J. Membrane Biol. 139, 15–29 (1994)

The Journal of
Membrane
Biology

The Topological Structure of Connexin 26 and its Distribution Compared to Connexin 32 in Hepatic Gap Junctions

J.-T. Zhang*, B. J. Nicholson
Department of Biological Sciences, SUNY at Buffalo, Buffalo, New York 14260-1300

Received: 23 April 1993/Revised: 3 January 1994

Abstract. Of the gap junction proteins characterized to date, Cx26 is unique in that it is usually expressed in conjunction with other members of the family, typically Cx32 (liver [Nicholson et al., *Nature* **329:**732–734, 1987], pancreas, kidney, and stomach [J.-T. Zhang, B.J. Nicholson, *J. Cell Biol.* **109:**3391–3410, 1989]), or Cx43 (leptomeninges [D.C. Spray et al., *Brain Res.* **568:**1–14, 1991] and pineal gland [J.C. Sáez et al., *Brain Res.* **568:**265–275, 1991]). We have used specific antisera both to investigate the distribution of Cx32 and Cx26 in isolated liver gap junctions, and empirically establish the topological model of Cx26 suggested by its sequence and analogy to other connexins. Antipeptide antisera were prepared to four of the five hydrophilic domains which flank the four putative transmembrane spanning regions of Cx26. Antibodies to N-terminal residues 1–17 (αCx26-N), to residues 101–119 in the putative cytoplasmic loop (αCx26-CL), and to C-terminal residues 210–226 (αCx26-C) were all specific for Cx26. An antibody to residues 166–185 between hydrophobic domains 3 and 4 of Cx32 had affinity for both Cx26 and Cx32 (αCx32/26-E2). The antigenic sites Cx26-N, -CL and -C were each demonstrated to be cytoplasmically disposed, although the latter was conformationally hidden prior to partial proteolysis. The antigenic site for αCx32/26-E2 was only accessible after exposure of the extracellular face by separation of the junctional membranes in 8 M urea, pH 12.3. This treatment also served to reveal the region between residues 45 and 66 to Asp-N protease. The topology thus demonstrated for Cx26 is consistent with that deduced for other connexins (i.e., Cx32 and Cx43). Comparison of immunogold decorated gap junctions reacted with antibodies specific to Cx26 (αCx26-N and -CL), or to Cx32 [αCx32-CL], indicates that these connexins do not aggregate in subdomains within a junction, at least within the resolution provided by the labeling density (one antibody per 15–22 connexons). Although the presence of both connexins within a single channel could not be distinguished, possible interactions between channels is discussed.

Key words: Membrane—Protein—Folding—Antibodies—Channels

Introduction

Gap junctions are membrane channels that allow molecules up to 1,000 daltons to pass directly between neighboring cells (Hertzberg, Lawrence & Gilula, 1981; Loewenstein, 1981; Sáez et al., 1989; Bennett et al., 1991). Rapid propagation of electrical signals and synchronization of activity are postulated as roles of gap junctions in excitable tissues (e.g., DeHaan et al., 1981; Joyner, 1982; Spach & Kootsey, 1983; Fozzard & Arnsdorf, 1986). In nonexcitable tissues, gap junctions are believed to mediate the exchange of metabolites and signal molecules between cells and regulation of cell growth and development (Loewenstein, 1979, 1981; Sheridan & Atkinson, 1985; Guthrie & Gilula, 1989; Klaunig & Ruch, 1990).

Gap junctions have been isolated and their protein constituents (connexins) characterized from liver (Henderson, Eibl & Weber, 1979; Hertzberg & Gilula, 1979; Nicholson et al., 1981, 1987); heart (Gros, Nicholson & Revel, 1983; Manjunath, Goings & Page, 1984); and lens (Kistler, Christie & Bullivant, 1988). Original

* *Present address:* Dept. of Physiology & Biophysics, University of Texas Medical Branch, Galveston, Texas

Correspondence to: B.J. Nicholson

studies suggested that gap junctions from a given source were comprised of a single connexin, a proposal that was supported directly in heart, where highly purified junctional fractions contain a single protein component, e.g., Cx43 (Manjunath et al., 1984, 1987). However, as more connexins were identified by cDNA and genomic cloning (*see* Bennett et al., 1991; Willecke et al., 1991) it became evident that most tissues express multiple proteins. Even in a quite homogeneous tissue like the eye lens, several proteins (Cx43, Cx46, and an M_r 70,000 protein -Cx50?), including the abundant MIP26 (Bok, Dockstader & Horwitz, 1982; Paul & Goodenough, 1983), which is not related to the connexin family (Nicholson et al., 1983), have been associated with junctions. However, the only clear demonstration of multiple proteins within a single gap junction remains the liver system, where both Cx32 and Cx26 are colocalized to the same junctional plaques between hepatocytes (Nicholson et al., 1987; Traub et al., 1989).

Cx32 and Cx26 are quite homologous to one another (64% amino acid identity overall) and somewhat less so to other members of both the "β" (most similar to Cx32) and "α" (most similar to Cx43) classes of connexins (Gimlich, Kumar & Gilula, 1990; Hoh, John & Revel, 1991). Several members of this family, including both Cx32, (Dahl et al., 1987) and Cx26 (Barrio et al., 1991), have been shown to form intercellular channels between paired oocytes. Antibodies specific for both Cx26 and Cx32 have also been shown to effectively block coupling between hepatocytes (Traub et al., 1989). Although these studies strongly implicate both proteins as playing a role in intercellular channel structure, Cx26 is unique among the other well-characterized connexins in that, with the possible exception of pinealocytes (Sáez et al., 1991), it is consistently coexpressed with other members of the family. This raises the question of the specific role played by Cx26 in channel formation, since none of the previous analyses precludes a possible accessory, rather than integral role in vivo. A first step towards examining this issue was to determine the folding of the protein within the membrane *in situ*. This would also serve as a basis for future comparisons of both wild type and mutant Cx26 expressed in exogenous systems (e.g., oocytes; cell-free systems) and for comparison with other members of the connexin family.

A topological structure for gap junction proteins has been demonstrated biochemically and immunologically for Cx32 (Nicholson et al., 1981; Revel, Nicholson & Yancey, 1984; Zimmer et al., 1987; Goodenough, Paul & Jesaitis, 1988; Hertzberg et al., 1988; Milks et al., 1988) and Cx43 (Yancey et al., 1989). Each has four transmembrane domains with N- and C-termini exposed on the cytoplasmic face of the membrane. Hydrophobicity analyses show that Cx26 has a similar profile to Cx32 and Cx43, leading us to propose a comparable model (Zhang & Nicholson, 1989). However, the recent history of failed topological models for several membrane proteins (e.g., acetylcholine receptor—*cf.* Finer-Moore & Stroud, 1984 with Leonard et al., 1988; K^+ channel—*cf.* Noda et al., 1984 with Yellen et al., 1991) strongly argues for an empirical approach to testing all models.

In this study, we have developed and characterized peptide antibodies specific to Cx26. Using these antibodies, combined with proteolysis-membrane protection assays, we have confirmed this model in isolated liver gap junctions. In conjunction with an antipeptide antibody specific to Cx32, we also demonstrate, through electron microscope immunolabeling, that no differential distribution of Cx26 and Cx32 in a given gap junction plaque can be discerned.

Materials and Methods

Synthesis of Peptides

Five peptides, three corresponding to portions of the amino acid sequence of rat Cx26 [Zhang & Nicholson, 1989: amino acids 1–17(N); 101–119(CL); 210–226(C) in the Table] and two corresponding to portions of the amino acid sequence of rat Cx32 [Paul, 1986: amino acids 110–128(CL); 166–185(E2) Table], were synthesized on a Biosearch Model 9500 automated peptide synthesizer using standard Merrifield chemistry and hydrogen fluoride cleavage, deprotection, and ether extraction to remove protecting groups. Purity of each peptide was assessed by high performance liquid chromatography (HPLC) on a C-18 analytical column using a Beckman 340 dual pump system with Kratos spectrophotometer. Purification of peptides for use in affinity purification of antisera utilized a 0–60% acetonitrile gradient in 0.1% trifluoroacetic acid on a Beckman C-18 preparative column.

Preparation and Affinity Purification of Antipeptide Antibodies

Rabbits were immunized intradermally and intramuscularly with 1 mg of crude peptide in complete Freund's adjuvant (mixed two parts with one part aqueous peptide solution). Animals were boosted intramuscularly near the popliteal lymph nodes two months later with 0.5 mg of crude peptide in complete Freund's adjuvant and bled weekly after boosting. Further boosts were spaced one to three months apart, using the same bleeding schedule.

