Attempts to Define Functional Domains of Gap Junction Proteins with Synthetic Peptides

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ABSTRACT To map the binding sites involved in channel formation, synthetic peptides representing sequences of connexin32 were tested for their ability to inhibit cell-cell channel formation. Both large peptides representing most of the two presumed extracellular loops of connexin32 and shorter peptides representing subsets of these larger peptides were found to inhibit cell-cell channel formation. The properties of the peptide inhibition suggested that the binding site is complex, involving several segments of both extracellular loops. One of the peptides (a 12-mer) did not inhibit but instead was found to form channels in membranes. Both in oocyte membranes and in bilayers, the channels formed by the peptide were asymmetrically voltage dependent. Their unit conductances ranged from 20 to 160 pS. These data are discussed in the form of a model in which the connexin sequence represented by the peptide is part of a β structure providing the lining of the channel pore.

INTRODUCTION

Gap junctions are assemblies of cell-cell channels (see review by Hall et al., 1993). Each channel is formed by the docking of two hemichannels located in apposing cell membranes. There is good evidence that a complete channel can be formed by a single type of protein subunit (connexin) (Paul, 1986; Dahl et al., 1987). Thus the interaction between hemichannels involves homophilic binding. Each connexin molecule is thought to consist of four transmembrane segments, three cytoplasmic domains, and two extracellular loops (Gilula, 1987; Goodenough et al., 1988; Hertzberg et al., 1988; Laird and Revel, 1990). It is these loops where the homophilic docking of hemichannels must occur that eventually results in the opening of a cell-cell channel. To map the binding sites responsible for the connexin interactions, we used a competition assay with synthetic oligopeptides representing segments of the extracellular loops. If the peptide, or more likely a fraction thereof, assumes a configuration compatible with that in the protein, it could mimic connexin-connexin binding and thereby inhibit channel formation. In addition, it is conceivable that peptide binding to a hemichannel might open the hemichannel. Mapping of protein binding involved in homophilic protein-protein interaction by synthetic peptides has been used previously for cell adhesion molecules (Kamboj et al., 1989).

We prefer the use of peptides over antibodies that have been used by others (Meyer et al., 1992) because of concerns about steric hindrance. Nonjunctional membrane glycoproteins have been shown to interfere severely with the channel formation process due to their bulky structure (Levine et al., 1991). Antibodies binding to any site on the extracellular loops, therefore, are likely to interfere with channel formation regardless of whether the antibody binding site plays a role in connexin-connexin interaction.

Whereas most of the tested peptides inhibited channel formation, one peptide induced a membrane conductance as if it were opening hemichannels. Further analysis of this effect revealed, however, that rather than opening hemichannels the peptide formed channels by itself. If that property reflects the function of the peptide in the protein, a modification of the current model of transmembrane configuration of connexins may be required.

MATERIALS AND METHODS

Materials

The various peptides (Table 1) were obtained from the Peptide Synthesis Core Facility at the University of Miami. They were synthesized by solid-phase methods using g-fluorenylmethoxycarbonyl chemistry with an Advanced Chemical Tech, Inc. (Louisville, KY) multiple peptide synthesizer. 1-Palmitoyl-2-oleyl-*sn*-glycerophosphoethanolamine and 1-palmitoyl-2-oleyl-*sn*-glycerophosphocholine were obtained from Avanti Biochemicals (Alabaster, Alabama).

Oocyte assay

The oocyte assay was performed as described (Dahl, 1992). Briefly, female *Xenopus laevis* were obtained from Xenopus I (Ann Arbor, MI). Oocytes were defolliculated with collagenase (Worthington Biochemical Corp., Freehold, NJ) in Ca-free OR2 medium. In vitro-transcribed mRNA was injected at the equator 16–20 h before pairing. Immediately before pairing the vitelline membrane was removed by forceps. Junctional conductance was determined 2 h after pairing with the dual voltage-clamp procedure (Spray et al., 1981). Peptides were added to the devitellinized oocytes 10 min before pairing and were present until conductance measurements.

Reconstitution in lipid bilayers

Bilayers were formed on the tip of patch pipettes according to Suarez-Isla et al. (1983). 1-Palmitoyl-2-oleyl-*sn*-glycerophosphoethanolamine and 1-palmitoyl-2-oleyl-*sn*-glycerophosphocholine, mixed at a 4:1 ratio, were taken up in hexane at 5 mg/ml. The aqueous compartments contained OR2 medium (pH 7.5).

