclamide treatment of mice and rats, which causes an increase of insulin secretion and of the size as well as the number of gap junction plaques in cells of Langerhans islets. Furthermore, the affinity purified anti-liver 26 K antibodies were shown by immunoblot to react with proteins of similar mol. wt. in pancreas and kidney membranes. Taken together these results suggest that gap junctions from several, morphogenetically different tissues have specific antigenic sites in common. The different extent of specific immunoreactivity of anti-liver 26 K antibodies with different tissues is likely due to differences in size and number of gap junctions although structural differences cannot be excluded.

Key words: antibodies to 26K gap junction protein/connexons/integral membrane proteins/immunoelectron microscopy/immunofluorescence

Introduction

Gap junctions represent specialized domains of plasma membranes which mediate, *via* protein channels, direct intercellular communication (Loewenstein, 1981), i.e., the so-called ionic and/or metabolic coupling of contiguous cells. This form of intercellular communication appears to be well conserved during the course of evolution. Gap junction plaques, i.e., aggregates of cell-cell channels, are present in phylogenetically ancient multicellular organisms as well as in almost all organs of higher vertebrates (Staehelin, 1974). In mammalian tissues gap junctions show a striking structural homology (cf. Griepp and Revel, 1977; Gilula, 1978). The molecular basis for this morphological homology could only recently be explored when purified gap junction proteins and antisera against them became available. Biochemical, immunochemical and immunohistochemical comparison of gap junctions from rat liver and bovine lens fibers indicated that the two (gap) junction proteins, the 25 K main intrinsic polypeptide (MIP) of lens fibers and the liver gap junction protein which has an apparent mol. wt. of 26 – 27 K must be largely different (Hertzberg *et al.*, 1982; Hertzberg and Gilula, 1982; Paul and Goodenough, 1983). Based on weak immunochemical cross-reaction of affinity-purified anti-mouse liver 26 K antibodies with lens fiber MIP, Traub and Willecke (1982) concluded that the liver and lens gap junction polypeptides may share some structural homology. Recently, Gros *et al.* (1983) isolated a 28 K protein from purified gap junctions of rat hearts and compared it by two-dimensional peptide mapping with the rat liver gap junction protein. No homology between these proteins was found.

During the last few years we have characterized rabbit antiserum raised against the SDS-denatured 26 K protein from mouse liver gap junctions (Traub *et al.*, 1982, 1983). Immunochemical studies with this antiserum indicated specific binding to purified liver 26 K protein, to urea/detergent-treated liver gap junction plaques and to 'native' gap junctions in isolated hepatic plasma membranes (Janssen-Timmen *et al.*, 1983). We wanted to define more precisely by immunocytochemistry the localization of the liver 26 K protein in gap junctions in comparison with other intercellular junctions as well as with non-junctional areas of plasma membranes. Furthermore, we used affinity-purified

Table I. Immunoreactivity of tissues screened with affinity-purified antibodies to the liver 26 K protein

Tissue		Immunoreactivity
Liver		+
Pancreas	Exocrine part	+
	Endocrine part	+/–
Small intestine	Epithelium	+
	Circular smooth muscle	+
Kidney	Tubules	+
	Glomeruli	+/–
Myocardium		–
Ovary	Granulosa cells	–
	Stroma	–
Lens	Epithelium	–
	Fiber cells	–
Fallopian tube	Epithelium	+
	Smooth muscle	+
Uterus	Endometrium	+
	Myometrium	+/–

The results obtained by immunofluorescence microscopy are reported in relative terms in order to compare the results of different experiments. Positive immunoreactivity as defined in the text is represented by +, no immunoreactivity is represented by –. The results of +/– immunoreactivity are discussed in the text.

