Antisera Directed against Connexin43 Peptides React with a 43-kD Protein Localized to Gap Junctions in Myocardium and Other Tissues

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Abstract. Rat heart and other organs contain mRNA coding for connexin43, a polypeptide homologous to a gap junction protein from liver (connexin32). To provide direct evidence that connexin43 is a cardiac gap junction protein, we raised rabbit antisera directed against synthetic oligopeptides corresponding to two unique regions of its sequence, amino acids 119–142 and 252–271. Both antisera stained the intercalated disc in myocardium by immunofluorescence but did not react with frozen sections of liver. Immunocytochemistry showed anti-connexin43 staining of the cytoplasmic surface of gap junctions in isolated rat heart membranes but no reactivity with isolated liver gap junctions. Both antisera reacted with a 43-kD polypeptide in isolated rat heart membranes but did not react with rat liver gap junctions by Western blot analysis. In contrast, an antiserum to the conserved, possibly extracellular, sequence of amino acids 164–189 in connexin32 reacted with both liver and heart gap junction proteins on Western blots. These findings support a topological model of connexins with unique cytoplasmic domains but conserved transmembrane and extracellular regions. The connexin43-specific antisera were used by Western blots and immunofluorescence to examine the distribution of connexin43. They demonstrated reactivity consistent with gap junctions between ovarian granulosa cells, smooth muscle cells in uterus and other tissues, fibroblasts in cornea and other tissues, lens and corneal epithelial cells, and renal tubular epithelial cells. Staining with the anti-connexin43 antisera was never observed to colocalize with antibodies to other gap junction proteins (connexin32 or MP70) in the same junctional plaques. Because of limitations in the resolution of the immunofluorescence, however, we were not able to determine whether individual cells ever simultaneously express more than one connexin type.

Gap junctions are membrane specializations that permit the exchange of small metabolites and ions between neighboring cells. While gap junctions and intercellular communication have been studied in a number of cell and tissue types, the structure of gap junctions has been best studied in rat liver where the interhepatocyte gap junctions have been shown to be composed of collections of membrane channels, called connexons, joined in mirror symmetry with connexons in the membrane of the adjacent cell (5, 24). The complete cDNA corresponding to a structural protein of the connexon has been cloned from rat and human liver (22, 32). We have suggested the name connexin32 for this protein, based on its predicted molecular mass of 32 kD (2). Connexin32 has been confirmed as a gap junction protein by structural and functional criteria (6, 32).

Data from cDNA and protein sequencing suggest that there is a family of related connexin proteins (2, 3, 21, 30). We have used low-stringency hybridization to clone an homologous cDNA from a rat heart library, which predicts a protein of 43 kD called connexin43 (2). The size of connexin43 and its amino-terminal sequence closely match those of the major protein in isolated preparations of rat heart gap junctions (23, 25, 26). Comparison of the predicted connexin43 polypeptide with connexin32 shows both conserved and divergent amino acids and suggests that (*a*) conserved amino acids lie in transmembrane and extracellular regions and (*b*) nonconserved amino acids face the cytoplasm.

To provide a direct evidence that the protein corresponding to connexin43 cDNA is a cardiac gap junction protein and to test the topological model, we have raised antisera directed against two synthetic oligopeptides from unique regions of the predicted connexin43 sequence, and we have demonstrated immunoreactivity with a 43-kD protein structurally localized to cardiac gap junctions.

Previous studies have examined the distribution of connexin32 expression by immunohistochemistry and have shown its presence in a number of different tissues but not in all locations where gap junctions are known to occur (8, 19, 31).

Northern blot experiments have shown that the mRNAs for connexin32 and connexin43 have different, but overlapping, distributions in multiple organs (2, 32). In this paper, we have used the specific antipeptide antisera to examine connexin43 distribution in many locations where its mRNA has been detected or where gap junction biology has been studied.

The availability of antisera specific for different connexin molecules permits study of the codistribution of connexin molecules within the same organ, tissue, and cell. Previous studies have shown the intermixing of two different connexin molecules in the same junctional plaques joining hepatocytes by immunofluorescence (30). Miller and Goodenough (28) showed that lens epithelial cells simultaneously express structurally and functionally unique gap junctions with their homologous and heterologous neighbors. In studies reported here, we take advantage of other connexin-specific antibody reagents which have been developed. Polyclonal antipeptide and monoclonal antibodies which specifically recognize connexin32 have been raised (14). A monoclonal antibody has been produced which specifically recognizes MP70, a protein that has been localized at lens fiber junctions and has a related NH_2-terminal sequence, suggesting that it is a member of the connexin family (16, 20, 21). We have used these reagents to determine the distributions of these gap junction proteins.

Materials and Methods

Reagents

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise specified.

Keyhole limpet hemocyanin (KLH)[1] was purchased from Pacific Biomarine Laboratories Inc. (Venice, CA). A peptide representing amino acids 119–142 of connexin43 (DGVNVEMHLKQIEIKKFKYGIEEH) was synthesized by Bachem, Inc. (Torrance, CA). This peptide was demonstrated to be >97% pure by HPLC and amino acid analysis. A peptide representing amino acids 252–271 of connexin43 (GPLSPSKDCGSPKYAYFNGC) was synthesized by Biosearch, Inc. (San Rafael, CA). HPLC analysis of this peptide demonstrated a single major peptide component but purity was not quantitated. HPLC was conducted on a Vydac C18 column after reduction with DTT; elution was with a gradient of 5–100% acetonitrile/0.1% trifluoroacetic acid over a 20-min period.

Preparation of Antisera

The peptides were coupled to KLH using the following procedure in an attempt to achieve a 30:1 molar ratio of peptide to KLH. 780 nmol of peptide were reconstituted in 400 μl PBS. 26 nmol of KLH in 250 μl of PBS were added. 5 μl of 25% glutaraldehyde (Sigma Chemical Co.) were added, and the resultant solution was incubated overnight at room temperature. Some turbidity developed. No efforts were made to further purify the conjugates.

For primary immunizations, peptide–KLH conjugate containing 200 μg peptide was emulsified in Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY) and was injected in duplicate into preimmune bled, female New Zealand white rabbits. All injections were made in multiple paravertebral intradermal sites. After 30 d, the rabbits were boosted with KLH-peptide in incomplete Freund's and were bled 7–10 d later. Rabbits were boosted thereafter at monthly intervals.

Antisera were screened for reactivity with the peptides by a modified ELISA. Unconjugated peptide was adsorbed to small squares of polyvinylidene difluoride membrane (Immobilon; Millipore Continental Water Systems, Bedford, MA). These squares were then blocked with BLOTTO (10% nonfat dry milk in PBS) and reacted with dilutions of test antisera, peroxidase-conjugated protein A (Boehringer Mannheim Biochemicals, Indianapolis, IN) diluted 1:2,000 in PBS, and 0.05% 3,3′-diaminobenzidine, 0.01% H_2O_2 in PBS.

For most experiments, crude antiserum was used. For some experiments, affinity-purified anti-connexin43 252–271 was prepared using peptide attached to activated CH-Sepharose 4B (Pharmacia Fine Chemicals, Uppsala, Sweden) according to manufacturer's directions.

Preparation and characterization of mouse monoclonal antibodies to connexin32 (M12.13) and to the lens protein MP70 (6-4-B2-C6) have been described previously (14, 21).