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TABLE 1 Peptides used in inhibition studies

Name	Sequence
E1	A A E S V W G D E K S S F I C N T L Q P G C N S V C Y D H F F P I S H V R
E1a	E S V W G D E K S S F I
E1b	I C N T L Q P G C N S V
E1c	S V C Y D H F F P I S H
E2	R L V K C E A F P C P N T V D C F V S R P T E K T
E2a	L Y P G Y A M V R L V K
E2b	V K C E A F P C P N T V
E2c	V D C F V S R P T E K T
C2	K M L R L E G H G D P L H L E E V K R

In vitro transcription

Connexin32 mRNA was transcribed in vitro from wildtype and mutant cDNA clones as described previously (Werner et al., 1989). Before transcription, plasmids were linearized with *Sspl*, located 649 base pairs downstream from the cDNA insertion site. mRNA concentrations were determined by agarose gel electrophoresis and adjusted to yield equal concentrations for in vitro translation experiments.

In vitro translation

Rabbit reticulocyte lysate (nuclease-treated), supplemented with canine pancreatic microsomal membranes (Promega Biotec, Madison, WI) were used to translate the connexin32 mRNAs. A typical reaction mixture consisted of 17 μl reticulocyte lysate, 0.5 μl 1 mM amino acid mixture (minus methionine), 2 μl [^{32}S]methionine at 10 mCi/ml, 1.2 μl of water, 1.8 μl microsomal membranes, and 2 μl of the in vitro-transcribed mRNA. The mixture was incubated at 30°C for 1 h. 5 μl of the reaction mixture was loaded onto 45 μl of 3% sucrose in a microcentrifuge tube and centrifuged at 14,000 × g for 1 h at 4°C. The pellet was resuspended in 25 μl of SDS sample buffer (1% glycerol, 2% SDS, 0.2% bromphenol blue), the sample was heated for 2 min at 80°C, and 5 μl were loaded onto a 15% polyacrylamide gel for analysis.

Mutagenesis

Mutant connexin32 cDNA clones were constructed from a connexin32 cDNA clone (Paul, 1986) in pGEM-3Z(f+), modified at the 5′ end to include the Kozak consensus sequence for efficient translation (Kozak, 1989) as described previously (Werner et al., 1991), by the Kunkel (1985) method using synthetic oligonucleotides as primers. All mutants were verified by complete DNA sequence analysis.

RESULTS

Peptide inhibition assay

Oocytes injected with connexin32 mRNA accumulate a pool of channel precursors. Upon pairing of such oocytes, cell-cell channels can form rapidly from this pool at rates of up to 40 channels per second (Dahl et al., 1992) (Table 2). When synthetic oligopeptides, representing either most of the first extracellular loop (E1, 38-mer,) or most of the second extracellular loop (E2, 26-mer) were added to the oocytes just before pairing, the junctional conductances determined 2 h after pairing were significantly reduced compared with untreated oocyte pairs (Fig. 1). There are six cysteines in the extracellular loops, all of which have been shown to be critically involved in the channel-formation process (Dahl et al., 1991, 1992). To test for unspecific thiol effects by the synthetic peptides, oxytocin was used as a control and found not to interfere with the channel-forming process. Another control peptide, representing the sequence of the cytoplasmic loop (amino acids 104–122) was also found to be inactive in inhibiting channel formation when applied extracellularly to oocytes. The inhibitory effect of E1 and E2 peptides appears to be at the level of channel formation because the peptides did not affect the conductance of existing channels (tested by adding peptides to oocytes paired 24 h before peptide addition, data not shown).

TABLE 2 Glycosylation mutants of connexin32

Site	Original sequence	Mutated sequence	Glycosylated	Conductance (μS)
159–161	GYA	NYS	−	20.88 ± 1.15
162–164	MVR	NVS	−	0.41 ± 0.15
165–167	LVK	NVT	+	0.09 ± 0.02
169–171	EAF	NAS	−	26.29 ± 2.61
175–177	NTV	NTT	+	0.01 ± 0.003*
183–185	RPT	NPT	−	26.25 ± 2.71
186–188	EKT	NKT	−	0.46 ± 0.16
189–191	VFT	NFT	−	0.20 ± 0.08
Wildtype connexin32				20.06 ± 3.10
Uninjected oocytes				not detectable (<0.01)

Junctional conductance, expressed as the mean ±SE (n = 9), was determined 2 h after pairing of the oocytes.

* This value was obtained in a different experiment in which wildtype connexin32 gave a conductance of 9.75 ± 2.10 μS.

To map the binding site more precisely, smaller partially overlapping peptides were used in the inhibition assay. If the binding site comprises only part of the extracellular loop, then one would expect some smaller peptides to exhibit equivalent or even stronger inhibitory effects than the larger peptides, whereas other small peptides should be ineffective. If, on the other hand, the binding site is large or if it is comprised of many small units, then several of the small peptides should inhibit to the same or to a lower extent than the large peptides.