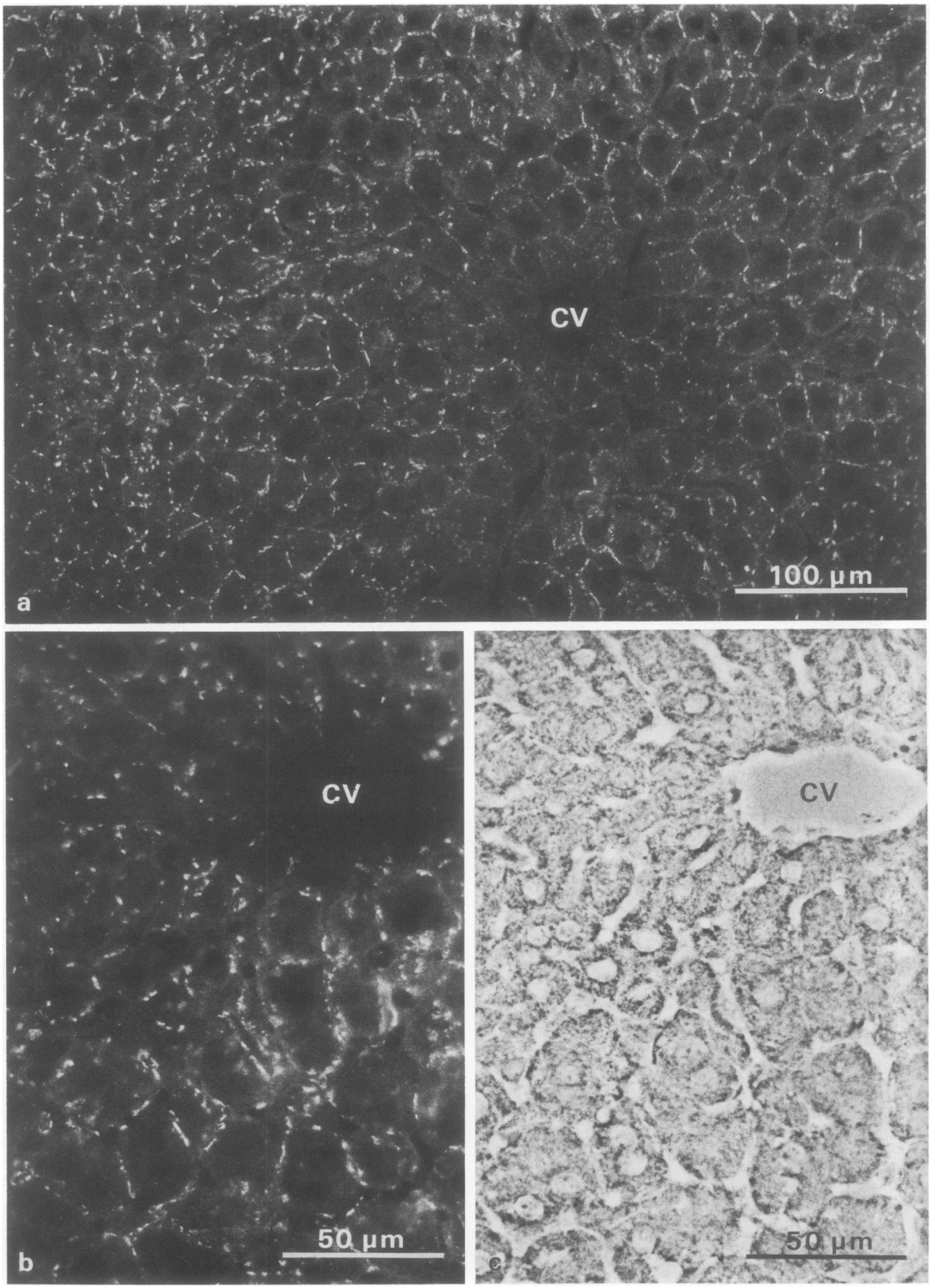

Fig. 1. Immunofluorescence in liver sections stained with affinity-purified anti-26 K antibodies (**a**,**b**). Note the variability in length of the fluorescent membrane zones. Fluorescence is located in contact plasma membranes of contiguous cells. (**c**) Phase contrast micrograph of (**b**) for better identification of structural details. CV, central vene.

anti-liver 26 K antibodies to study their cross-reactivity with gap junction plaques and proteins from different tissues.

Results

Immunofluorescence: affinity-purified anti-liver 26 K antibodies react specifically with discrete areas on apposed membranes of contiguous cells in several tissues

Indirect immunofluorescence was performed on sections through various tissues. The characteristics of the labelling pattern of some of them will be described in detail below. Table I presents a summary of the results, including negative findings. The immunofluorescence reaction was concluded to be positive and specific when discrete fluorescent spots were found on membranes of contiguous cells after incubation with affinity-purified anti-26-K antibodies. The pattern of these fluorescent spots was characteristic for each tissue studied. Occasionally autofluorescence and weak immunofluorescence after incubation with the IgG fraction of normal rabbit serum was noticed. Both artefacts, however, could be clearly discriminated from the pattern of specific labelling.

For comparison with tissues described below, Figure 1 illustrates the immunofluorescence obtained with affinity-purified anti-liver 26 K antibodies on liver tissue. Considerable variation in the extent to immunolabelling was noticed. The fluorescent spots, however, were always located on apposed plasma membranes of contiguous cells. Besides small dots, fluorescent zones were found covering maximally ~1/5 of the perimeter of the cell. This pattern of immunofluorescence is in agreement with the extension and localization of liver gap junctions described for ultrathin-sectioned and freeze-fractured material (Goodenough, 1976; Griepp and Revel, 1977). All the results of immunofluorescence reported in this paper were obtained with affinity-purified anti-26 K antibodies which showed much lower unspecific binding compared with the crude antiserum (Janssen-Timmen *et al.*, 1982).

Pancreas (exocrine and endocrine)

The exocrine pancreas exhibited an immunofluorescence pattern analogous to that of liver. The fluorescent spots, however, appeared to be more dispersed and did not show the extensive variability in size as seen in liver (Figures 1 and 2). In the endocrine part of the pancreas we failed to demonstrate recognizable immunolabelling (Figure 2). Islets of Langerhans appeared as 'black holes' in the surrounding fluorescent 'firmament' of the exocrine pancreas. To ascertain whether the lack of labelling in the islets depended on the rare occurrence and minute size of gap junctions in this tissue (Orci *et al.*, 1973) we checked the immunofluorescence pattern after glibenclamide treatment of the animals. This treatment has been shown by Meda *et al.* (1979, 1983) to stimulate insulin secretion in rats and to cause a 2.3-fold increase in size and number of individual cap junctions between Langerhans islets cells. In our studies of immunofluorescence after glibenclamide treatment tiny fluorescent spots occasionally became visible on apposed membranes of some islet cells. Apparently the detection of gap junctions under these conditions is near the limit of sensitivity of this method.

Small intestine, kidney and female genital tract

In the epithelium of the small intestine, fluorescent spots were consistently found along lateral areas of the cell membrane. They resided mostly in the perinuclear region of the cell membrane and became more prominent in oblique sections through the epithelium (Figure 3a–c). In some cases, when grazing sections along the epithelium passed through apical regions, an ordered arrangement of fluorescent spots was seen close to the sites of the junctional complexes (which consists of tight junctions, intermediate junctions, desmosomes) (Figure 3A). Immunoreactivity in the small intestine was not restricted to the epithelial layer. Strong fluorescent spots were also found in the submucous stratum and in the smooth muscle layers. Here the spots occurred preferentially along the long axis of the smooth muscle cells with variable size and intensity (Figure 3e). Clear dominance of immunoreactivity was found in the circular muscle layer, as expected from earlier electron microscopic investigations of the corresponding gap junctions (Gabella and Blundell, 1979). In the longitudinal muscle layer immunofluorescence labelling occurred only scarcely (Figure 3e). In kidney, different intensities of specific fluorescent labelling were found in different parts of the epithelium of the tubules. Immunoreactivity was most prominent in the epithelium of the proximal tubules, while the distal parts and the collecting tubules showed scarce and infrequent labelling. Tiny spots occasionally occurred within the glomeruli. Again, as in the epithelium of the small intestine, fluorescence became most apparent when the epithelium of the tubules was cut tangentially (Figure 3d).

Furthermore we examined the endometrium and myometrium of rat and mouse for anti-26 K immunoreactivity. Gap junctions occur only infrequently in the myometrium of non-pregnant animals of both species. After stimulation with estrogen or during delivery, a dramatic increase of gap junction frequency had been reported (Dahl and Berger, 1978; Garfield *et al.*, 1980). Our immunofluorescence findings confirm these observations. Non-pregnant rats showed only very rare immunolabelling of the myometrium whereas relatively strong immunofluorescence was detected in delivering animals. The results of freeze fracture studies confirmed the increase in the number of gap junctions at that particular stage. In the myometrium of delivering mice, however, we did not see the expected increase in immunofluorescence. The reasons for this discrepancy (see Dahl and Berger, 1978) are not clear but could be due to masking of the corresponding antigens.