For purposes of affinity purification, crude [Cx26 (1–17) and Cx32 (110–128)] or HPLC-purified (all others used) peptides were conjugated, according to manufacturer's instructions, via Schiff's base to the aldehyde groups on a MAC-25 cartridge (Memtec: now sold by Nalgene). After blocking unreacted sites with 0.1 M sodium borohydride at pH 9.0 for 30 min at room temperature, 700 μl of serum was passed through the filter which was then washed with phosphate-buffered saline (PBS: 10 mM sodium phosphate, 150 mM NaCL, pH 7.4) to remove unbound material. Specific antibodies were eluted with 0.1 M glycine at pH 2.3. Eluted antibody, which was collected in 2 × 500 μl fractions, was immediately equilibrated with PBS

by passage over a Sephadex G-50 spun column. Aliquots were then stored at 4°C for subsequent use and analysis. IgG fractions from preimmune serum were prepared on either a MAC-25 cartridge with bound protein-A, or on a protein-A HPLC column (Pierce, CA) using similar binding and elution buffers as for the cartridge.

ELISA Assay of Antipeptide Antibodies

Fifty microliters of crude peptide solution (50 μg/μl), or mouse liver gap junction fraction (0.1 μg/ml of protein: preparation described below) in 0.5 M Na_2CO_3 (pH 9.3), was absorbed overnight onto a polystyrene microtitre plate. Additional binding sites on the plate were then blocked with 0.5% bovine serum albumin (BSA) in TBS (10 mM Tris, HCl pH 7.4, 150 mM NaCl) for 2 hr at 37°C before three additional washes with TBS containing 0.2% BSA and 0.05% Tween-20. Primary antibody reactions were carried out at 37°C for 2 hr in TBS containing 0.2% BSA. After washes in TBS/0.2% BSA/0.05% Tween-20, protein A-horseradish peroxidase (HRP) conjugate (Cappel, NC; 2 μg/ml) was added and incubated for 1 hr at room temperature. After further washes with TBS, 0.2% BSA, 0.05% Tween-20, color was developed in 0.2% *O*-phenyldiamine, 0.015% H_2O_2, 16 mM citric acid, and 60 mM sodium phosphate (pH 6.3). The reaction was terminated by addition of 10 μl of 4 M H_2SO_4. Data were quantitated by reading the absorbance of each sample at 490 nm.

Immunohistochemistry

Cubes of rat or mouse liver (~1 mm square) were embedded in OCT mounting medium (Miles, IN) and flash-frozen in liquid propane. Sections (3 μM) were fixed in 4% paraformaldehyde in PBS (freshly prepared) for 15 min, washed in PBS and blocked in 0.1 M glycine in PBS for 1 hr. Nonspecific binding sites were then blocked for 30 min in 0.2% BSA in PBS followed by primary antibody incubation (IgG concentration ~5 μg/ml) for 2 hr at room temperature in 0.1% BSA in PBS. After three washes in the same buffer, FITC-conjugated goat anti-rabbit IgG-F'ab fragment (Chemicon, CA) at a dilution of 1:00 in the same buffer was added for 20 min at RT. Final washes in 0.1% BSA in PBS and H_2O preceded mounting in 90% glycerol in PBS and viewing on a Zeiss Axiovert epifluorescence microscope.

Immunoblot and Electron Microscopic Immunolabeling of Intact and Proteolysed Gap Junctions

Gap junctions were isolated using a modified procedure of Hertzberg (1984) in which the isolation of plasma membrane using a sucrose gradient was omitted. Typically, about 100 μg of purified gap junction proteins was obtained from 50 mice. Approximately 0.2 μg of junctional protein from these isolated fractions was separated by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membrane (Immobilon by Millipore). Electroblotting was performed according to Towbin, Staehelin and Gordon (1979) with the modification that gels were not presoaked prior to transfer. The blots were blocked for 1.5 hr at 37°C in TBS containing 3% BSA. Reactions with primary antibodies at an IgG concentration of about 4 μg/ml in TBS with 0.2% BSA were done at 37°C for 2 hr. After three washes in TBS containing 0.2% BSA, the blots were treated with 2 μg/ml alkaline phosphatase-protein A conjugate (Cappel, NC) in TBS/0.2% BSA for 1 hr at room temperature. Color development was achieved with nitroblue tetrazolum chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate *p*-tolnidine salt (BCIP) according to the vendor's (Bethesda Research Labs, MD) instructions.

Proteolysis/membrane protection assays of isolated gap junctions were carried out on resuspended fractions in PBS at a concentration of 0.01 μg/μl of protein. Trypsin (TPCK treated from Sigma, MO) and V8 (Worthington, NJ) proteolysis, at a substrate: enzyme ratio of 5 to 1, was allowed to continue for variable times at 37°C. The reaction was stopped by centrifugation at 14,000 × *g* for 15 min to pellet gap junctions. In the case of trypsin digestion, soybean trypsin inhibitor (Sigma, MO) was added prior to pelleting. Junctional pellets were then rinsed twice with PBS before analysis by SDS-PAGE or immunogold labeling.

For immunogold decoration of gap junctions, 10 μl of a crude fraction of mouse liver gap junctions (0.01 μg/μl of protein) were applied to copper grids and air-dried. The grids were then incubated in 0.5% BSA in PBS at 37°C for 30 min. After three 5 min washes in PBS with 0.2% BSA, they were incubated for 1 hr at 37°C with affinity-purified immune serum (IgG concentration of 0.08 mg/ml) or equivalent concentration of an IgG fraction of preimmune serum in PBS containing 0.2% BSA. The grids were then washed three times for 5 min in the same buffer before incubating for 30 min at room temperature with goat anti-rabbit IgG conjugated to 10 nm gold particles (Janssen Scientific, Belgium, 1:5 dilution in PBS/0.2% BSA). After a further three rinses with water, the grids were stained with 0.2% phosphotungstic acid (pH 7.2) or 2% uranyl acetate before viewing in a Hitachi transmission electron microscope.

Separation of Gap Junctional Membranes

Gap junction suspensions (100 μg gap junction protein each) were pelleted using a Brinkman microfuge for 15 min at max speed (14,000 × *g*). Each pellet was resuspended in a starting volume (500 μl) of either 8 M urea, 10 mM Tris (pH 12.3); or 8 M urea, 10 mM Tris, HCl (pH 7.2) and incubated for 3 hr at 37°C with occasional shaking. Gap junctions were then pelleted as before and resuspended in either 0.5 M Na_2CO_3 (for ELISA assay) or PBS (for electron microscope (EM) analysis and proteolysis/membrane protection assay). For EM analyses, treated junctions were fixed in 4% glutaraldehyde (Electron Microscopy Sciences, PA) in 0.1 M Na cacodylate (pH 7.2) for 30 min on ice before embedding in 2% noble agar and post fixation with 1% OsO_4 (Electron Microscopy Sciences, PA) in 0.1 M Na cacodylate (pH 7.2). The membranes were then dehydrated through increasing concentrations of ethanol and embedded in epon. Thin sections were strained with 2% uranyl acetate before viewing in the EM.

Abbreviations

PBS-phosphate buffered saline; TBS-Tris buffered saline; BSA-Bovine serum albumin; HRP-horseradish peroxidase; SDS-PAGE-sodium dodecylsulfate polyacrylamide gel electrophoresis; NBT-nitrobluetetrazolium chloride; BCIP-5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt; EM-electron microscopy.

Results

Characterization of Antipeptide Antibodies

Specificity

The portions of Cx26 represented by the peptides synthesized are shown in the Table, along with a designation to be used throughout the manuscript. They com-

Table 1. Summary of site-specific antibodies to Cx26 and Cx32

Antibody	Peptide	Peptide sequence
Cx26-N	Cx26 (1–17)	M D W G T L Q S I L C G V N K H S
Cx26-CL	Cx26 (101–119)	E K K R K F M K G E I K N E F K D I E
Cx26-C	Cx26 (215–226)	I R Y C S G K S K R P V
Cx32-CL	Cx32 (110–128)	G H G D P L H L E E V K R H K V H I S
Cx32/26-E2	Cx32/26 (166–185)[a]	V K C E A F P C P N T V D C F V S R P T

[a] While the peptide corresponds to Cx32 (166–185), it also corresponds to Cx26 (167–186) with only three differences. $E_{169} \rightarrow N$; $F_{171} \rightarrow W$; $V_{181} \rightarrow I$.