Preparation and characterization of rabbit antisera to peptides representing amino acids 98–124 (QQHIEKKMLRLEGHGDPLHLEEVKRHK) and 164–189 (RLVKCEAFPCPNTVDCFVSRPTEKTV) of connexin32 have been described elsewhere (14). The antiserum to peptide 98–124 specifically reacts with the cytoplasmic face of isolated rat liver gap junctions. It will be referred to as anti-connexin32 antiserum throughout this paper. The antiserum to peptide 164–189 does not react with native rat liver gap junctions unless they have been split apart using either a urea/alkali or a hypertonic sucrose procedure, suggesting a possible extracellular orientation of this epitope in the junction protein. The peptide used as immunogen for this antiserum contains 59% identical residues to the corresponding portion of the connexin43 sequence (residues 182–208, SAYYTKCRDPCPHQKDCFLSRPTEKTI) (3), suggesting that it might react with both proteins.

Western Blot Analysis

Rat liver gap junctions were isolated as described by Fallon and Goodenough (9) with the modification of the addition of 0.5 mM diisopropylfluorophosphate (Sigma Chemical Co.) to the initial homogenization buffer and the sarcosine solutions. Preparations enriched for intercalated discs were isolated from rat hearts as described by Green and Severs (15), except for the addition of 0.5 mM diisopropylfluorophosphate and 1 mM PMSF (Sigma Chemical Co.) to all solutions. Urea-washed calf lens membranes were isolated according to Paul and Goodenough (33). For analysis of other tissues, freshly dissected rat tissues were homogenized in 50 vol or more of 1 mM $NaHCO_3$/0.5 mM diisopropylfluorophosphate/1 mM PMSF and centrifuged at 10,000 *g* for 10 min; the resulting pellet was resuspended in boiling SDS sample buffer; insoluble material was removed by centrifugation; and the supernatant was applied to the SDS-polyacrylamide gels.

SDS-PAGE was performed using 12% minigels. Immunoblots were prepared essentially as described by Paul and Goodenough (33) except that transfer buffer did not contain SDS, and transfer to nitrocellulose membranes was conducted at 75 V for 1 h. Antibody binding was performed using primary rabbit antisera diluted 1:1,000 and secondary peroxidase-conjugated protein A as described for the ELISA dot assay above. For some experiments, ^{125}I-protein A (Amersham Corp., Arlington Heights, IL) was used, as described by Goodenough et al. (14). In some experiments, to demonstrate the specificity of immunoblot reactivity, diluted antibodies were preincubated with pure peptide at a concentration of 100 μg/ml.

Immunohistochemistry

Immunofluorescence microscopy was performed on rat tissues that were either directly frozen unfixed or were fixed in 1% paraformaldehyde in PBS for 1 h followed by a 1-h incubation in 0.5 M sucrose in PBS for cryoprotection before freezing. Most tissues were obtained from adult rats of either sex. Ovarian tissue was obtained from 21-d-old female rats primed for two successive days with intraperitoneal injections of 10 IU of pregnant mare's serum gonadotropin (Sigma Chemical Co.) in PBS. Uteri were obtained either from pregnant rats in labor or from 21-d-old females who had received five consecutive daily subcutaneous injections of 500 μg of estradiol (Sigma Chemical Co.) in sesame oil. Previous studies have shown a dramatic morphological increase in gap junction structures after these hormonal treatments (1, 13, 23). Small pieces of tissue were rapidly frozen in liquid freon cooled with liquid nitrogen. 5–10-μm cryostat sections were cut and placed on gelatin-coated slides. Sections were reacted with rabbit antipeptide antisera diluted 1:200 or 1:500 in PBS or with straight culture supernatants for the mouse monoclonal antibodies followed by rhodamine- or fluorescein-conjugated goat anti-rabbit or -mouse antisera (Boehringer Mannheim Biochemicals) as appropriate. In some experiments, to demonstrate the specificity of immunolabeling, diluted primary antisera were preincubated with pure peptide at a concentration of 20 μg/ml.

Both anti-connexin43 antisera were used for staining of heart, liver, ovary, and uterus and gave identical results. Anti-connexin43 252–271 was used for all other tissues. Anti-connexin32 98–124 was used for all tissues except the double-labeling experiments in which the anti-connexin32 mono-

1. *Abbreviation used in this paper*: KLH, keyhole limpet hemocyanin.

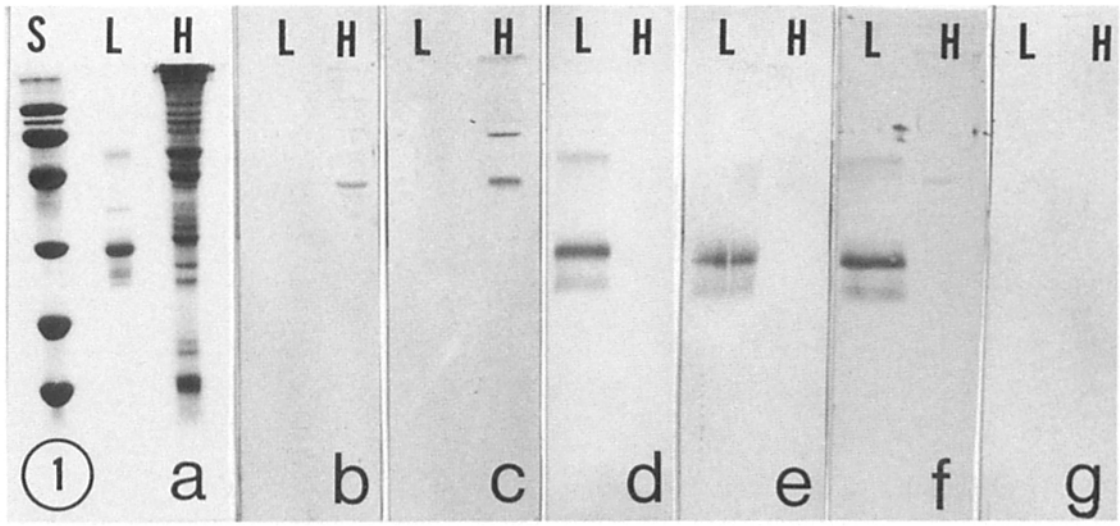

Figure 1. Immunoblot analysis of isolated liver gap junction and enriched cardiac intercalated disc preparations using anti-connexin peptide antisera or monoclonal antibodies. (*a*) Coomassie Blue–stained SDS-PAGE of (lane *S*) molecular mass standard proteins (phosphorylase B, 97 kD; BSA, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD; lysozyme, 14 kD). (Lanes *L* and *H*) Isolated rat liver gap junctions and enriched heart intercalated disc preparations. (*b*) Immunoblot of a gel identical to that in *a*, but without standards, reacted with anti-connexin43 119–142. This antiserum reacts strongly with a 43-kD band in the heart preparations, and weakly with other bands. It does not react with the liver junctions. (*c*) Identical immunoblot reacted with anti-connexin43 252–271. This antiserum also reacts strongly with a 43-kD band in the heart preparations; it also recognizes a band of ~60 kD that is not seen by the other antiserum. This antiserum does not react with the liver junctions. (*d*) Immunoblot reacted with monoclonal anti-connexin32 M12.13. 5% as much liver gap junction protein was loaded as in *a*. This antibody specifically reacts with several forms of the rat liver gap junction protein, including monomer, dimer, and some proteolysis fragments. It does not react with any proteins in the heart preparation. (*e*) Immunoblot reacted with anti-connexin32 98–124. Lane *L* contained 0.5% as much liver gap junction protein as in *a*. This antiserum also reacts specifically with all forms of the liver gap junction protein, but not with any heart proteins. (*f*) Immunoblot reacted with anti-connexin32 164–189. Lane *L* contained 5% as much liver gap junction protein as in *a*. This antiserum reacts with the liver gap junction polypeptides and with a 43-kD polypeptide from the heart, suggesting common structure. (*g*) Immunoblot prepared as in *a* reacted with preimmune serum from a rabbit which was subsequently immunized with connexin43 peptide 252–271. This serum shows no reactivity with any polypeptides from liver or heart.

clonal M12.13 was used to permit the use of different species-specific secondary antisera.