The affinity-purified anti-26 K antibodies were also used for analyzing the immunoreactivity on mouse and rat ovary, myocardium and lens tissue. None of these tissues proved to be positive although different concentrations of the antibodies, different times of incubations and different fixations of the tissues were used (Figure 5c, d, e). Masking of the corresponding antigenic determinants, however, possibly due to different configurations, cannot be excluded. Table I summarizes the different tissues assayed for immunoreactivity with affinity-purified anti-26 K antibodies. Some other positive results (i.e., endometrium, epithelium and muscle layer of the Fallopian tube) are not documented on the figures since the strong yellow autofluorescence within these tissues rendered the specific green FITC-fluorescence on black and white photographs ambiguous.

Immunocytochemistry on ultrathin frozen sections of liver and the exocrine part of pancreas

We wanted to investigate the distribution of the 26 K protein in gap junctions, other intercellular junctions and in non-junctional areas of sectioned tissues. Ultrathin frozen sections of liver (70–100 nm) exposed to affinity-purified anti-26 K antibodies and Au_5-protein A exhibited consistent labelling of

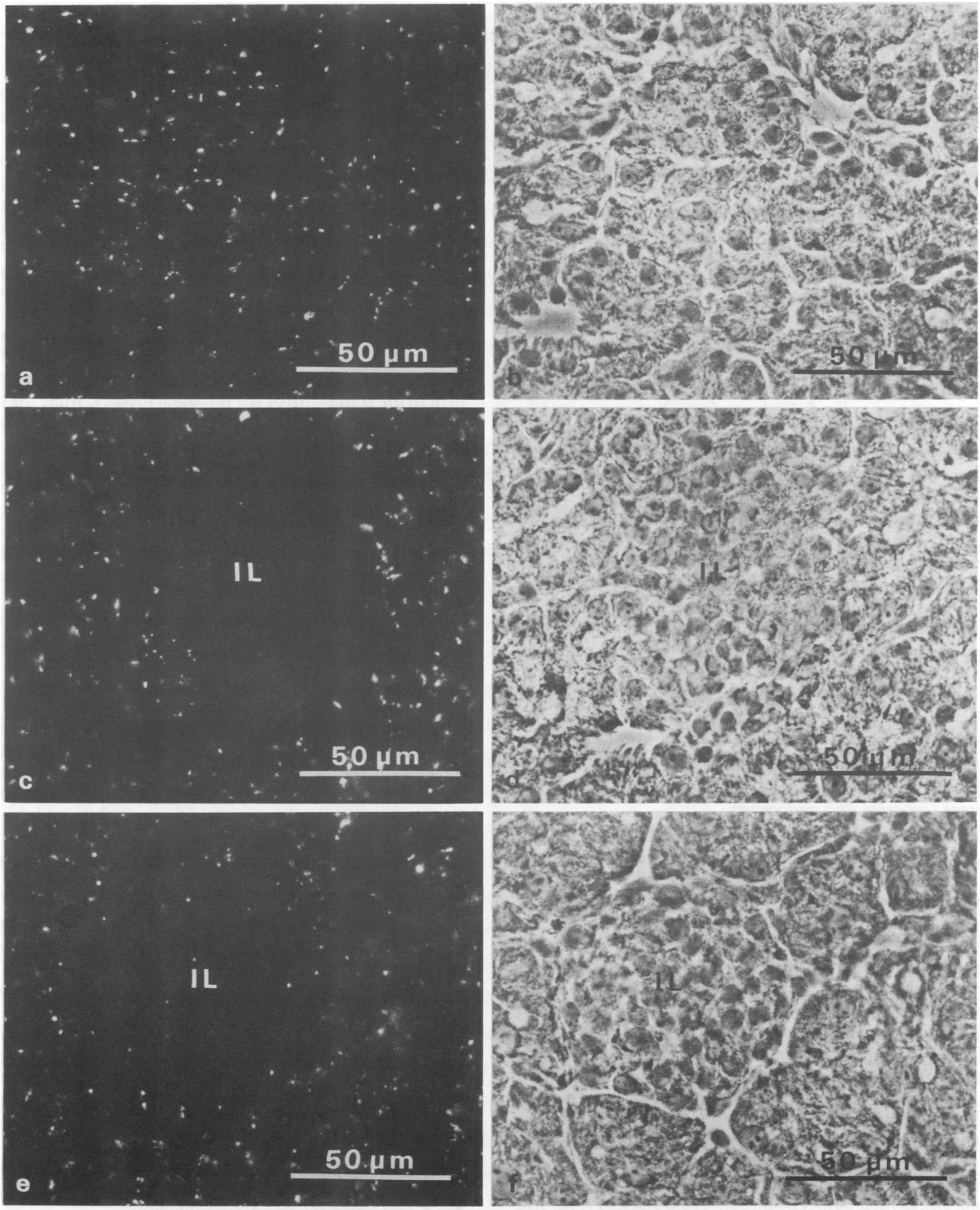

Fig. 2. Anti-26 K immunoreactivity in the exocrine (**a**) and endocrine part (**c,e**) of pancreas. Size and number of fluorescent spots in the exocrine part is smaller than in liver tissue but confined to the cell membrane. (**c**) Islet of Langerhans (IL) lacking any detectable immunoreactivity. (**e**) Treatment with glibenclamide (see Materials and methods) causes some immunoreactivity on islet cell membranes. (**b,d,f**) Represent phase contrast micrographs corresponding to the left hand side immunofluorescence pictures (**a,c,e**).

gap junctions. Gold particles were found to be localized along the cytoplasmic side of the gap junction domains (Figure 4). The intensity of labelling varied according to the incubation protocol and the reagents used, but the pattern of labelling never changed. The use of affinity-purified antibodies led to a drastic reduction of background labelling. The intensity of