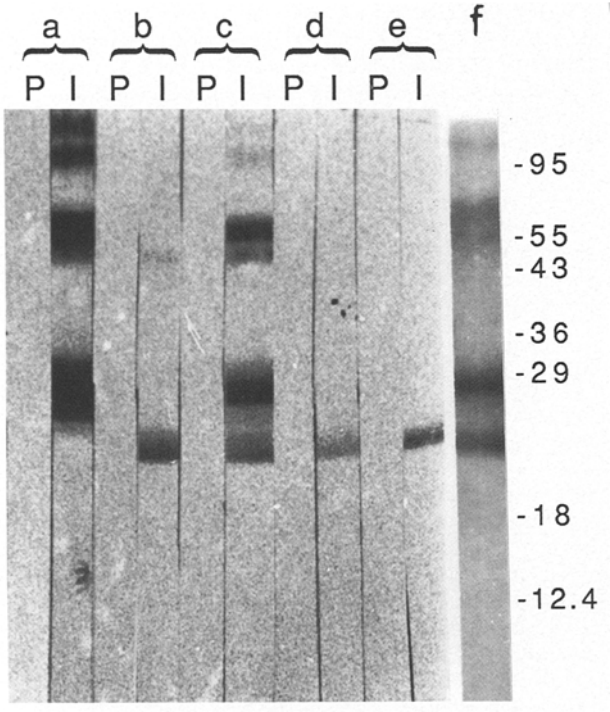


Fig. 1. Immunoblots of isolated mouse liver gap junction fractions probed with the site-specific antibodies listed in the Table. Mouse liver gap junction fractions isolated according to Hertzberg (1984) were separated on a 15% SDS polyacrylamide gel and either stained with Coomassie blue (*f*: 1 μg of junction protein) or electroblotted onto PVDF membrane (0.2 μg of junctional protein per lane) and probed as described in the text with preimmune (*P*) or immune (*I*) sera (IgG concentration, 4 μg/ml): (*a*) αCx32-CL; (*b*) αCx26-N; (*c*) αCx32/26-E2; (*d*) αCx26-CL; (*e*) αCx26-C. αCx32-CL specifically recognizes Cx32 (~28 kD mobility on the gel) and its degradation and aggregation products (*a*). All antibodies raised to Cx26-specific peptides recognized a single band of ~25 kD, (*b,d* and *e*) and, in one case, an aggregation product (*b* only). αCx32/26-E2 directed against a conserved epitope, recognizes both proteins (*c*). Molecular weight markers, with their size in kilodaltons, are shown on the right.

prise three peptides (from rat Cx26) representing residues 1–17, 101–119, and 215–226 (Zhang & Nicholson, 1989), one peptide representing amino acids 110–128 of rat Cx32 (Paul, 1986), and another based on a highly homologous sequence of both Cx26 and Cx32 (amino acids 166–185 in Cx32). In the latter peptide, three mismatches occur between the sequences of the two proteins. In each case, the Cx32 sequence was used in synthesizing the peptide.

All antibodies have similar reactivities against their corresponding peptides in ELISA's (positive at titres of $>10^{-4}$, *data not shown*), and titres ranging from 10^{-2} to 10^{-3} against gap junction proteins on Western blots (Fig. 1). In Western blots of purified mouse liver gap junctions, containing both Cx26 and Cx32, antibodies Cx26-N (1–17), Cx26-CL (101–119) and Cx26-C (210–266) each react exclusively with Cx26 (Fig. 1). An additional band at ~50 kD is also detected in some cases (e.g., Fig. 1*b*), consistent with previous demonstrations of connexin aggregation in SDS-PAGE (Henderson et al., 1979; Nicholson et al., 1981). αCx32-CL (110–119) reacts only with Cx32, its degradation products, and higher MW bands consistent with dimers and higher aggregation products (Fig. 1*a*). An antibody to the second extracellular domain, as might be expected based on the conserved nature of its site, recognizes both Cx32 and Cx26 (Fig. 1*c*). Hence, this polyclonal antibody is designated αCx32/26-E2. Indeed, faint reactivity with the more distantly related Cx43 has also been detected with this antibody (*data not shown*). Thus, with the exception of the latter, each of the antibodies is quite specific to connexin type. However, Western blots of more complex mixtures of proteins (e.g., whole tissue homogenates) revealed reactivity with several other bands. This appears to reflect the presence of common peptide motifs in several non-junctional proteins of the cell—most of which appear significantly more abundant than connexins.

Localization to Gap Junctional Structures

Immunohistochemistry of liver sections with αCx26-N (Fig. 2*a, b*), αCx26-CL (Fig. 2*c, d*) and αCx32-CL (Fig. 2*e, f*) produced a punctate pattern of staining delineating each hepatocyte characteristic of previously published labeling of gap junctions in this tissue (Dermietzel et al., 1984; Hertzberg, Spray & Bennett, 1985; Nicholson et al., 1987). No labeling of other membrane or cytoplasmic features was evident. In the case of rat