Six dodecapeptides, comprising amino acids 41–52 (cx32: 41–52), 52–63 (cx32:52–63), and 62–73 (cx32:62–73) for E1 and 156–167 (cx32:156–167), 166–177 (cx32:166–177), and 177–188 (cx32:177–188) for E2, were tested. Of these six peptides, five were found to be inhibitory at similar or lower levels than the larger peptides (Fig. 1). The other peptide, spanning amino acids 156–167, did not inhibit at all. Instead, it produced a large nonjunctional membrane conductance. No such change in membrane conductance was observed with any of the other five dodecameric peptides nor with the larger E1 and E2 peptides.

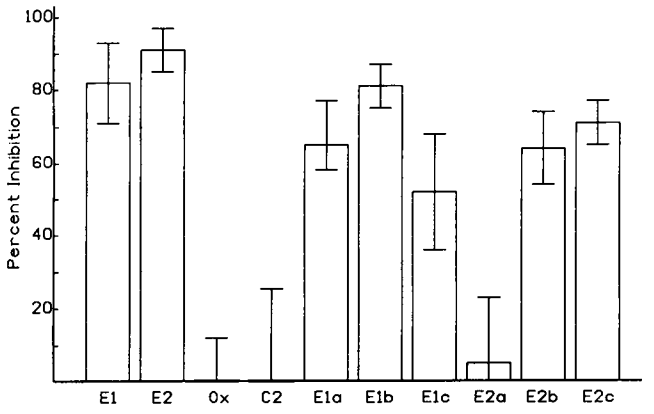

FIGURE 1 Inhibition of cell-cell channel formation by synthetic peptides representing part of the extracellular loops of connexin32. The sequences of the peptides used are listed in Table 1. Percent inhibition is calculated from the conductance observed in oocyte pairs in the presence and absence of the peptide. This normalization was done to be able to compare data from experiments using different oocyte batches and different mRNA preparations. Each data point represents the normalized mean obtained from nine oocyte pairs ± SE. As controls, oocytes were paired in the presence of oxytocin (Ox) or C2 (intracellular connexin32 loop). All peptides were present at a concentration of 5×10^{-4}M.

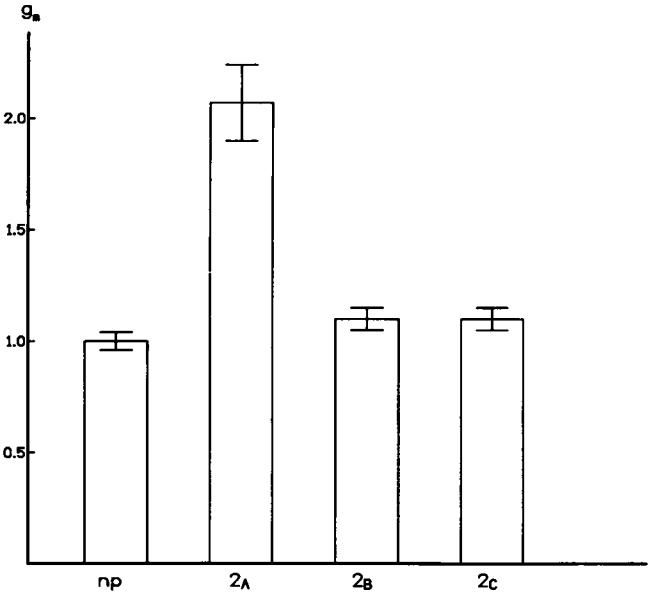

FIGURE 3 Membrane conductance (μS) of single oocytes with no peptide (np) and oocytes exposed to peptides E2a, E2b, or E2c, determined 2 h after application. Each bar represents the mean of 17 oocytes ± SE.

Channel formation by an E2 peptide (cx32:156–166)

The peptide that was found to be inactive in the inhibition assay had other drastic effects on the oocytes. Within 2 h of application a change of pigment distribution could be observed and within 24 h the oocytes had died (Fig. 2). Oocytes exposed to this peptide were depolarized (data not shown), and nonjunctional membrane conductance, measured early after peptide application, was significantly increased over that of untreated oocytes or of oocytes treated with the other two E2 peptides (Fig. 3). None of the E1 peptides caused a detectable change in the nonjunctional membrane conductance (data not shown).