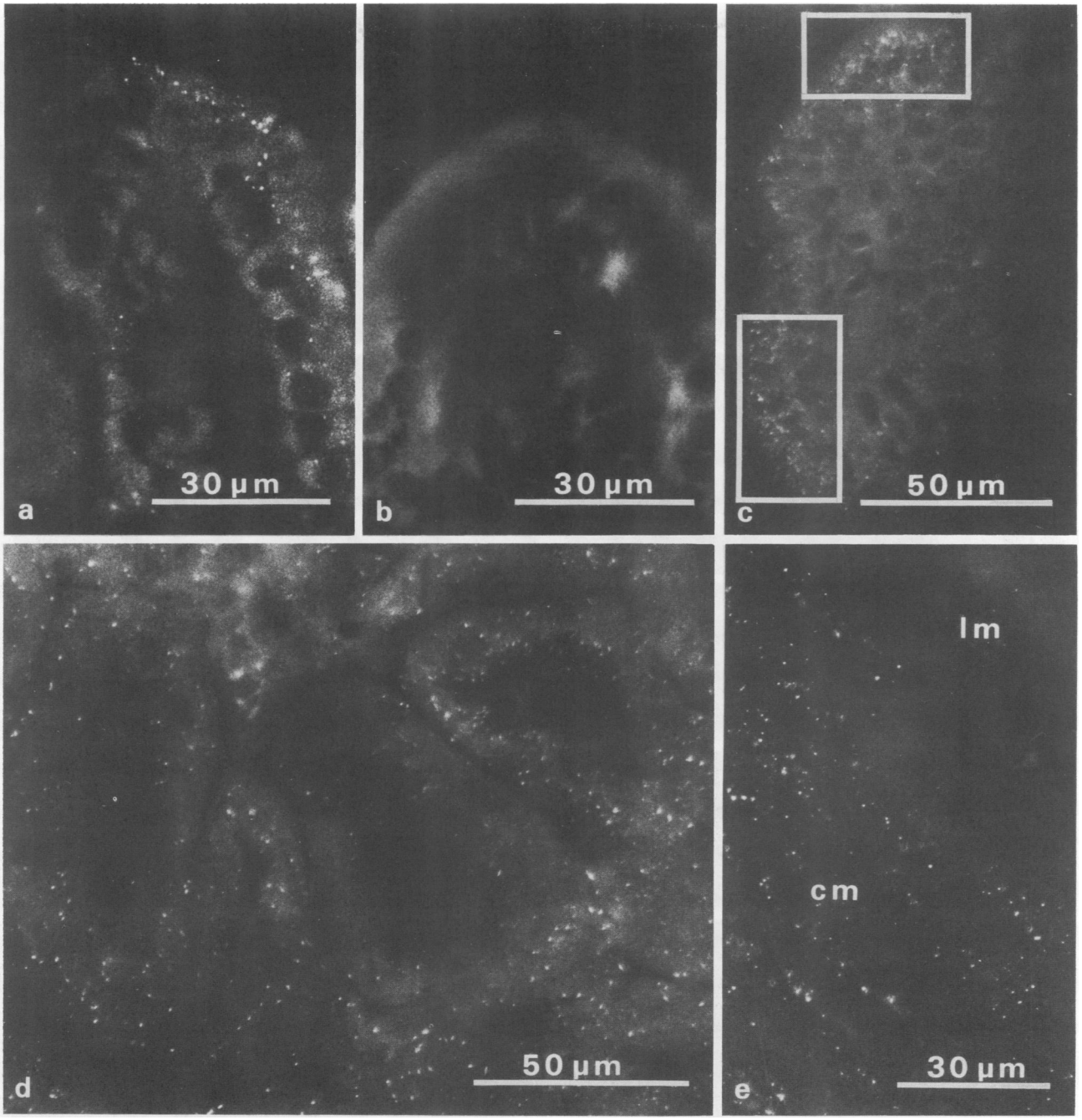

Fig. 3. Immunoreactivity in the epithelium of the small intestine (**a**,**c**) and control incubation with normal rabbit IgG (**b**). Fluorescent spots are located in the apical and perinuclear zone of cell membrane. Cross-section of a villus (**c**), outlined areas show tangentially cut apico-lateral membrane zones with a variable amount of tiny fluorescent spots. Control incubation (**b**) shows no immunoreactivity. (**d**) Cross-section of kidney tubules. Distinct immunoreactivity is prevalent in the epithelial lining of the tubules. (**e**) Positive immunoreactivity in the circular muscle layer (cm) of small intestine (duodenal part). The fluorescent spots often occur in chains orientated along the longitudinal axis of the smooth muscle cells. Note that the longitudinal muscle layer (lm) is devoid of immunofluorescence.

labelling was quantified by counting the gold particles per μm^2 over gap junction plaques and over non-junctional membrane areas. Table II summarizes the data used to compute the density of particles in gap junctions and non-junctional areas. Relative high numbers of gold particles were found within 40 nm wide areas along the cytoplasmic leaflets of the junctional membranes. This could be due to the spacing effect of the IgG protein-A complexes which become located between the antigenic determinants and the gold beads and to the fact that the gold beads can penetrate into ultrathin frozen sections (cf. Roth, 1982). Presumably this effect leads to binding of Au_5-protein A complexes to antigenic sites in deeper layers of the sections and thus could account for the scattering of the gold beads along their antigenic targets. Therefore all gold beads in 40 nm wide areas along both sides of the membrane were counted. In addition gap junctions cut obliquely were brought in a perpendicular position by tilting the sections with the use of a goniometer cartridge. Our data indicate that the affinity-purified anti-26 K antibodies react with liver gap junction plaques 8-fold better than with non-junctional areas on plasma membranes of ultrathin sectioned tissue. Under certain experimental conditions gap junction