EM immunocytochemistry was performed on a crude preparation of rat heart membranes obtained according to the Green and Severs (15) protocol by homogenization in bicarbonate buffer and low-speed centrifugation, but without KCl extraction. The methods of Paul and Goodenough (33) were followed. Gold-labeled secondary antisera were obtained from Janssen Life Sciences Products (Piscataway, NJ) and used at full strength after exhaustive absorption with the isolated rat heart membranes.

Results

Western Blot Analysis

Immunoblot analysis was performed to determine the specificity of the different antisera (Fig. 1). Neither of the antisera to peptides from connexin43 reacted with the liver gap junctions; both reacted specifically with a polypeptide migrating at 43 kD in the intercalated disc preparations, exactly matching the mass predicted from the cDNA for the cardiac gap junction protein. Each of these crude antisera also reacted less intensely with various other polypeptides in the heart preparations, but no other common bands were seen. Anti-connexin43 252–271 reacted with a polypeptide of ~60 kD as well as the 43-kD band in many heart preparations (Figs. 1 and 4); affinity purification of this antiserum did not abolish this reaction (data not shown), but it was substantially reduced by absorption of the serum with a crude rat liver homogenate (Fig. 4). Absorption of anti-connexin43 252–271 with liver was determined empirically and was not attempted on anti-connexin43 119–142, because no bands other than that at 43 kD appeared so prominent. Preimmune sera or sera absorbed with the corresponding peptides showed no reactivity. The antiserum to connexin32 peptide 98–124 and the monoclonal antibody M12.13 both reacted with the 28-kD principal polypeptide in the rat liver gap junctions as well as its aggregate and proteolysis fragments, but did not react with any proteins in the heart lanes, confirming their specificity for connexin32. The antiserum to connexin32 peptide 164–189 reacted with all forms of the rat liver gap junction protein and reacted weakly with a polypeptide of 43 kD in the heart samples that co-migrated with the bands reactive with the anti-connexin43 antisera, suggesting that both connexin43 and connexin32 shared common structure in a potentially extracellular epitope. Monoclonal antibody 6-4-B2-C6, which reacts with a 70-kD protein from lens fibers, did not react with either the heart or liver samples (data not shown).

Antibody Localization in Heart

Antisera were used for immunohistochemistry on frozen sections of rat heart (Fig. 2). Both anti-connexin43 sera gave

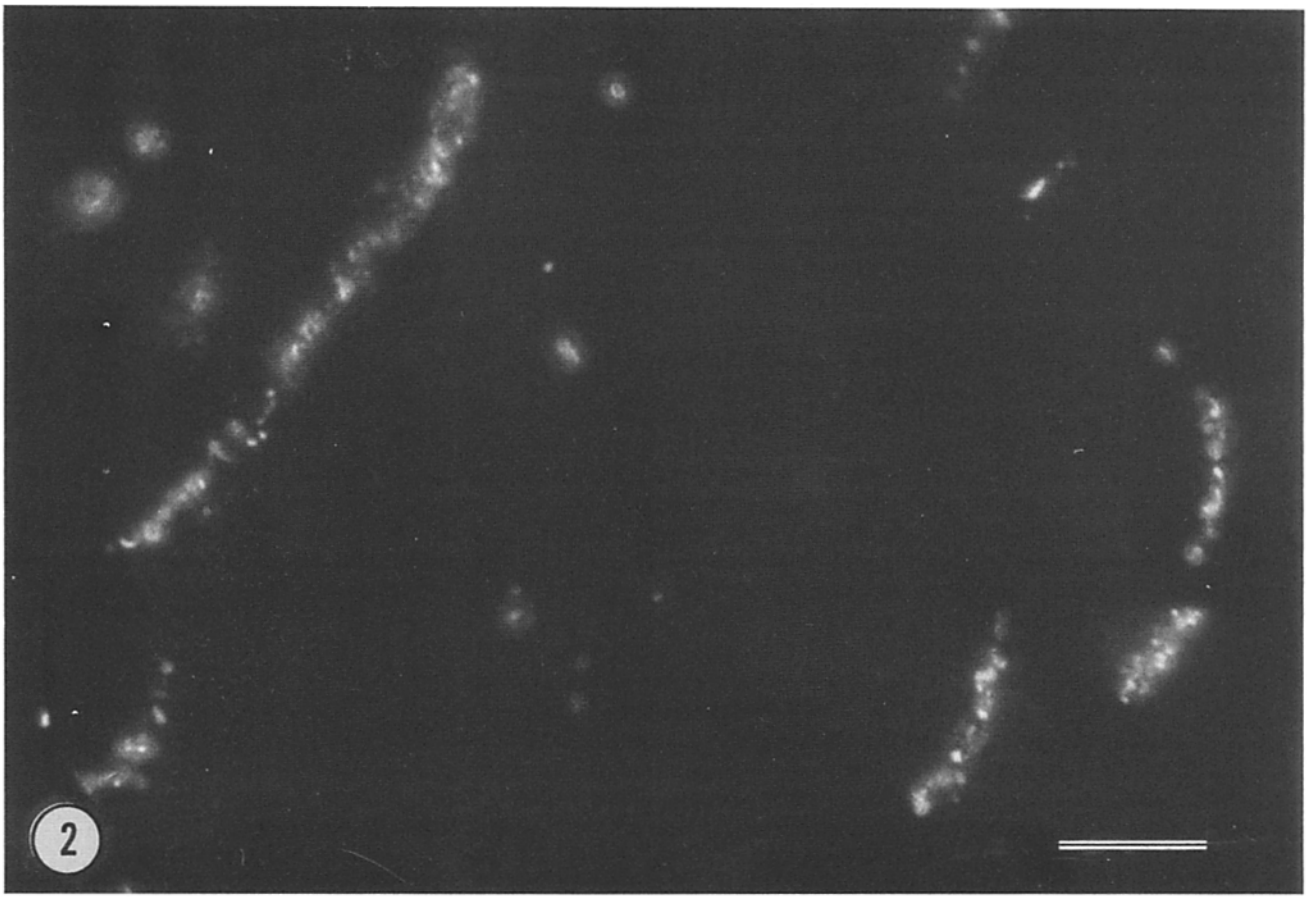

Figure 2. Immunohistochemical localization of connexin43 in frozen section of rat heart. Anti-connexin43 252–271 specifically stains the intercalated discs, which contain the cardiac gap junctions. Staining appears punctate. Bar, 10 μm.

identical staining consisting of bright staining of the intercalated disc structures, which was composed of discrete spots. This staining was consistent with the known distribution of gap junctions in the heart (10, 11, 27). The staining was completely abolished by preincubation of the antisera with 20 μg/ml of the corresponding peptide before staining (not shown). Reaction with a nonhomologous peptide had no effect on staining. Preimmune sera showed no reactivity. Neither of the connexin32 antisera nor the monoclonal antibodies showed any reactivity with the heart.