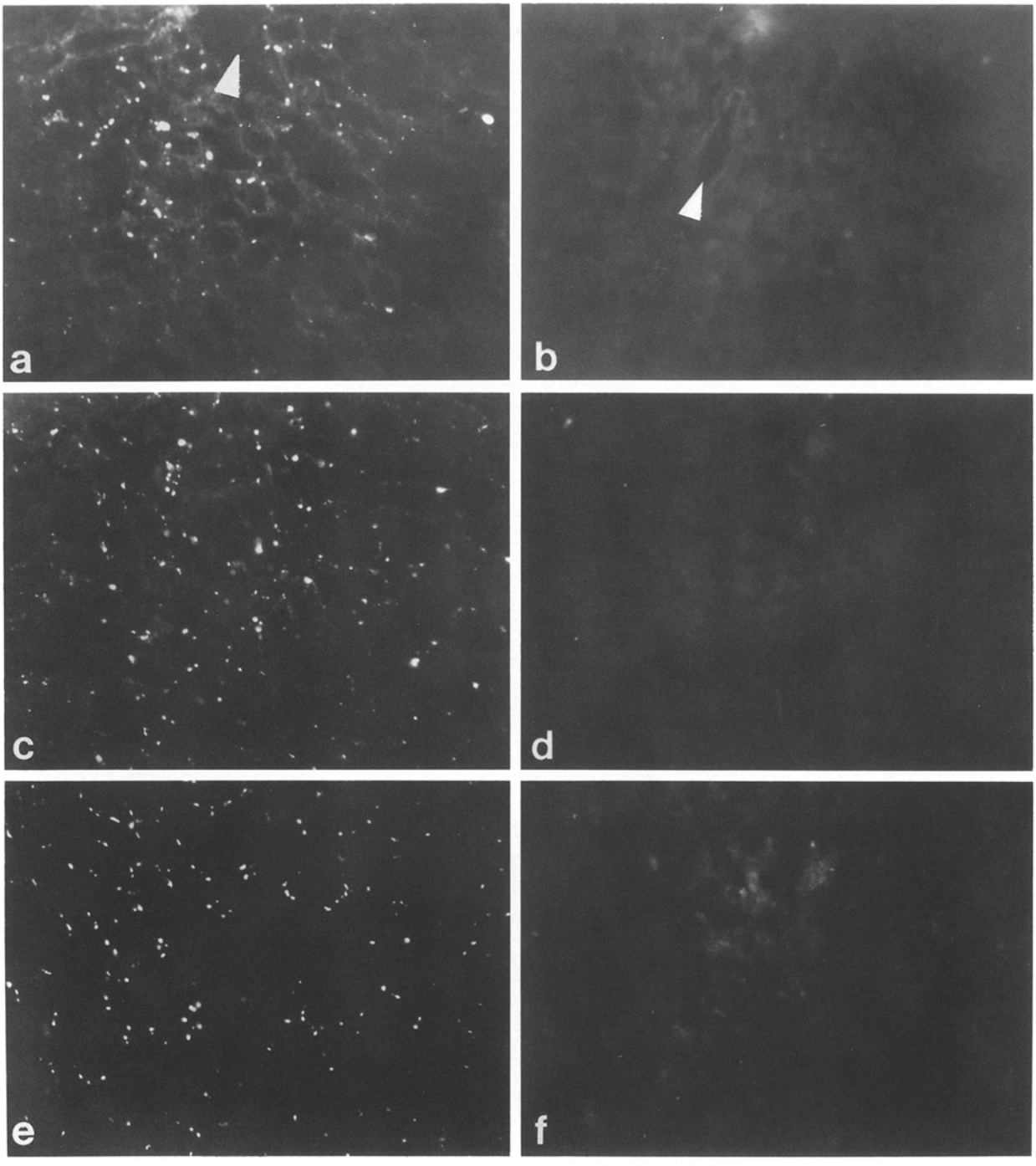
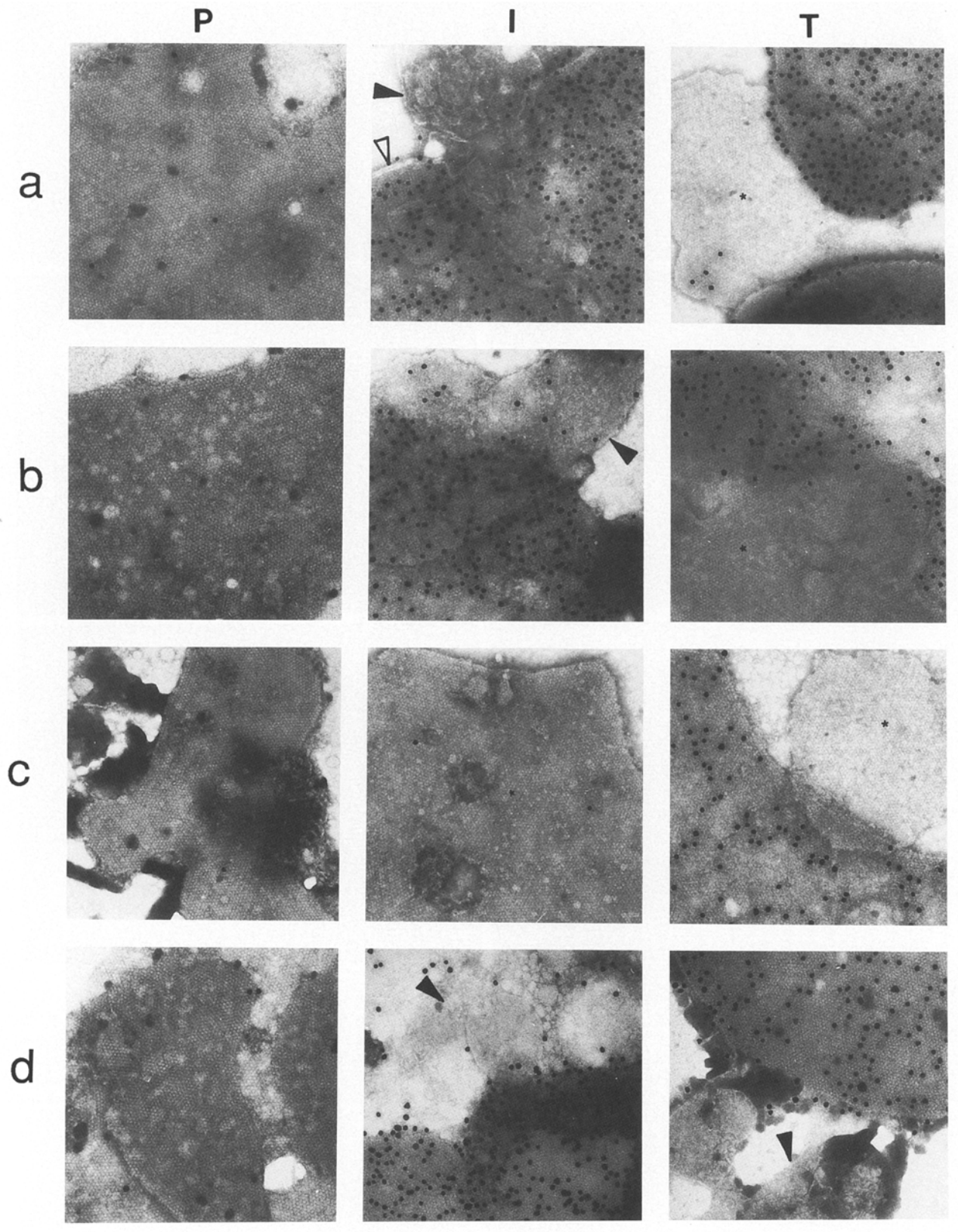


Fig. 2. Immunohistochemistry of liver demonstrates the specificity of αCx26-N, αCx26-CL and αCx32-CL for junctional structures *in situ*. Affinity-purified fractions of αCx26-N(*a*), αCx26-CL(*c*) and αCx32-CL(*e*), or equivalent concentrations of IgG fractions from the respective preimmune sera (*b, d* and *f,* respectively), were used to label frozen sections of either rat (*a* and *b*) or mouse (*c–f*) liver. Each of the antibodies revealed the punctate pattern surrounding hepatocytes characteristic of the distribution of gap junctions in this tissue. In the case of rat liver, Cx26-N reveals a decreasing gradient of label within each liver acinus from the portal (arrowhead) to the central vein—consistent with earlier reports of Traub et al. (1989). Surprisingly, in our hands, αCx26-N did not recognize Cx26 in mouse liver sections, despite its greater abundance and the ability of this antibody to bind to mouse Cx26 on Western blots (Fig. 3). This suggests that some other conformational factors must play a role in *in situ* labeling. Magnification is: 1,900×.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liver, αCx26-N revealed a graded distribution of this protein from portal to central vein within the acinus (Fig. 2*a*). This pattern has been previously observed for Cx26 by Traub et al. (1989). Neither αCx32/26-E2 nor αCx26-C produced specific labeling of liver tissue. The former result is consistent with the extracellular disposition of E2 in Cx32 (Nicholson et al., 1981; Zimmer et al., 1987; Goodenough et al., 1988; Milks et al., 1988), based on restricted access to the 2 nm extracellular "gap" between membranes. The likely explanation of the latter result was only evident in subsequent experiments (Fig. 3*c*; Fig. 4). Specific localization of the various site-specific antibodies to gap junctional structures was demonstrated more directly by gold decoration of negatively stained gap junction fractions (Fig. 3), the protein composition of which is shown in Fig. 1*f*. Labeling by αCx32-CL and αCx26-N and αCx26-CL, in all cases, was restricted to junctional structures which could be recognized by the hexagonal arrays of connexons on their surface and the occasional double membrane profile evident when the flat sheets curl upwards at their edges (Fig. 3*a*, open arrowhead). Fibrous and membranous material not showing these features were not stained significantly above preimmune levels with any of the antibodies (Fig. 3*a,b*, and *d* filled arrowheads). Consistent with the immunohistochemical studies, no labeling of intact junctions was seen with αCx26-C (Fig. 3*c*) or αCx32/26-E2 (Fig. 5). This result demonstrates that at least amino acids 1–17 and 101–119 of Cx26, and amino acids 110–128 of Cx32 are located on the cytoplasmic face of the junctions, since IgG molecules (smallest dimension approx. 5 nm) are too large to have access to extracellular domains in the gap between membranes (about 2 nm wide). The density of labeling varies from antibody to antibody. While this may be partially attributed to the abundance of the proteins (Cx32:26 ratio in mouse liver is 2:1, Nicholson & Zhang, 1988), it also seems likely to be affected by antibody titre. Actual labeling varied from 1 in every 12 connexons decorated in the case of αCx32-CL, to 1 in every 15 and 22 connexons for αCx26-N and -CL, respectively. Although no double-labeling of the same preparation was performed, both Cx32- and Cx26-specific antibodies stained all identifiable junctional structures evenly across their surface.

Exposure and Removal of Antigenic Sites

In contrast to the Cx26-N, Cx26-CL, and Cx32-CL antibodies, αCx26-C did not react with intact gap junction plaques, but required prior trypsinization of the gap junctions to expose its antigenic site (Fig. 3*c*). A time course study (Fig. 4*a*) showed that the reactivity of gap junctions to αCx26-C, as measured by ELISA of immobilized junctional plaques, increases with time of trypsinization, reaching a maximum at 30 min, and dropping thereafter. Overall junctional morphology, including the paired membrane profiles, remained unaffected over the time studied (Fig. 3*c*, column *T*, *see also* Goodenough & Stoeckenius, 1972 and Zimmer et al., 1987). Western blots of these same samples show that by 10 min of trypsinization, most gap junction proteins were cleaved into 10–15 kD fragments (Fig. 4*b*) with the resulting loss of the αCx26-CL site. Reactivity with αCx26-C on Western blots was still evident after 10 min but not after 2 hr of digestion. A reduction in binding of αCx26-C to the 10 kD trypsinized product may indicate the loss of a portion of this site during trypsinization. Thus, despite a reduction in net binding sites on the denatured protein, partial trypsinization causes increased reactivity of intact junctional structures. This suggests that the C-terminal end of Cx26 is also located on the cytoplasmic face, but is protected by other protease-sensitive components of the junction (e.g., cytoplasmic loop of Cx26, or possibly Cx32). Once exposed, the antigenic site for αCx26-C is then in turn subject to proteolysis, leading to a loss of binding in more extensively digested junctions (Fig. 4*a*). At an ultrastructural level, the loss of antigenic sites with proteolysis was also observed for αCx26-N, -CL and αCx32-CL (Fig. 3*a,b* and *d*, column *T*). This demonstrates that these antigenic sites are also accessible to trypsin and independently confirms that these domains are located cytoplasmically. Of particular interest is the pattern of labeling loss in response to trypsinization. Instead of a general decrease in density of gold particles, as seen for Cx32 in several independent experiments with αCx32-CL (e.g., Fig. 3*d*), Cx26 antigenic sites are preferentially cleared from whole domains, often whole junctions (Fig. 2*a–c*, starred areas in column *T*).