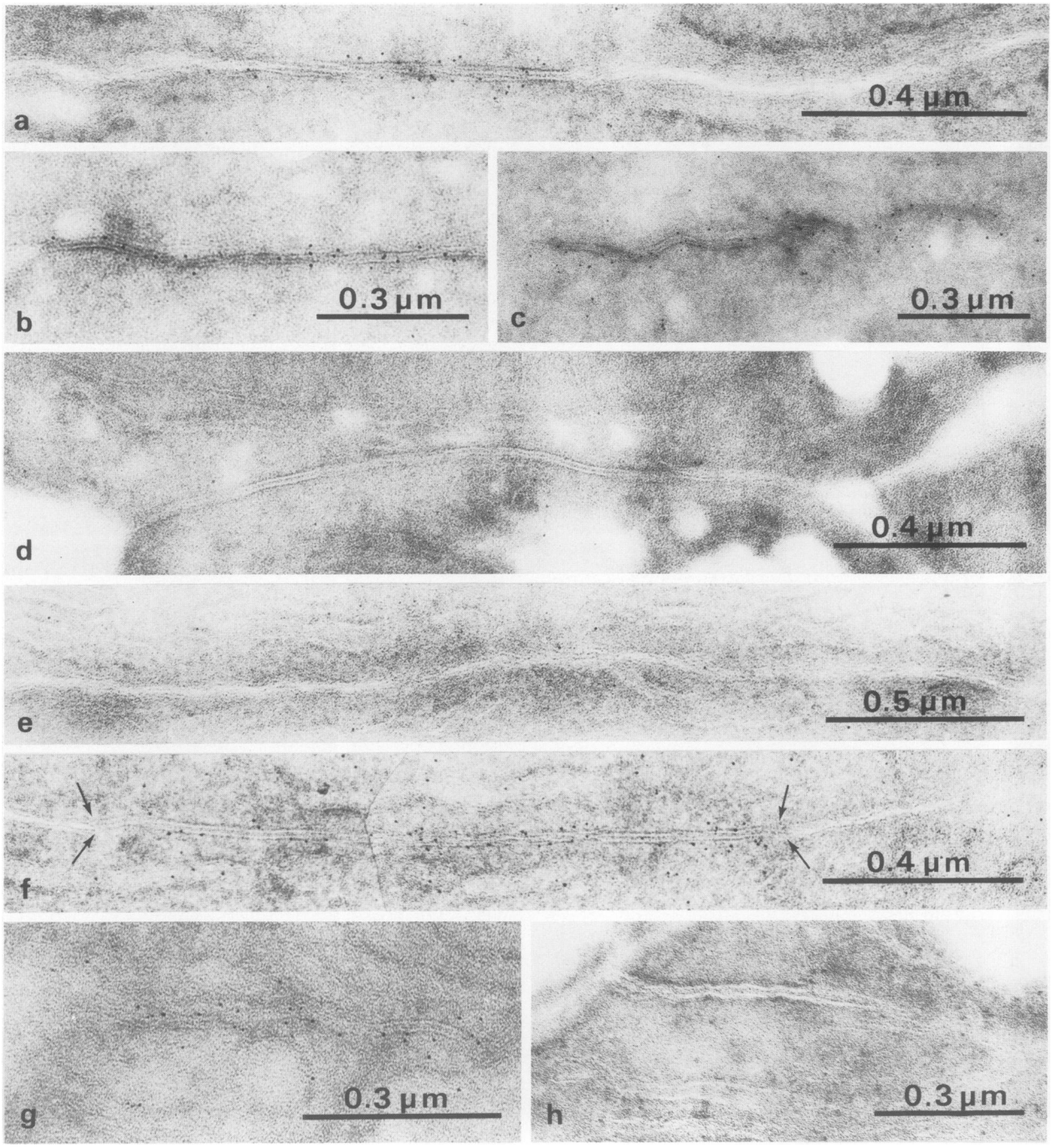

Fig. 4. Ultrathin cryo-sections of liver (**a**–**d**) after incubation with the antibodies and Au_5-protein A. (**a,b,c**) Representative samples of Au_5-protein A labelling of gap junctions after incubation with affinity-purified anti-26 K antibodies. Significant labelling is apparent on cross-sectioned gap junction plaques when compared with the non-junctional membrane area. (**d**) Control incubation with rabbit IgG, lack of labelling. (**e**–**h**) Ultrathin cryo-sections of exocrine pancreas. (**e**) No labelling in the non-junctional area after incubation with affinity-purified anti-26 K IgG. (**f,g**) The gold particles are concentrated along the cytoplasmic side of cross-sectioned gap junctions. Arrows indicate the transition from the junctional to the non-junctional area; note the abrupt change in labelling density. (**h**) Control incubation with normal rabbit IgG.

areas appear to have a low affinity even for control IgG, a fact which may have contributed to the findings by Willingham *et al.* (1979). The extraordinary variability in length of labelled gap junction domains furthermore substantiated our immunofluorescence results (Figure 1). Frozen thin sections that included tight junction areas or desmosomes showed that no 26 K protein was immunochemically detectable in these regions. It had previously been reported after freeze fracture analysis that tiny gap junctions are located within the facets of tight junction contact zones (Yee and Revel, 1978). Since we did not see any specific immunogold label in this region our experimental approach is presumably too intensive to label relatively small gap junction plaques. Alternatively these small gap junctions may not be accessible to the antibodies.

Frozen thin sections of the exocrine part of the pancreas showed a similar pattern of immunolabelling as seen in liver. Again immunoreactivity was solely seen along gap junction domains (Figure 4f, g). Although the labelling index was slightly lower relative to liver gap junctions, binding to pan-

Table II. Binding of affinity-purified anti-26 K antibodies labelled with gold-protein A to gap junction plaques and membrane areas on ultrathin frozen sections of liver and exocrine pancreas

	Liver		Pancreas	
Gap junction area (μm^2)	2.22	(0.65)	0.66	(1.56)
Number of gold beads counted per μm^2 ± SD	300 ± 90	(26 ± 24)	265 ± 80	(10 ± 9)
Non-junctional membrane area (μm^2)	2.36	(0.59)	2.31	(1.62)
Number of gold beads counted per μm^2 ± SD	37 ± 19	(1 ± 1)	17 ± 11	(8 ± 8)

Numbers in parentheses represent results of controls obtained with normal rabbit IgG.

creas gap junctions is significantly higher when compared with non-junctional membrane areas (Table II). In addition to immuno-gold labelling of gap junctions in the exocrine part of the pancreas, similar results were obtained by immunoperoxidase staining (D. Drenckhahn, unpublished experiments).

Immunoblot: affinity-purified anti-liver 26 K antibodies react with 26 K proteins in plasma membranes of pancreas and kidney

Figure 6 shows autoradiographs of immunoblots after incubation with [^{125}I]protein A. The affinity-purified anti-liver 26 K antibodies recognized proteins of similar apparent mol. wt. in plasma membranes of pancreas and kidney. Based on comparisons of several immunoblots we conclude that the cross-reaction of the anti-liver 26 K antibodies with the 26 K band in pancreas and kidney membranes is ~20-fold weaker than with the liver 26 K protein. This can be due to less gap junction protein in pancreas or kidney and/or it may reflect structural differences between gap junction protein in different tissues. We do not know why at least two non-26 K proteins react with affinity-purified anti-26 K antibodies as well as with normal IgG. However, normal IgG did not react with the 26 K proteins in liver, pancreas, or kidney membranes (Figure 6).

Discussion

The patterns of indirect immunofluorescence seen on cross-sections of several tissues clearly reflect the arrangement and frequency of gap junction plaques known from electron microscopy of freeze-fractures and ultrathin-sections of the respective tissue (cf. Griepp and Revel, 1977; Orci, 1982). The characteristic pattern of immunofluorescence was not seen when the anti-liver 26 K antibodies were absorbed with purified mouse liver gap junction plaques. This result underlines the specificity of the immunoreaction. Further supportive evidence for the specificity was obtained by immunolabelling of ultrathin cryosections with gold-protein A.