EM localization of antibody binding was used to demonstrate that gap junctions were responsible for the staining pattern observed by light microscopy. The EM immunohistochemistry study was performed on crude membranes isolated from rat heart. Anti-connexin43 antibody binding was detected using colloidal gold coupled to goat anti-rabbit IgG (Fig. 3). Gold particles were observed only on the cytoplasmic aspects of the heart gap junctions; no specific staining was observed on any surface of the fascia adherens or of any nonjunctional membranes. Preimmune antisera and anti-connexin32 showed only a low, nonspecific background binding of gold.

Although the anti-connexin32 98–124 antiserum and the monoclonal antibody M12.13 both brightly stained punctate areas between hepatocytes, as published in studies reviewed in the introduction, the anti-connexin43 antisera showed no reaction with liver by immunofluorescence. The anti-connexin43 antisera showed no immunogold staining of isolated rat liver gap junctions (data not shown). The antiserum to the conserved region connexin32 164–189 did not react with either liver or heart by immunofluorescence and was not used for any further studies.

Connexin43 in Other Tissues

Other rat organs were examined by Western blot analysis for immunoreactive polypeptides with the connexin43 antisera (Fig. 4). Cardiac intercalated discs, urea-washed lens membranes, and whole tissue homogenates from kidney, uterus, ovary, and cornea all contained polypeptides of ~43 kD that reacted with both anti-connexin43 antisera. There were some variations in the intensity of reaction of the two sera. For instance, the calf lens membranes reacted more strongly with anti-connexin43 119–142 than with anti-connexin43 252–271.

These rat organs were also examined by immunofluorescence with the anti-connexin43 sera for staining consistent with gap junctions. Northern blot studies had shown that pregnant mare's serum gonadotropin–primed ovary contains large amounts of connexin43 mRNA (2). Immunofluorescent localization with anti-connexin43 produced large, bright spots of fluorescence along the borders of adjacent granulosa

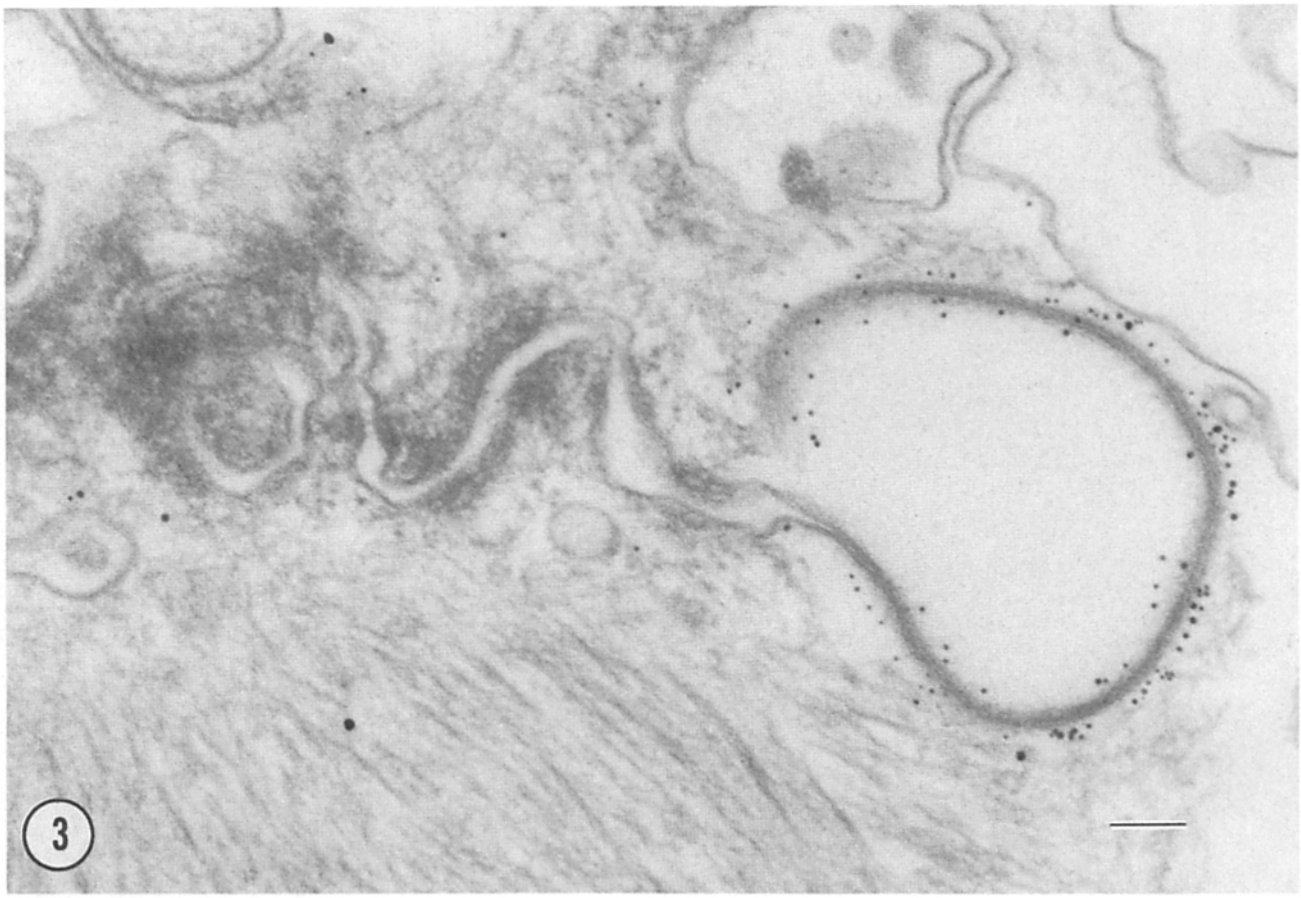

Figure 3. Immunocytochemical localization of connexin43 in rat heart membranes. Thin section electron micrograph of crude rat heart membranes incubated with anti-connexin43 252–271 antiserum and colloidal gold–conjugated goat anti-rabbit antibodies before fixation and embedding. Gold particles decorate the cytoplasmic surfaces of the gap junction. No significant labeling is detectable on any other structures, including other junctional or nonjunctional membranes. Bar, 0.1 μm.

cells (Fig. 5). Some staining was also observed between connective tissue cells of the theca externa, and small spots were occasionally present over the oocytes in locations consistent with the junctions between granulosa cell processes and the oocytes (1). The anti-connexin32 sera showed no reaction with ovary.

Smooth muscle derived from the large intestine, stomach, and pregnant uterus in labor all showed punctate labeling at cell borders (data not shown). Uterine smooth muscle from nonpregnant or preterm animals showed little staining. However, treatment of juvenile female rats with pharmacologic doses of estradiol produced an intense area of reactivity in a circular band of smooth muscle (Fig. 6 *a*). These estrogen-treated animals also showed immunofluorescent staining consistent with gap junctions in longitudinal smooth muscle, in the connective tissue, and on the serosal epithelium. Endometrium showed no anti-connexin43 reactivity in any of the uterine samples.