Fig. 3. Immunogold labeling of crude preparations of mouse liver gap junctions. Mouse liver gap junctions, isolated as described in the text, were applied to EM grids (~0.1 μg of junctional protein per grid) prior to (columns *P* and *I*) or after (column *T*) treatment with trypsin. They were then reacted, as described in the text, with preimmune IgG fractions (column *P*) or affinity-purified immune sera (columns *I* and *T*)—all at IgG concentrations of 0.08 mg/ml—against the following epitopes: Cx26-N (*a*), Cx26-CL (*b*), Cx26-C (*c*) or Cx32-CL (*d*). Gap junctional structures were then identified by negative staining with phosphotungstic acid which reveals the typical, semicrystalline array of connexons on the face of the membranes and occasional double membrane profiles visible at the edge of some gap junctions (open arrowhead in *a*). Amorphous nonjunctional membranes, unlabeled by immune IgG, are indicated by filled arrowheads. Gap junctions unlabeled by immune IgG, indicated by stars, are found in partially trypsinized junctional fractions (column *T*). All photographs are at a magnification of 100,000.

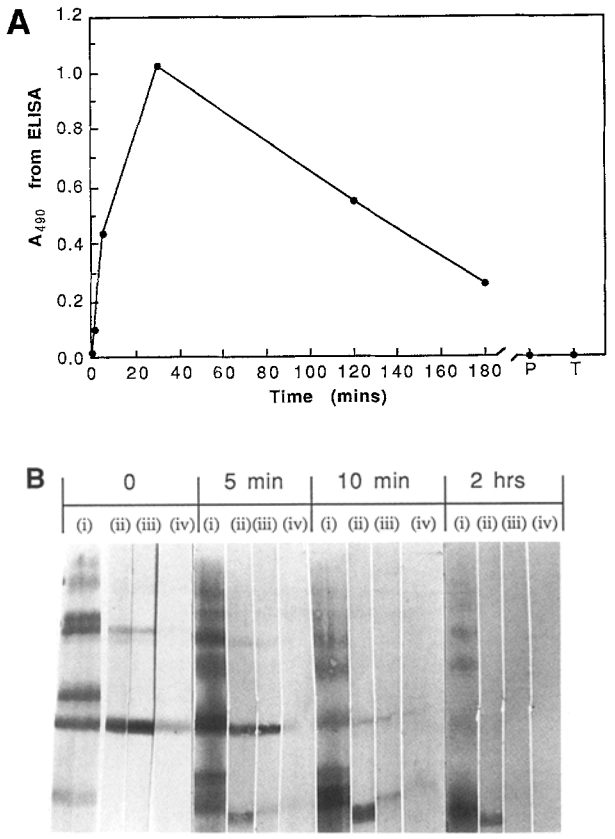


Fig. 4. Immunoreactivity of gap junctional structures: a time course study. (*A*) Reactivity of isolated mouse liver gap junctions with αCx26-C. Mouse liver gap junction fractions were treated with trypsin at 37°C at a substrate/enzyme ratio of 5. After stopping the reaction at the times indicated by addition of soybean trypsin inhibitor, aliquots containing approx. 1 μg of junctional protein were bound overnight to microtitre wells before reaction with crude αCx26-C at a dilution of 1/100 (*see text* for details of quantitation). Junctional aliquots reacted with preimmune sera (*P*), or trypsin alone reacted with immune sera (*T*) gave no significant signal. Maximal antibody binding occurs after 30 min of trypsinization at which time the junctional protein has largely been cleaved to 10–15 kD fragments (Fig. 4*b*). After this, reactivity steadily declines. (*B*) Western blot of trypsinized junctions assayed with αCx32-E2, Cx26-N, Cx26-C and Cx26-CL. Aliquots containing approximately 0.3 μg of junctional protein from every other time point (0 min; 5 min; 10 min and 2 hr) assayed in *A* were separated by 15% SDS PAGE, blotted onto PVDF membranes and reacted with αCx32-E2(*i*), αCx26-N(*ii*), Cx26-C(*iii*) and Cx26-CL(*iv*). The former recognizes both Cx32 and Cx26 proteins and their C-terminal degradation products while the latter three antibodies only recognize Cx26. The C-terminus of Cx32 is truncated within the first 5 min of digestion. By 10 min, most of both Cx32 and Cx26 have been cleaved into 15–9 kD fragments, with the loss of the Cx26-CL site (lane (*iv*)). A diffuse band at ~10 kD is recognized by Cx26-C, (lane (*iii*)) although an apparent reduction in staining intensity compared to the 0 and 5 min time points suggests that a portion of the antigenic site may have been lost. By 2 hr, all of the junctional protein has been reduced to fragments of approximately 10 kD, although immunoreactivity with αCx26-C or αCx26-CL is no longer detectable. Molecular weight markers in kilodaltons are indicated on the right.

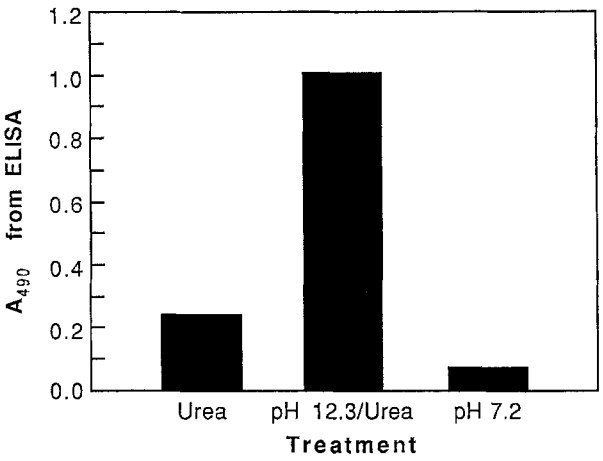


Fig. 5. Reactivity of isolated mouse liver gap junctions with αCx32/26-E2 before and after separation of the paired membranes. Isolated junctions were incubated, as described in the text, at pH 7.2 in the presence (1st bar) or absence (3rd bar) of 8 M urea, treatments which failed to cause any changes in the gap junction morphology (Fig. 7*a*), or at pH 12.3 in the presence of 8 M urea (2nd bar). The latter treatment separates the junctional membranes (Fig. 7*b; also* Zimmer et al., 1987). Following these treatments, the fractions were bound to microtitre plates and reacted with αCx32/26-E2 at 1/100 dilution. Only minimal binding to intact gap junctions, even after denaturing urea treatment, was detected. In contrast, strong reactivity was evident in gap junctions in which the extracellular surface was exposed by separation of the membranes in alkaline urea solution.

As proteolysis proceeds, antigenic sites are eventually removed from all junctions (e.g., Fig. 4*a*).

In contrast to the other antibodies, αCx32/26-E2 shows no reactivity with gap junction fractions, intact or proteolysed. However, this antigenic site can be exposed, as demonstrated by ELISA (Fig. 5), if junctional fractions are first treated with 8 M urea at pH 12.3. This treatment, as reported previously (Manjunath et al., 1984; Zimmer et al., 1987), causes the paired gap junctional membranes to separate (*see* Fig. 7*b*). The most direct interpretation of these data is that amino acids 166–185 are located between the two adjacent membranes and can be exposed only after the two membranes are separated. Denaturation of the protein by 8 M urea at neutral pH, a treatment which fails to separate the membranes, does not expose this antigenic site (Fig. 5).