Significant binding of gold particles to the cytoplasmic surface of gap junctions from pancreas and liver was obtained. Non-junctional membrane areas showed no cross-reactivity with the anti-26 K antibodies. These findings extend our previous studies which showed binding of anti-26 K antibodies to urea/detergent-treated purified gap junction plaques and to native gap junction domains on isolated liver membranes (Janssen-Timmen *et al.*, 1983). The lack of binding to non-junctional areas of plasma membranes indicates either that the 26 K protein exists in these areas in too low an amount to be detected or that it exists in a conformation which differs from that of the same protein within the plaques. Recently, Paul and Goodenough (1982) and Fitzgerald *et al.* (1983) demonstrated by immunocytochemistry that the putative gap junction protein (MIP) of lens fibers is distributed throughout the plasma membrane of the lens fiber cell with no apparent distinction between junctional and non-junctional membrane areas. The relative high concentration of MIP in non-junctional areas of fiber cell membranes compared with liver membranes may be due to the >10-fold higher concentration of gap junction plaques on lens fiber membranes. Although gap junction proteins from liver and lens must be different polypeptides it is not clear at present to what extent the corresponding gap junctions are structurally and functionally homologous (Hertzberg *et al.*, 1982; Hertzberg and Gilula, 1982; Traub and Willecke, 1982). Antibodies against proteins associated with adhaerens junctions and desmosomes have been used for characterization of the corresponding intercellular junctions in epithelial cells (Kartenbeck *et al.*, 1982).

Our immunofluorescence and immunochemical demonstration of cross-reactivity of anti-liver 26 K antibodies with gap junctions of different tissues suggests structural homologies of gap junctions in different tissues. Homology has previously been proposed on the basis of findings that cultured cells from different tissues can readily establish cell-cell communication (Michalke and Loewenstein, 1971; Azarnia and Loewenstein, 1971). Although we have so far tested only a limited number of tissues for cross-reactivity with anti-liver 26 K antibodies, it is significant that the tissues tested are morphogenetically different. Immunoreactivity was found in tissues of endodermal as well as of mesenchymal origin. The lack of a clear immunoreactivity in Langerhans islets appears to be due to a lack of sensitivity rather than that of immunogenicity. Weak immunofluorescence spots were seen on membranes of Langerhans islets after glibenclamide treatment of mice and rats.

The lack of cross-reactivity with some other tissues which are known to possess a considerable amount of gap junction plaques, for example heart muscle and granulosa cells, is noteworthy. Recently, Hertzberg and Skibbens (1983) reported that a sheep antiserum directed against the 27 K gap junction protein from rat liver cross-reacted with a protein of intercalated discs in rat heart. This protein co-migrated with the rat liver gap junction polypeptide. If it holds true that this polypeptide from heart muscle represents the major gap junction protein of these cells the apparent difference in reactivity of rabbit anti-26 K antibodies used in this study and those of Hertzberg and Skibbens (1983) could be due to the recognition of additional antigenic determinants by the latter antibodies. Alternatively we cannot exclude that the gap junction protein in our preparations of myocardium and ovaries was masked and not accessible to the anti-26 K antibodies. The 28 K protein isolated by Gros *et al.* (1983) from rat heart gap junctions does not react with the rabbit anti-liver 26 K antibodies when analyzed by immunoblot (O. Traub and D. Gros, unpublished results). At present we do not know the extent of structural homology represented by immunoreactive sites common to gap junctions in different tissues. Qualitative as well as quantitative differences between gap junction plaques in different tissues could explain the observed differences in immunoreactivity. Although this problem can be further studied by monoclonal and oligopeptide-specific anti-