In contrast, anti-connexin32 showed no reactivity with any of the smooth muscle preparations and little staining of connective tissue. However, the endometrium of pregnant rats showed large spots of anti-connexin32 staining along the lateral epithelial cell membranes (Fig. 6 *b*). Such staining was weak or absent in nonpregnant or estrogen-stimulated animals.

The apparent localization of connexin43 in connective tissue in a number of organs led us to examine the cornea, which contains a connective tissue composed mainly of stroma and fibroblasts (18). In the corneal stroma (Fig. 6 *c*), anti-connexin43 stained discrete spots on the fibroblast cell bodies in a distribution consistent with that demonstrated previously for corneal fibroblast gap junctions (17). In addition, anti-connexin43 stained numerous contacts between corneal epithelial cells (Fig. 6 *c*), showing the highest density between the basal cells and a graded reduction as the cells approached the free surface of this stratified epithelium. Anti-connexin43 also stained corneal endothelial contacts (data not shown). Anti-connexin32 showed no reactivity in frozen sections of cornea.

Multiple Connexins in the Same Organ or Tissue

Immunofluorescence was used to examine several rat organs where previous immunological, physiological, biochemical, or Northern blot experiments have suggested the presence of more than one gap junction protein. In the lens, anti-con-

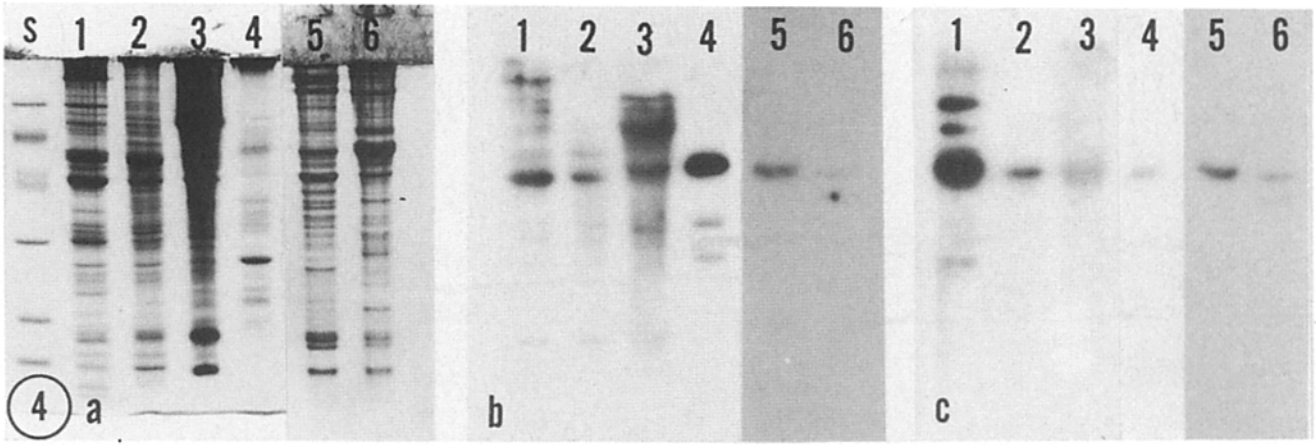

Figure 4. Immunoblot of crude membrane preparations of multiple organs with anti-connexin43 sera. (*a*) Coomassie Blue–stained SDS-polyacrylamide identical to those transferred to nitrocellulose. (Lane *S*) Molecular mass standard proteins (phosphorylase B, 97 kD; BSA, 66 kD; ovalbumin, 43 kD; carbonic anhydrase, 31 kD; soybean trypsin inhibitor, 21.5 kD; lysozyme, 14 kD). (Lanes *1–6*) Preparations of membranes from heart, kidney, ovary, lens, estrogen-stimulated uterus, and cornea, respectively. (*b*) Immunoblot of a gel identical to that in *a*, but without standards, reacted with anti-connexin43 119–142. This antiserum reacts strongly with a 43-kD band in all the preparations, and weakly with other bands. (*c*) Identical immunoblot reacted with anti-connexin43 252–271 which had been absorbed with a crude homogenate of rat liver. This antiserum also reacts strongly with a 43-kD band in all preparations. Since this antiserum does not react with liver gap junctions, absorption with the liver homogenate was used empirically and found to reduce reactivity with polypeptides other than that of 43 kD.

nexin43 stained spots along the most apical region of the epithelial cells' lateral borders (Fig. 7, *a* and *b*) in locations consistent with the distribution of epithelial–epithelial junctions (28), and consistent with previous Northern blots of mRNA fractions from capsulated and decapsulated lenses (2). Anti-connexin43 showed no reaction with the fiber cells. In contrast, 6-4-B2-C6, an antibody to MP70, gave punctate staining between all the lens fiber cells (Fig. 7, *c* and *d*), and no interpretable interepithelial staining.

In pancreas, anti-connexin32 gave bright staining in spots between the acinar cells (data not shown). A few spots were also seen in islets. In contrast, anti-connexin43 showed little or no reactivity with the exocrine or endocrine pancreas but produced bright spots between the cells of ductules and in the connective tissue between lobules.

Northern blots (2) have suggested the presence of both connexin32 and connexin43 in kidney. Double immunofluorescence of kidney sections with the rabbit antipeptide connexin43 antiserum and the mouse monoclonal anti-connexin32 antibody M12.13 was performed. Connexin43 reactivity (Fig. 8 *a*) localized to spots between the epithelial cells in virtually all tubules and to some spots in the glomeruli and connective tissue. Connexin32 reactivity (Fig. 8 *b*) also localized to spots in glomeruli and between epithelial cells in proximal tubules, but showed no reactivity with the distal tubules. Although both connexins were localized to many of the same proximal tubules, few or none of the immunofluorescent spots coincided, suggesting that the two proteins were present in different gap junction plaques. The complex interdigitation of the lateral borders of these cells denied the determination by immunofluorescence of whether an individual cell coexpressed both connexin types.

Discussion

Gap Junction Antibodies and Connexin Topology

Antisera directed against gap junction proteins have been raised in several laboratories and show different tissue specificities. Several antisera raised against the isolated rat liver gap junction protein, connexin32, show immunofluorescent staining consistent with gap junctions between hepatocytes, pancreatic acinar cells, proximal renal tubule cells, and various gastrointestinal epithelial cells (8, 19, 31). However, while most antisera to this protein do not react with heart, one stained intercalated discs (19). Another single report suggested reactivity with lens fiber cells (34). Zervos et al. (36) described an antiserum directed against an NH_2-terminal peptide that reacts on immunoblots with proteins from liver, heart, and uterus. However, no immunohistochemistry was performed with this antiserum.