Proteolysis and Immunolabeling of Gap Junction Fractions

The earliest indication that gap junction proteins span the membrane multiple times came from the demonstration that exhaustive proteolysis of either Cx32 or Cx43 yields two 10–12 kD membrane protected fragments (Nicholson et al., 1981; Gros et al., 1983). As an independent means of analyzing Cx26 topology, we

conducted similar studies on mouse liver gap junctions. However, unlike previous analyses, Cx26 represents the minor component of a mixture of two related connexins which colocalize to the same junctions (Nicholson et al., 1987). To generate fragments from both Cx32 and Cx26 which we could differentiate by SDS-PAGE, we chose to use a highly specific protease. As predicted from the primary sequences and the above studies (summarized for Cx26 in Fig. 8), treatment with *Staphylococcus* V8 protease under conditions which cleave the polypeptide chain after aspartate and glutamate residues produced four polypeptides ranging from 9 to 14 kD in SDS-PAGE [Fig. 6*a* (*vi*)]. Bands of higher M_r, evident in both V8 (Fig. 6*a*) and tryptic digests (Fig. 6*b*), represent aggregates of the proteolysed fragments—predominantly dimers, but also higher order aggregates in the case of Cx32 (Fig. 6*a* (*ii*) and (*iv*)). The failure of αCx26-CL (Figs. 6*a* and *b*) and αCx32-CL (Fig. 6*b*) to recognize these bands demonstrates that they do not represent incompletely digested Cx26 or Cx32.

The origins of the major proteolytic fragments were ascertained using specific antisera. αCx26-N reacted with the 11-kD band only [Fig. 6*a* (*iii*)], indicating that this fragment contains the N-terminal end of Cx26. Recognition of the 9 kD fragment by αCx26-C [Fig. 6*a* (*i*)] suggests that it represents the C-terminal end of Cx26. Binding of αCx32/26-E2 to this same fragment [Fig. 6*a* (*iv*)] is consistent with this assignment and further demonstrates the inaccessibility of this domain in protease digestion of intact junctions. A 14 kD band is also recognized by αCx32/26-E2, as well as αCx32-CL, [Fig. 6*a* (*iv*) and (*ii*), respectively], indicating that this polypeptide must represent the C-terminal half of Cx32 which still retains the antigenic site from residues 110–128. The 13 kD band, poorly resolved from the 14 kD band in Fig. 6*a* (*iv*), is likely to represent a further degradation product of the 14 kD polypeptide which has lost at least a portion of the αCx32-CL site from its N-terminus, but retains the protected extracellular site. This peptide cannot arise from Cx26, as it is not recognized by αCx26-C. Given the distribution of acidic residues which define potential V8 cleavage sites in Cx32 (*see* Fig. 8), this result also localizes the antigenic site of αCx32-CL to the N-terminal 9 residues of its peptide (i.e., between amino acids 110–119). Although we have no antibodies specific to the N-terminal half of Cx32, it seems possible that it may comigrate at 9 kD with the C-terminal half of Cx26, since this band has approximately twice the intensity of other bands in a Coomassie blue-stained gel [Fig. 6*a* (*vi*)], whereas all major fragments would be expected to be approximately equimolar. Since the V8 cleavage sites at positions 101, 110, 114, and 119 of Cx26 are all located inside the antigenic site of αCx26-CL (Fig. 8), it is to be expected that this antibody fails to identify digested junctions.

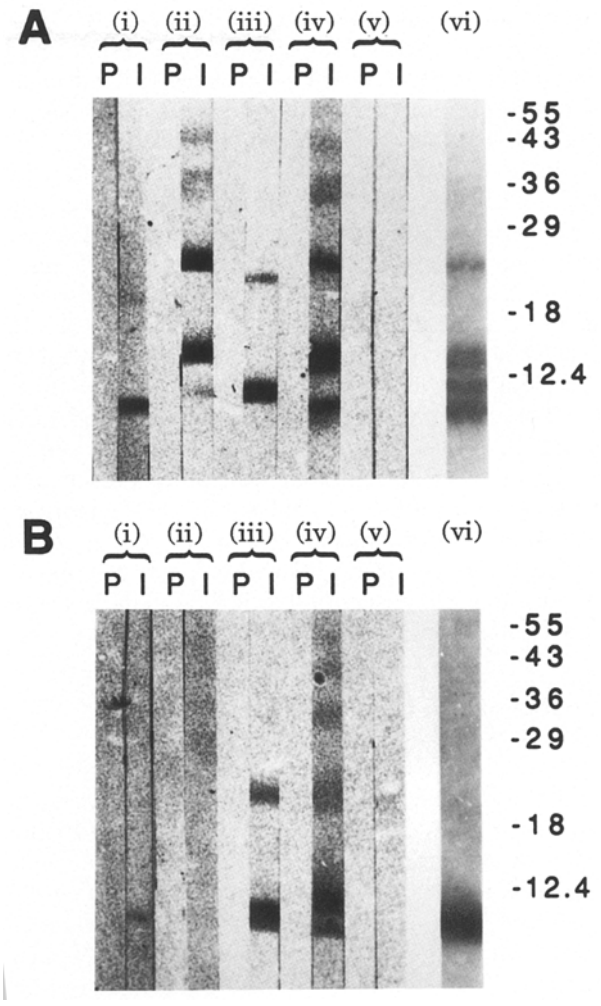


Fig. 6. Immunoblot of proteolyzed mouse liver gap junction fractions. Isolated mouse liver gap junctions were treated with V8 protease (*A*) or trypsin (*B*) for 2 hr as described in experimental procedures. Lane (*vi*) in each panel shows the Coomassie-stained pattern of peptides produced. The same material was loaded on parallel lanes and electroblotted onto PVDF membranes. Immunoblots were probed with a preimmune IgG fraction (lanes *P*) or affinity-purified immune (lanes *I*) sera: (*i*) αCx26-C; (*ii*) αCx32-CL; (*iii*) αCx26-N; (*iv*) αCx32/26-E2; (*v*) αCx26-CL. In addition to the major proteolytic products of 9–14 kD, antibodies also recognize aggregated products higher on the gel. Molecular weight markers are shown on the right of each panel in kilodaltons.

In contrast, no sites for V8 protease are found in the N- and C-terminal peptides of Cx26 (*see* Fig. 8).

A parallel set of studies using partial trypsin digestion yielded similar results. On Coomassie-stained gels, trypsin digests yielded a broad band of M_r 10,000 (Fig. 6*b* (*vi*)), consistent with previous observations (Goodenough & Stoeckenius, 1972; Henderson et al., 1979; Nicholson et al., 1981). C- and N-terminal antibodies to Cx26 bind to two fragments of slightly different mobility [Fig. 6*b* (*i*) and (*iii*), respectively]. The

lower relative binding of αCx26-C to the Cx26 C-terminal tryptic fragment compared to the V8 fragment in Fig. 6*a* could reflect loss of portions of the C-terminus after trypsinization (*see* Fig. 8 for trypsin and V8 sites). αCx32/26-E2 binds to two fragments [Fig. 6*b* (*iv*)], one of which corresponds to the fragment recognized by αCx26-C [Fig. 6*b* (*i*)]. The other fragment is likely to represent the C-terminal half of Cx32 although this cannot be independently confirmed with αCx32-CL since this site is apparently removed by trypsin [Fig. 6*b* (*ii*)]. The presence of the N-terminal half of Cx32 in the 10 kD band, however, has previously been demonstrated by sequencing (Nicholson et al., 1981).

Thus, both trypsin and V8 protease cleave Cx26, like Cx32, into two fragments containing the N- and C-termini of the native protein, which are presumably protected from further proteolysis by the membrane. Solubilization of the membranes in detergent prior to proteolysis results in much smaller fragments, apparently due to the exposure of tryptic sites in the putative extracellular loops (*see* Fig. 8).