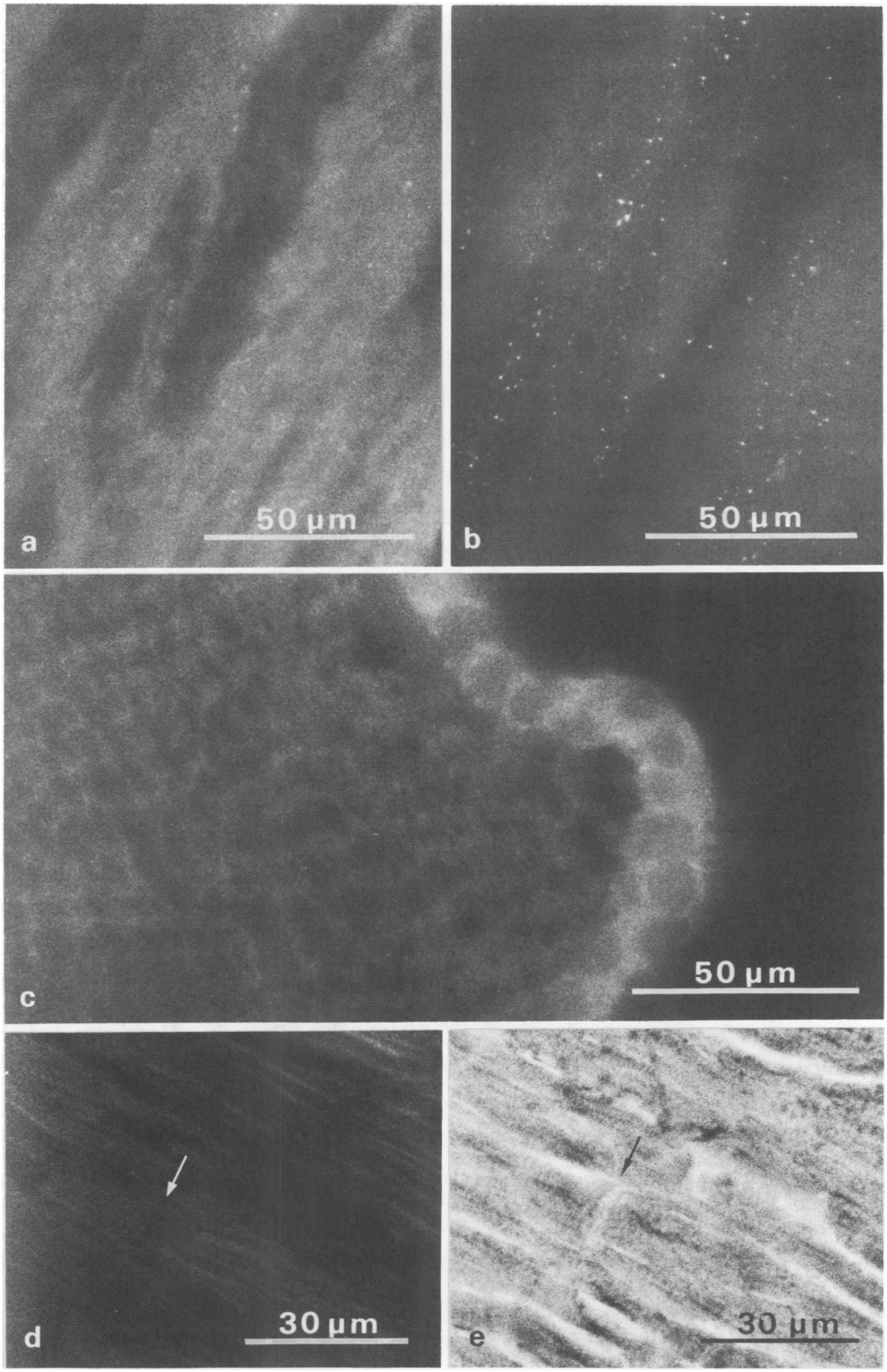

Fig. 5. Immunofluorescence in myometrium sections of a non-pregnant rat (**a**) compared with a pregnant rat under delivery (**b**). Notice the increase of the fluorescent spots under the latter condition. (**c**–**e**) Negative findings of specific immunoreactivity: in lens fiber cells (left hand part of **c**), lens epithelium (**c**), myocard immunofluorescence (**d**) and the corresponding phase contrast photomicrograph (**e**). The arrows mark the position of an intercalated disc in the myocardium which does not show any immunoreactivity after incubation with affinity-purified anti-liver 26 K antibodies.

bodies, final answers can only be expected when total amino acid sequences of gap junction proteins from different tissues become available.

Materials and methods

Animals

BALB/c or NMRI-mice (12–14 weeks old) and Wistar rats were used in this study. Animals had free access to food and water. They were anaesthetized

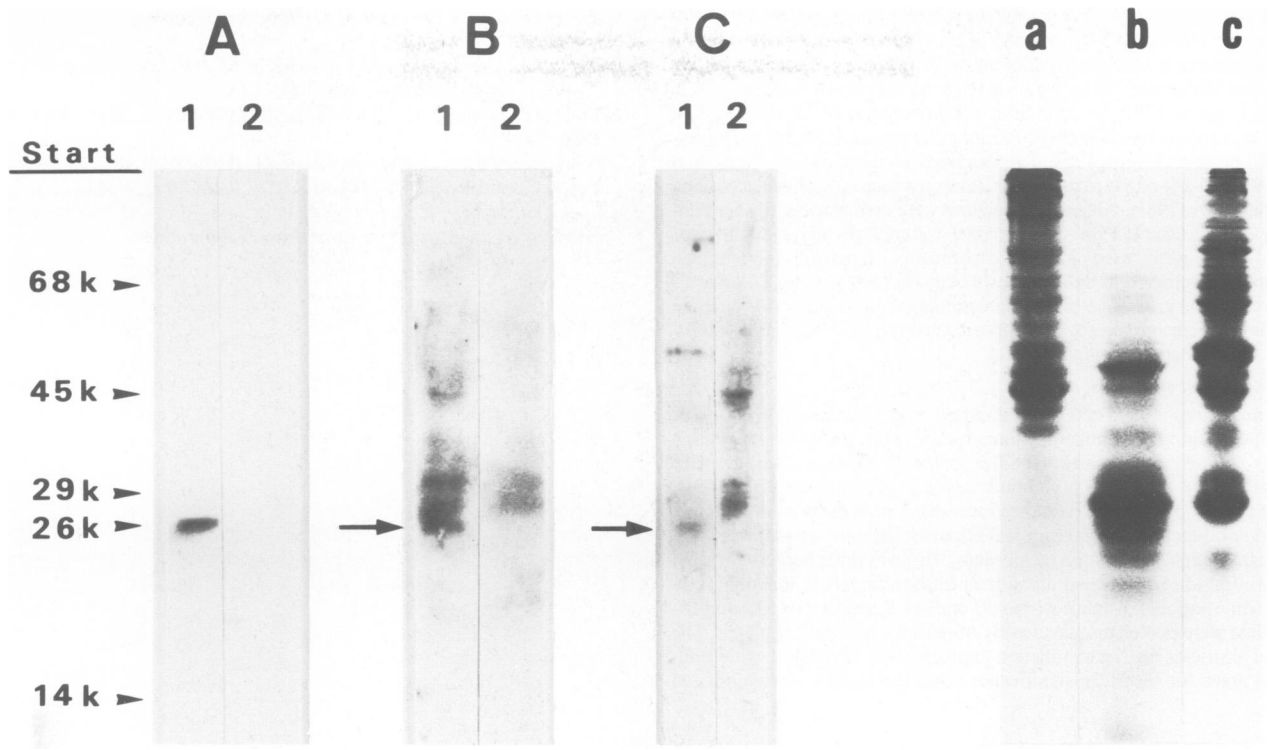

Fig. 6. Immunoblot of total membrane proteins from mouse liver (**A**), pancreas (**B**) and kidney (**C**) using affinity-purified anti-liver 26 K antibodies (**1**). Controls (**2**) were performed with IgG from non-immunized rabbits. Each lane was incubated with 5–20 μg affinity-purified antibodies or normal IgG, respectively, after SDS-electrophoresis of 150–200 μg membrane protein. After incubation with [^{125}I]protein A the nitrocellulose paper was processed for autoradiography. Due to the different intensities of the 26 K bands from different tissues, the autoradiographs of **B** and **C** were exposed for 48 h and 120 h, respectively, compared with an exposure time of 3 h for autoradipgraph **A**. Arrows indicate the position of 26 K protein band which only react with anti-liver 26 K antibodies and not with normal IgG. For comparison with the bands on the autoradiographs, **lanes a, b, c** illustrate Coomassie blue stained gels of the electrophoretic runs **A, B** and **C**, respectively, after capillary blot to nitrocellulose paper.

with chloroethyl ether and perfused with cold phosphate buffered saline (PBS, pH 7.2), *via* the left ventrical until the efflux of the right ventrical was blood-free. Some of the animals were kept pregnant and killed during delivery. Various organs (see Table I) were dissected, briefly rinsed in cold PBS and quenched in liquid propane cooled by liquid nitrogen.

Glibenclamide treatment of mice and rats

Injections of glibenclamide were given under conditions similar to those described by Meda *et al.* (1979). Mice (25 g each) and rats (200 g each) were intraperitonally injected with Euglucon N (Boehringer/Hoechst), which contained the effective substance glibenclamide, at a concentration of 0.2 mg glibenclamide/100 g body weight of the animal. The application time was 2.5 days with injections given every 12 h. After glibenclamide treatment the animals were perfused as described above and their pancreates were dissected.