Recent amino acid sequence data from Edman degradation and cDNA cloning experiments have helped explain these discrepancies (2, 3, 21, 29, 32). Connexin32, connexin43, connexin46, and MP70 all have regions at their amino termini containing many identical amino acids. The connexins also have a second internal region of high homology (3). But they have other regions that are unique, containing few identical residues. Topological models of the connexin proteins (2, 14, 37) suggest that the unique regions may be located on the cytoplasmic face of the junctional membrane, and the conserved sequences may lie in transmembrane and extracellular areas. Thus, the connexins potentially contain many shared antigenic sites; but most of the shared epitopes resulting from conserved amino acid sequences may be inaccessi-

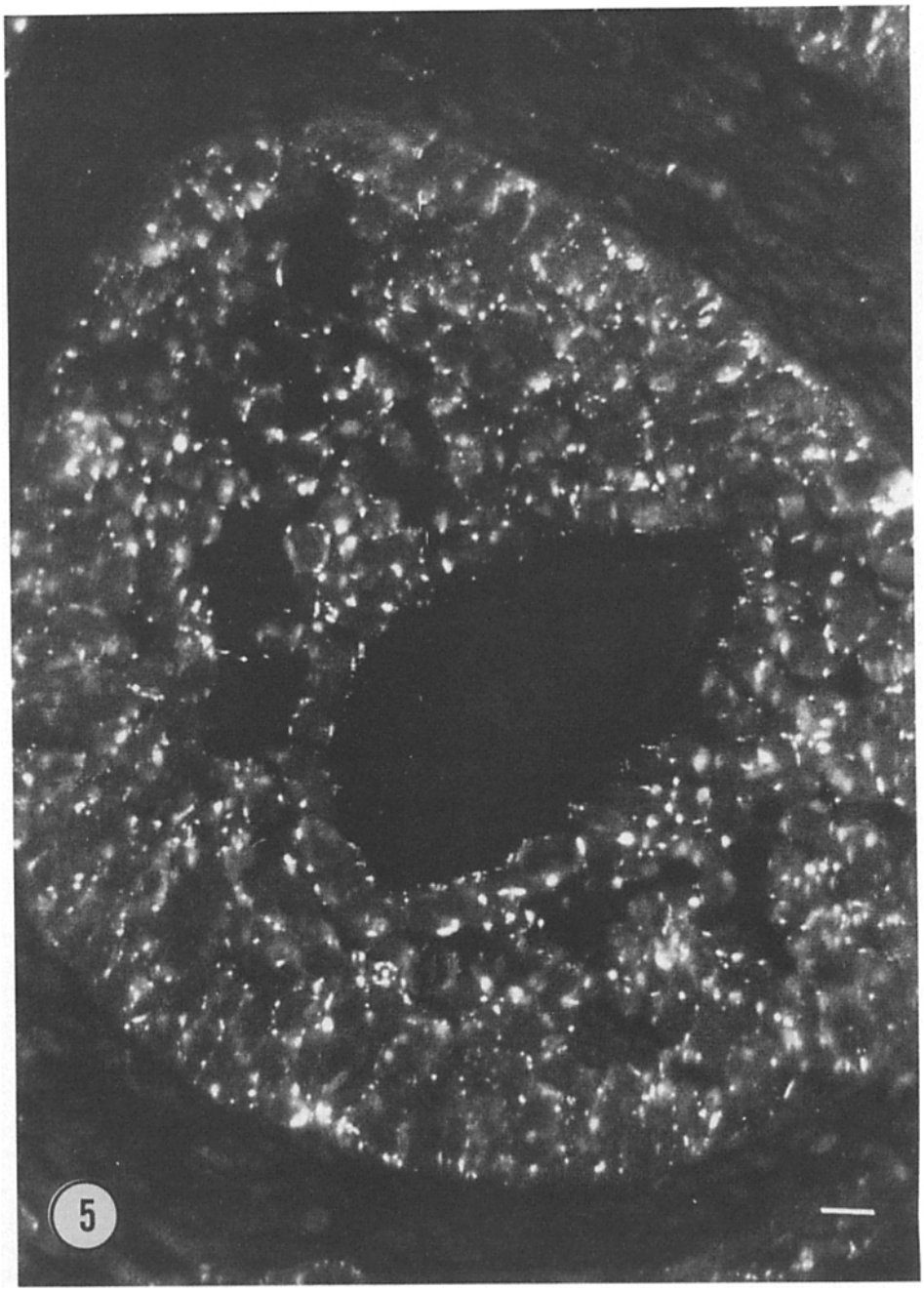

Figure 5. Immunohistochemical localization of connexin43 in a frozen section of rat ovary. Anti-connexin43 (252–271) staining is observed in many large bright spots between granulosa cells and in rare small spots over the oocyte and in the theca externa. Bar, 10 μm.

ble to antibodies applied to the cytoplasmic face of the native gap junction, if they are buried in the hydrophobic bilayer or sequestered within the minute extracellular space. Antisera may also recognize different connexin molecules in cytoplasmic areas without amino acid sequence homology if they recognize secondary or tertiary structures (conformational determinants). We have no evidence for such antibodies within our reagents; however, the broad species and tissue specificity reported for some anti-connexin32 antibodies suggests the existence of such antibodies (12, 35).

We have used the sequence data to choose peptides to elicit specific anti-connexin antisera. Localization of the reactivity of these antisera supports the model of connexin topology. The antiserum to connexin32 peptide 164–189, which may contain extracellular epitopes and which is predicted to be 60% conserved in connexin43, reacts on immunoblots with proteins of the correct size in both liver and heart. However, because the epitopes recognized by this antiserum are only accessible after experimental splitting of the junctional membranes (14), this antiserum shows no reactivity with the native gap junction structure in either liver or heart. Antisera directed against unique cytoplasmically located peptide se-