Proteolysis and Immunolabeling of Split Gap Junctions

A corollary of the above demonstration that three domains of the protein are exposed at the cytoplasmic face, with intervening segments protected by the membrane, is that at least two regions should be located extracellularly, per the currently proposed connexin model (Milks et al., 1988; Zhang & Nicholson, 1989). To determine if this is true, we treated gap junctions with 8 M urea at pH 12.3. Under these conditions, the paired gap junctional membranes can be separated (Manjunath et al., 1984; Zimmer et al., 1987), exposing the extracellular face to protease and antibody probes. Untreated fractions contained less than 10% single membranes (Fig. 7*a*) whereas, after treatment, approximately 50–90% of the membranes were separated (Fig. 7*b*). Efficiency of splitting was somewhat variable from experiment to experiment. Some residual double membrane profiles could be distinguished (arrowhead in Fig. 7*b*), clearly showing that the single membranes are derived from junctional structures. As a proteolytic probe of these structures, we chose Asp-N (Boehringer Mannheim, IN), an endoproteinase which cleaves on the amino side of aspartate residues, thus producing a limited number of predictable fragments (*see* Fig. 8). As with V8 protease, proteolysis of intact gap junctions with Asp-N produced three or four resolvable products ranging from 11 to 17 kD (Fig. 7*a*). Immunoblots probed with our antipeptide antibodies showed that the 17 and 16 kD fragments represent partial and complete digestion products of the C-terminal half of Cx32 (*cf.* Fig. 7*c* (*i*) and (*iii*)–(*v*)). The 13 kD fragment reacts only with αCx26-N (Fig. 7*c* (*iii*)), while the 11 kD fragment reacts only with αCx26-C (Fig. 7*c* (*iv*)). It is informative that αCx26-CL does not recognize the 13 kD N-terminal half of Cx26, since there is but one Asp-N cleavage site in the cytoplasmic loop which would only cleave the C-terminal 3 residues of the antigenic target for αCx26-CL (Fig. 8). This is apparently sufficient to destroy the site.

Asp-N proteolysis of junctions first split with 8 M urea at pH 12.3, produced further digestion of the junction proteins, yielding additional bands at 9 and 6 kD. In some experiments, only partial digestion was achieved (Fig. 7*c* (*ii*)) while others showed more complete proteolysis (Fig. 7*b*). The 9 kD fragment reacts with αCx32-CL (Fig. 7*c* (*viii*)) while the 6 kD fragment is recognized by αCx26-N (Fig. 7*c* (*vi*)). This suggests that the N-terminal half of Cx26, and the C-terminal half of Cx32 have at least one protease cleavage site exposed on the outside of the cell membrane.

While the modified pattern of proteolysis observed correlates with the exposure of the extracellular face of gap junctions to proteolysis, this requires denaturing conditions utilizing 8 M urea at pH 12.3. To ascertain whether the denaturation of the protein itself could reveal additional cleavage sites, we treated gap junctions with 8 M urea at neutral pH. While presumably causing similar disruption of secondary structure to that induced by 8 M at alkaline pH, no separation of junctional membranes was induced, and no modification in the pattern of proteolysis was detected (*data not shown*). This correlates with the failure to expose residues 166–188 of Cx32 or Cx26 by the same treatment discussed above (Fig. 5).

Discussion

Antibody Characterization

Although Cx26 has now been demonstrated to form intercellular channels independent of other connexins (Barrio et al., 1991), it is nonetheless generally found to be coexpressed with other connexins in vivo. Thus, to directly demonstrate its structure and relationship to other connexins *in situ,* specific reagents which distinguish between Cx26 and other connexins needed to be developed. In this way, one can empirically test the "established" models for the unique case of Cx26. By selecting hydrophilic domains which vary maximally between connexins (Table), antipeptide antibodies were produced which proved specific for Cx26-N, -CL and -C regions, or αCx32-CL (fig. 1). The only exception was αCx32/26-E2, raised against a peptide from the second extracellular domain of Cx32, a region which is quite conserved among connexins. Predictably, this la-

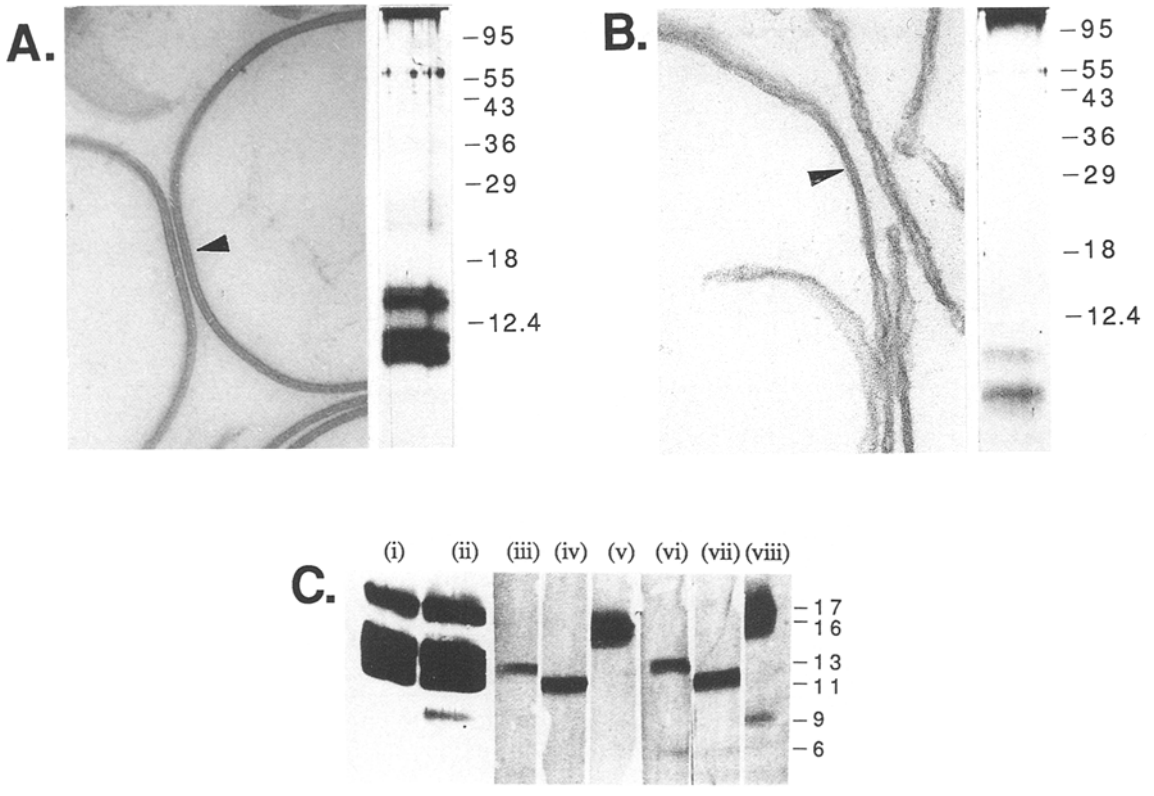


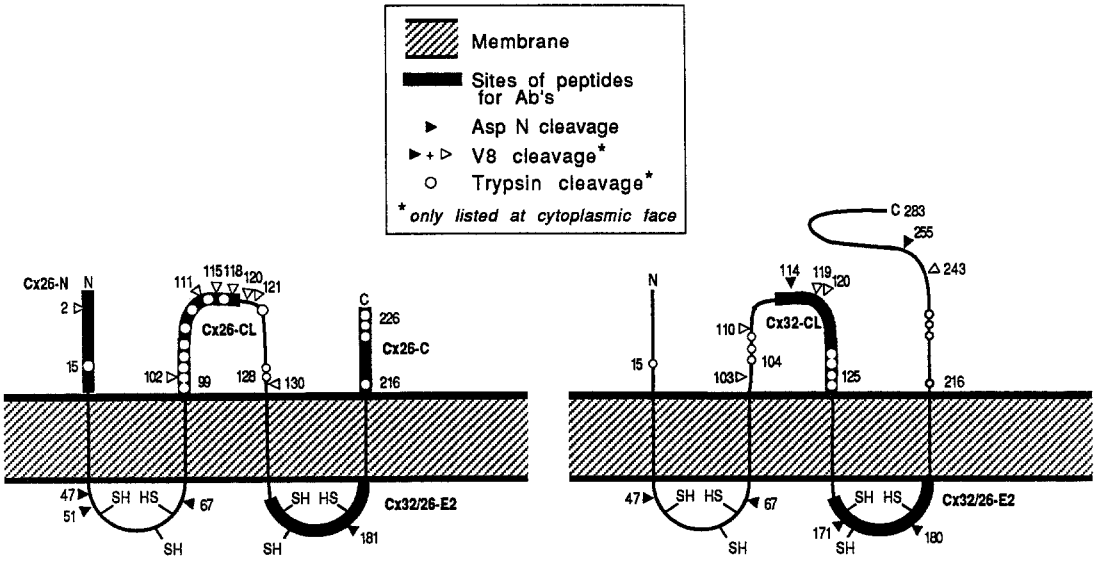
Fig. 7. EM analysis and proteolysis of intact and split liver gap junctions. Isolated mouse liver gap junctions were examined before (*A*) or after treatment with 8 M urea at pH 12.3 (*B*) as described in experimental procedures. Typical double membrane profiles of untreated gap junctions (indicated by a filled arrowhead in *A* were separated after treatment with 8 M urea at pH 12.3 into single membranes, although a small fraction of paired membranes remained (filled arrowhead in *B*). Thin sections are shown at a magnification of 100,000. Lanes adjacent to *A* and *B* show silver-stained gel patterns of intact and split gap junctions respectively treated with Asp-N protease and separated by 15% SDS-PAGE (molecular weight markers in kilodaltons are indicated to the right of each lane). In a separate experiment shown in *C*, isolated junctions were again treated with Asp-N before [(*i*), (*iii*)–(*v*)] or after [(*ii*), (*vi*)–(*viii*)] treatment with alkaline urea. In this case, only partial separation of the membranes was achieved. Products of proteolysis were then separated by SDS-PAGE and either silver stained [(*i*) and (*ii*)] or blotted to PVDF membrane for reaction with αCx26-N [(*iii*) and (*vi*)]; αCx26-C [(*iv*) and (*vii*)]; or αCx32-CL [(*v*) and (*viii*)]. Molecular weight markers are indicated on the right in kilodaltons. Immunoblots reacted with αCx26-CL are not shown as no reactivity with any bands was evident.