Affinity purification of rabbit anti-26 K antiserum

Rabbit antiserum against SDS-denatured mouse liver gap junction protein (26 K) was raised as described in an earlier report (Traub *et al.*, 1982). For the preparation of an affinity column, 1 mg of purified mouse liver gap junction plaques were dissociated in 200 μl $NaHCO_3$ buffer (0.1 M), pH 8.3, containing 0.5 M NaCl and 2%, w/v SDS. Undissociated gap junctions (cf. Henderson *et al.*, 1979) were centrifuged at 15 000 *g* for 35 min and the supernatant was diluted to reach a final SDS concentration of 0.2%. This solution (2 ml) was sonicated with a Branson sonifier (30 W, 5 x 5 s) and coupled to cyanobromide-activated Sepharose 4B (Pharmacia) according to the Pharmacia coupling protocol. The crude rabbit anti-26 K serum was applied onto this column and washed first with 0.5 M NaCl in order to eliminate unspecific binding and second with 3 M KSCN (pH 6.4) in order to desorbe specific anti-26 K antibodies. The eluted antibodies were dialyzed against PBS and concentrated with Aquacide II-a (Calbiochem) to a final volume of ~1 ml. For longer storage the antibodies were lyophilized and dissolved in smaller volumes in order to give protein concentrations of at least 1 mg/ml. 1 ml of crude anti-26 K serum yielded 500–1000 μg of affinity-purified antibodies. The affinity-purified anti-26 K antibodies showed 7- to 10-fold higher specific activity than the anti-26 K IgG fraction when binding to purified liver gap junction plaques was determined by an enzyme immunoassay (Traub *et al.*, 1982). Rabbit-IgG was purified by column chromatography on protein A-Sepharose (Pharmacia).

Immunoblot

Gel electrophoreses and immunoblots were initially performed as described by Traub *et al.* (1982). Later the incubations with antiserum were carried out in the presence of 0.5 M NaCl and 0.5% Triton X-100 or 1% bovine serum albumin (BSA) in order to decrease unspecific binding of antibodies.

Immunofluorescence microscopy

Cryosections (10 μm thick) were obtained on a Bright Cryostat at −25°C, placed on coverslips, fixed in absolute ethanol for 6 min at −25°C, and rinsed in PBS. After an additional washing in PBS with 0.1% BSA as an additive to the washing buffer, the sections were incubated with 35 μl of affinity-purified anti-26 K antibodies (Janssen-Timmen *et al.*, 1983). For controls, the anti-26 K antibodies were replaced by rabbit preimmune IgG (5 μg/ml), or by the anti-26 K antibodies previously absorbed to mouse liver gap junction plaques. For absorption, affinity-purified antiserum (2 μg) had been incubated with purified gap junction plaques (5 μg) for 1 h at room temperature with occasional gentle agitation. The absorbed antibodies were pelleted for 20 min at ~15 000 *g*. Aliquots of the supernatant were incubated with cryosections of liver, pancreas or kidney. The secondary antiserum (sheep anti-rabbit IgG) had been affinity purified, conjugated to fluorescein isothiocyanate, and was used as 3 μg in 35 μl PBS per tissue section on each side. Fluorescence microscopy of the labelled sections was done with a photomicroscope III (Zeiss) equipped with EP-illumination and appropriate filters. Kodak 3X-films served as photomaterial.

Immunoelectron microscopy

Immuno gold-labelling of ultrathin frozen sections, ultracryotomy of frozen liver and pancreas was performed as described by Tokuyasu (1980) with minor modifications. Briefly, fixation was done by vascular perfusion with 2% formaldehyde plus 0.1% glutaraldehyde in PBS (pH 7.4) for 5 min, and the pancreas was removed. Fixation was continued by immersion in 2% formaldehyde for 1 h. The tissue was immersed in a solution of gelatine (10%) at 40°C. This gelatine was solidifed on ice and cross-linked with PBS-buffered glutaraldehyde (0.5%) (Slot and Geuze, 1981). Blocks were stored in a mixture of 2% formaldehyde and 2.3 M sucrose in PBS at 4°C. Before freezing in liquid propane, fragments of the gelatine-embedded tissue were immersed in 2.3 M sucrose which served as an cryoprotectant. Sectioning was performed with a LKB-Cryokit attachment at −50°C to −70°C. Sections were taken

up with a droplet of 2.3 M sucrose containing 10% gelatine in PBS. After rinsing in glycine buffer (0.01 M in PBS, pH 7.4) sections were incubated on droplets of gelatine for 5 min to reduce unspecific labelling. Primary antibody labelling was performed using 10 μl droplets of affinity-purified anti-26 K antibodies (1 μg/m PBS) for 15 min at room temperature. Protein A-gold was used as a secondary marker for labelling the bound antibodies. The colloidal gold-protein A complexes were prepared as described by Faulk and Taylor (1971). Sizing of the particles was done on a sucrose gradient according to Slot and Geuze (1981). All particle fractions were used without further dilution after dialysis against PBS. Gold-protein A complexes with a diameter of 4–5 nm (Au_5) were used in our experiments. Contrast for electron microscopy was achieved as described by Tokuyasu (1980). After Tokuyasu's absorptive staining grids were picked up with platine loops and dried at room temperature. Electron microscopy was performed using a Philips EM 400 fitted with a goniometer cartridge.

Morphometry and statistics

Electron micrographs were taken at a standard magnification of 46 000-fold. Gold particles along gap junction domains and non-junctional membrane areas were counted after a final magnification of 80 000-fold. Morphometry and statistics were done with a computer-aided digitizer system (Basis 108). Counting of gold particles on tissue sections was done directly over gap junctional and non-junctional membrane leaflets and it included 40 nm wide lanes on both cytoplasmic sides of the membranes. This was done because the gold beads were found to be located along their antigen targets in junctional domains at some variable distance within 40 nm (see Results). For each experiment all data were pooled and the density of particles per μm^2 calculated. The density of particles near gap junction domains was compared with non-junctional areas for statistical significance using the Student's test unpaired samples.

Acknowledgement

The technical assistance of D. Schünke, P. Kalweit and B. Karow is gratefully acknowledged. The authors are indebted to Dr. D. Drenckhahn (Marburg) who contributed to the initial work by immunochemical investigations and by discussions and critical reading of the manuscript. This work was supported by grants of the Deutsche Forschungsgemeinschaft (SFB 114) to R.D. and by the Ministerium für Wissenschaft und Forschung, Düsseldorf, as well as by the Stiftung Volkswagenwerk to K.W.

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Received on 16 April 1984; revised on 29 June 1984