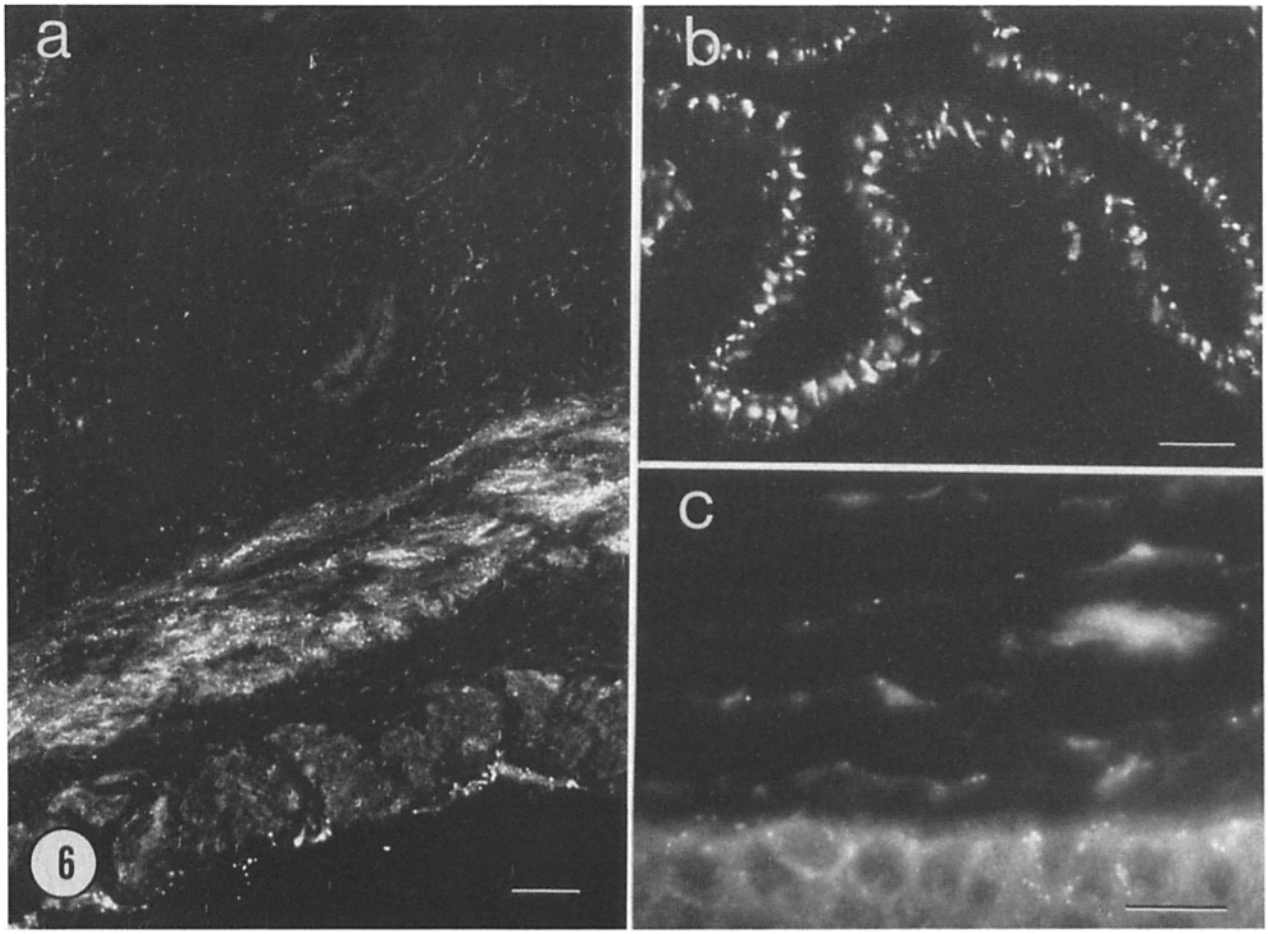

Figure 6. Immunohistochemical localization of connexins in uterus and cornea. (*a*) In uterine tissue from a juvenile female rat treated with estradiol, intense anti-connexin43 (252–271) staining is observed in a circular band of smooth muscle. Punctate staining with this antiserum is also present in longitudinal smooth muscle, in the connective tissue, and in the serosal epithelium. The endometrial epithelium shows no reactivity with the connexin43 antisera. Bar, 25 μm. (*b*) Endometrial epithelium from a pregnant rat near term stained with anti-connexin32 shows large bright spots between epithelial cells. Bar, 25 μm. (*c*) In rat cornea, intercellular borders between corneal epithelial cells and fibroblasts show punctate anti-connexin43 (252–271) reactivity. Bar, 10 μm.

quences from connexin32 and connexin43 are connexin specific and have been used in this paper to examine the distributions of these gap junction proteins.

Connexin43 Is a Cardiac Gap Junction Protein

The demonstration that a protein is a gap junction protein requires the satisfaction of rigorous morphological and functional criteria, as we have discussed previously (2). In this paper, we have prepared two antisera that are specific for peptide sequences unique to connexin43 and that recognize a 43-kD protein in intercalated disc preparations. These antisera do not react with liver gap junctions either on Western blots or by immunofluorescence. These antisera specifically label the cardiac gap junction structure as examined by immunohisto- and immunocytochemistry, providing a morphological demonstration that connexin43 is a gap junction protein. A functional demonstration might be provided by showing that that connexin43-specific antisera can block intercellular communication in pairs of cardiac myocytes; however, the performance and interpretation of such experiments is very difficult, as discussed previously (2). We have recently shown that connexin43 expressed in pairs of *Xenopus* oocytes from in vitro–synthesized mRNA will form functional cell-to-cell channels (Swenson, K. I., Beyer, E. C., Paul, D. L., Goodenough, D. A., manuscript in preparation).

Connexin43 Is a Gap Junction Protein in Other Tissues

Previous Northern blot experiments had suggested that connexin43 mRNA was expressed in many organs besides heart. In this paper, immunofluorescence studies have shown staining consistent with gap junctions in many tissues using the anti-connexin43 sera. The antipeptide antisera used in this paper have revealed intercellular, macular staining between many different cell types, consistent in size and distribution with gap junctions as visualized by thin section and freeze-fracture methodologies. We will refer to this fluorescence