beled both proteins (Fig. 1*c*) and was also found to weakly recognize Cx43. Specificity for gap junctional structures was also demonstrated for most of the antisera by immunohistochemistry of rodent liver or heart (Fig. 2) and/or by immunogold decoration of isolated gap junctional fractions (Fig. 3).

Distribution of Cx26 and Cx32 within Junctions

The best characterized case of coexpression of two connexins in the same cell has been that of Cx26 and Cx32 in mouse hepatocytes where both immunohistochemistry (Nicholson et al., 1987) and double immunogold labeling of thin sections (Traub et al., 1989) have been used in demonstrating the codistribution of these proteins within individual gap junctional plaques. Despite the fact that double-labeling was not used in this study, as our antibodies were all rabbit polyclonals, it is clear that the two antigens were colocalized to the same plaques since all junctions on a grid were labeled uniformly by both antibodies to Cx26 and Cx32. As in previous studies, the density of labeling achieved was insufficient to test the colocalization of the two proteins at the level of individual channels. However, the immunogold decoration of isolated junctional plaques used here does allow a more comprehensive analysis than earlier studies and indicates that specific subdomains enriched for Cx26 or Cx32 do not form within the plaque. This conclusion, of course, is limited by the density of our labeling, which could not have detected domains smaller than 10–20 connexons.

The density of labeling achieved in this study could



Cx 26 **Cx 32**

	αα's	Size (kD)	Ab Sites	Det[a]	αα's	Size (kD)	Ab Sites	Det
V8 Digest (intact GJ's) (Fig. 4A)	2-102	11	N	+	1-103	11.5	–	–
	130-226	10	E2; C	+; +	120-243	14	E2	+
Trypsin (intact GJ's) (Fig 4B)	15-99	9	–	–	15-104	10	–	–
	(1-99	11	N	+)	(1-104	12	–	–)
	128-216	10	E2	?	125-216	10	E2	?
	(128-226	11	E2; C	+; +)	(120-220 or 224	12	E2	+)
Asp-N (intact GJ's)	2-118	13	N; CL?	+;-	1-114	13	–	–
	118-226	12	E2; C	NT;+	114-225	16	CL	+
(split GJ's) (Fig 5)	2-47	5	N	+	1-47	5	–	–
	67-118	6	CL?	–	67-114	5	–	–
	118-181	7	E2?	NT	114-171	7	CL	+
	181-226	5	E2?;C	NT; –	180-255	9	E2?	NTs

Fig. 8. Topological maps and predicted proteolytic cleavage pattern of Cx26 and Cx32 based on their deduced sequences. Models are shown with the cytoplasmic face up and the extracellular face down. The locations of the peptides used for producing antibodies listed in the Table and the potential sites of proteolytic cleavage predicted from the primary sequence are indicated. The figure table shows the theoretical fragments produced from the proteolytic treatments shown on the left. Only peptides resolvable by our SDS-PAGE system are shown (i.e., >3 kD). Peptides in parentheses represent partial degradation products. Antigenic sites present on each peptide are indicated. An appended "?" indicates that a portion of the site has been removed by proteolysis with an unknown effect on immunoreactivity. ([a]) Detection of peptides—indicates peptides identified in Western blots shown in Figs. 6 or 7; (+) Band identified; (–) Band not found; (?) Band could not be resolved due to overlap with cross-reactive fragments; (NT) not tested.

not be increased, despite using higher antibody concentrations or differently sized gold beads conjugated to our secondary antibody. Therefore, it seems unlikely that higher labeling densities could be achieved *in situ* following intracellular injections of much lower concentrations of antibodies (e.g., Hertzberg, Spray & Bennett, 1985 and Traub et al., 1989). Since such injections have been demonstrated to uncouple cells, it would appear that closure of the junctional channels does not require 1:1 binding of IgG molecules, but apparently involves more cooperative phenomena. Cooperativity in gating of gap junctional channels has also been suggested from analyses of voltage gating of different connexins (Nicholson et al., 1992). Of potential relevance to this issue is our observation here that limited proteolysis of isolated junctions removes Cx26 antigenic sites from whole plaques, while adjacent plaques remain unaffected. This effect appears specific for Cx26, as the Cx32-CL site is removed uniformly in all plaques by trypsinization. These observations could also be explained by conformational cooperativity between Cx26 subunits in adjacent connexons within a plaque leading to preferential cleavage within a given gap junction structure.

Topology of Cx26

The specific antibodies generated from the peptides tested in the Table have allowed us to establish a comprehensive topological map of Cx26 which proves analogous to those established for Cx32 and Cx43 (Zimmer et al., 1987; Goodenough et al., 1988; Hertzberg et al., 1988; Milks et al., 1988; Yancey et al., 1989). This model, with the antigenic and potential proteolytic sites indicated, is shown in Fig. 8. It is determined from: (i) the labeling of isolated gap junctions by αCx26-N, -CL and -C, which would not have access to the extracellular surface of intact gap junctions because of their size; (ii) the cleavage of these sites by proteases, which would, similarly, not have access to extracellular surfaces of the junctions; and (iii) the accessibility of αCx32/26-E2 to its site and Asp-N protease to its potential cleavage sites (before residues 46, 50 and 66 in Cx26) after separation of the junctional membranes, but not after similar denaturing treatments which failed to separate the membranes. Since this effectively defines the disposition of each hydrophilic domain of the protein, the topological model shown in Fig. 8, in its basic features, is the only one consistent with the data. One additional refinement of this model can be made based on the masking of the C-terminus of Cx26, illustrated in Figs. 3*c* and 4*a*. Exposure of the site by mild proteolysis indicates that it is covered by other protein domains. In the absence of stoichiometric amounts of nonjunctional proteins in the preparations [*see* Fig. 1*f*], these masking domains must have been contributed by either Cx32 or Cx26. The specific sequences involved are likely to be in the cytoplasmic loops, since time course studies show that binding to the C-terminus coincides with the cleavage of these proteins into two 10–15 kD fragments, and not with the removal of the C-terminal domain of Cx32 (Fig. 4*b*).

These results clearly define a topology for the minor Cx26 component of liver gap junctions which is analogous to that previously deduced for Cx32 and Cx43. The combined use of proteolysis and site-specific antibodies has also proven useful as a probe of the conformational arrangement of this junctional protein. These models should facilitate interpretation of future structural and mutagenic studies on the Cx26 protein and its interaction with other connexins. Indeed, the value of empirically establishing the arrangement of channel proteins within the membrane is emphasized by recent results in other channel families where the models deduced from the sequence have proven to be flawed (*cf.* Finer-Moore & Stroud, 1984 with Leonard et al., 1988; and Noda et al., 1984 with Yellen et al., 1991). While our studies have also furthered our understanding of how Cx26 may interact with Cx32 in hepatic junctions, it is clear that higher resolution analyses will be needed to achieve a complete understanding of this potentially complex process.

We would like primarily to recognize the input and work of Alan Siegel in helping to achieve EM labeling of gap junctions and Ms. Feng Gao for characterization of the antibodies used. We would also like to thank Kathleen Sodaro and Marty Bartel for technical help in preparation of the antibodies, Linda Mack and Rose Stern for help in preparation of the manuscript and James Stamos for preparation of the figures. This work was supported by a grant from the U.S. Public Health Service, National Institutes of Health, CA 48049 and a Scholars award in the biomedical sciences from the PEW Charitable Trust (to B.J.N.).

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