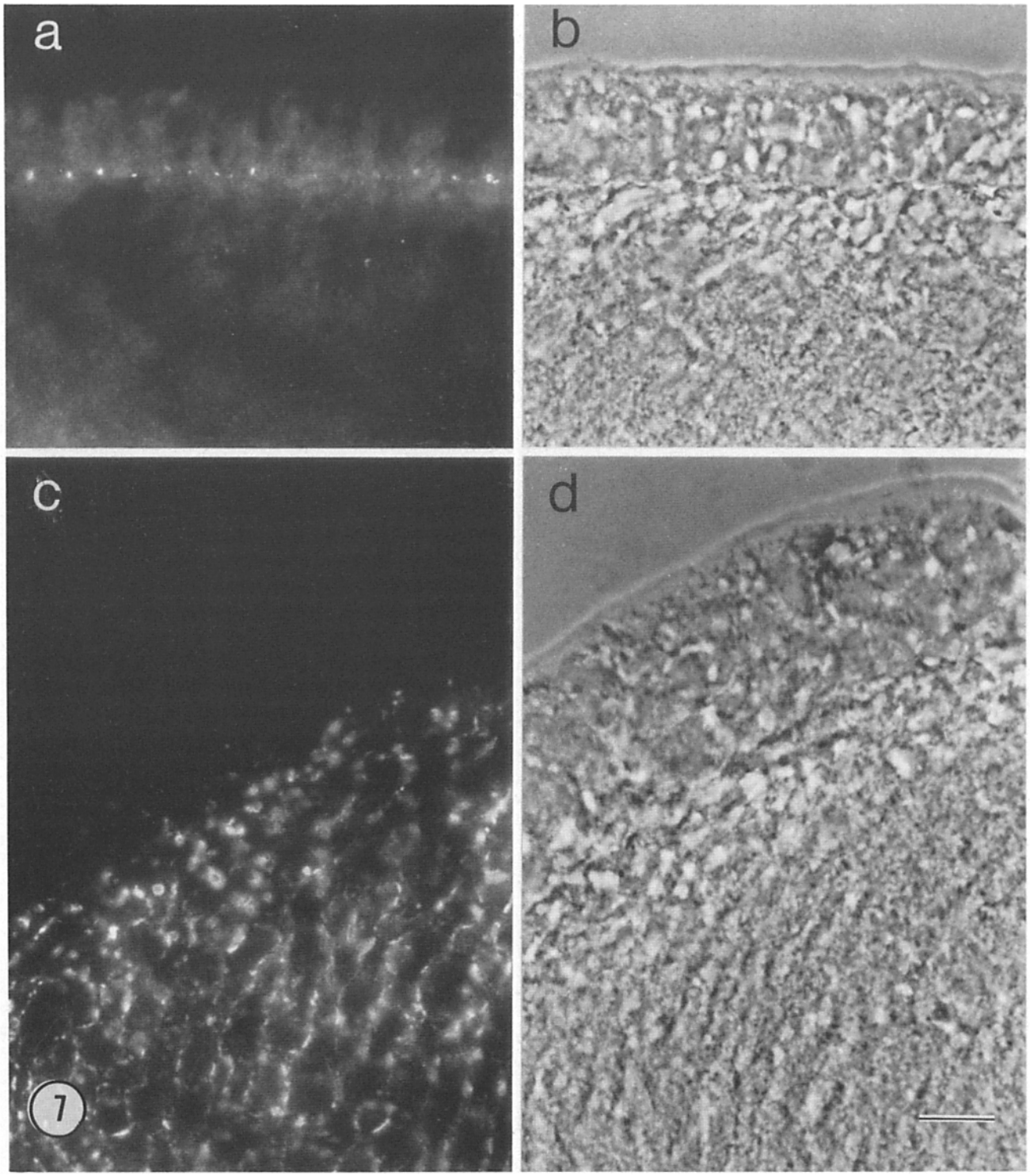

Figure 7. Immunohistochemical localization of connexins in frozen sections of rat lens. (*a*) Connexin43 (252–271) localizes to bright spots in the apical regions of the lateral contacts between adjacent lens epithelial cells in a distribution consistent with that of epithelial–epithelial gap junctions. (*b*) Phase-contrast micrograph of same field as *a*. (*c*) Immunohistochemistry using anti-MP70 antibody 6-4-B2-C6 produces staining between lens fiber cells in a distribution consistent with that of fiber–fiber gap junctions. (*d*) Phase-contrast micrograph of same field as *c*. Bar, 10 μm.

staining as gap junctional, mindful that electron microscopic localization has not yet been done.

Connexin43 appears to be associated with gap junctions between smooth muscle cells. It is found between cells in connective tissues in many organs, and since connexin43 can be localized between corneal stromal fibroblasts, part of this connective tissue staining probably arises from interfibroblast gap junctions. Connexin43 localizes in the gap junctions be-

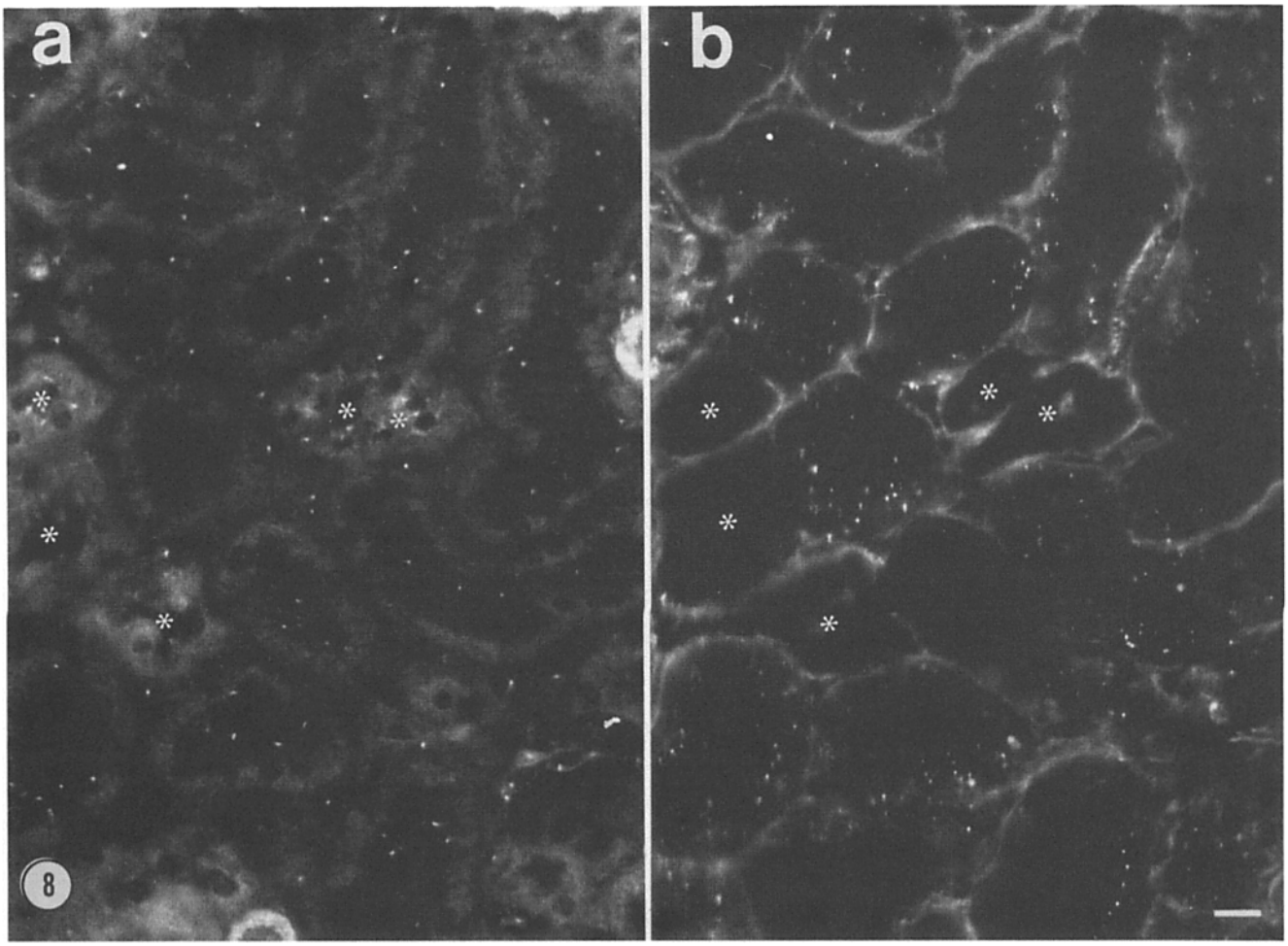

Figure 8. Double-label immunohistochemical localization of connexins in a frozen section of rat kidney. (*a*) Reactivity with anti-connexin43 252–271 antiserum. (*b*) Reactivity with anti-connexin32 monoclonal antibody M12.13. Both antibodies stain spots between renal tubular epithelial cells. However, few, if any, of the spots from the two antibodies coincide, suggesting that they are reacting with different gap junction plaques; the two connexins are not mixed in the same plaque. Distal tubules (*) only show connexin43 reactivity. Bar, 10 μm.

tween granulosa cells and possibly between the oocyte and granulosa cells. Connexin43 is contained in gap junctions between epithelial cells in the lens, cornea, and renal tubules.

In smooth muscle, as in the heart, gap junctions permit electrical coupling (7) but common properties of the other tissues that contain connexin43 are unclear. However, we now have developed appropriate connexin-specific antibody probes that will facilitate the study of gap junction function in these diverse locations.

Connexin43 Makes Different Junctions from Other Gap Junction Proteins

Nicholson et al. (30) showed intermixing of two different connexin molecules within the same gap junction plaques in mouse liver. While we have seen no evidence yet for intermixing of connexin43 with other connexins, we have found a few cases where connexin43 is localized in the same organ as another gap junction protein. In uterus and pancreas, for example, both connexin43 and connexin32 are present but in different cell types. In kidney, both connexin43 and connexin32 are present in the proximal convoluted tubule, but they are present in different plaques and likely in different cells. This suggests that adjacent renal tubular cells can differ in connexin expression. Interestingly, Brown et al. (4) have found similar local heterogeneity of H^+-ATPase expression in kidney tubules.

Finally, in the lens, connexin43 localizes to epithelial cells (probably epithelial–epithelial gap junctions) and the connexin-related protein MP70 localizes to fiber–fiber junctions. We do not yet know how the heterocellular junctions are made between epithelial and fiber cells.

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