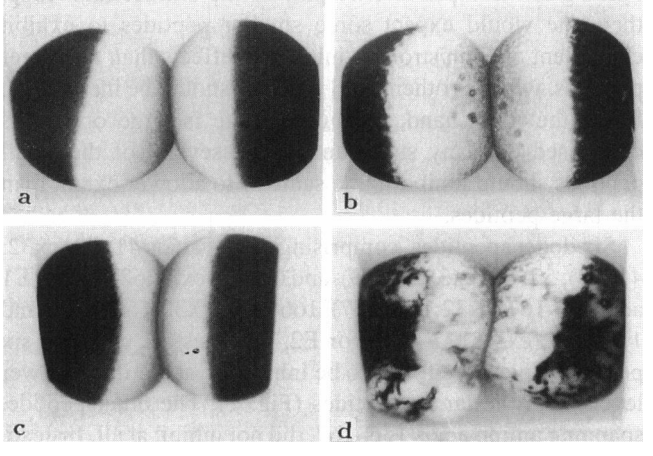

FIGURE 2 Oocyte pairs treated with peptide E2b (*a*, *c*) and E2a (*b*, *d*) at 2 h (*a*, *b*) or 24 h (*c*, *d*) after peptide application. Oocytes exposed to peptide E2b appear normal while oocytes exposed to peptide E2a exhibit a change in pigmentation within 2 h and at 24 h are disintegrating.

The current-voltage relationship of the peptide-induced conductance is shown in Fig. 4. There is a strong voltage dependence; depolarization of the membrane activates an outward current. The conductance activates slowly but deactivates fast. A similar conductance had been found in oocytes that express connexin46 and probably was due to open connexin46 hemichannels (Paul et al., 1991). This suggested the possibility that the peptide opened connexin32 hemichannels. Alternatively, the peptide could interact with another channel protein, thereby opening that channel, or it could be forming a channel by itself.

The first possibility is unlikely because the peptide acted to the same extent on uninjected oocytes as on oocytes expressing connexin32. Even if endogenous connexin38 were present at similar levels as connexin32 one would expect at least a quantitative difference between uninjected and connexin32-injected oocytes. Furthermore, in connexin38 the sequence equivalent to the one represented by the peptide is different. This makes analogous effects of the peptide on the two connexins unlikely.

To test whether the peptide by itself was capable of forming channels it was incorporated into lipid bylayers. A similar conductance was observed in bilayers as in oocytes (Fig. 5 *a*). An asymmetrically voltage-dependent conductance was seen, which became symmetric over time (>20 min). The disappearance of the asymmetry suggested that the peptide reoriented itself in the membrane after an initial asymmetric insertion. With a similar time scale as the loss of asymmetry, the conductance increased, probably due to the formation of larger aggregates.

At early times after peptide application to bilayers, and with low peptide concentrations, single channel activity was observed (Fig. 5 *b*). The unit conductances of these single-channel events varied over a broad range, between 20 and

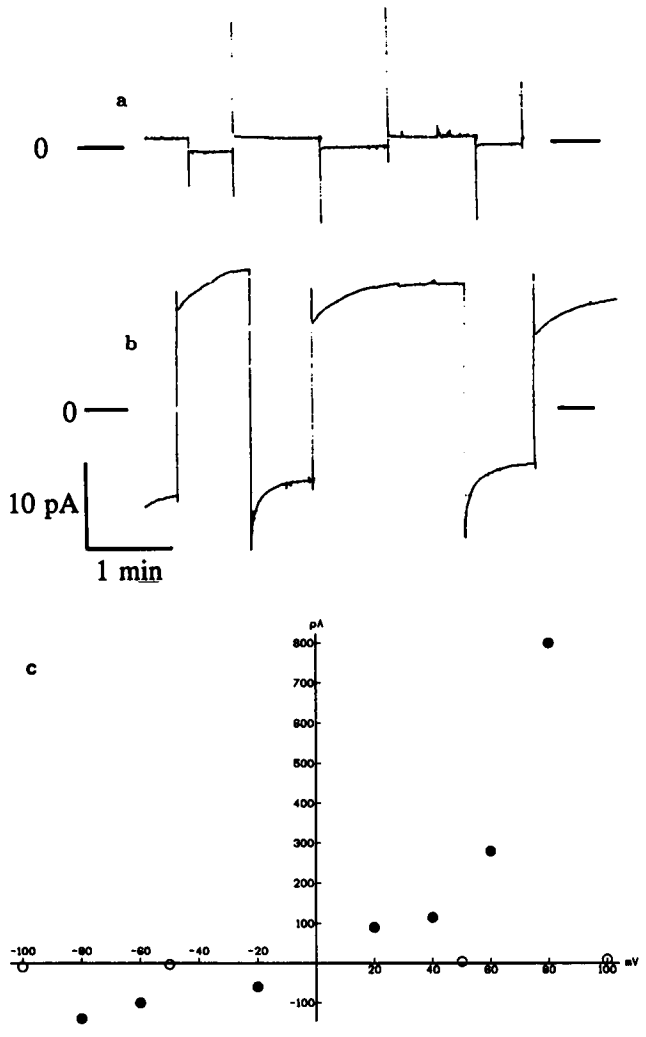

FIGURE 4 Membrane currents in excised oocyte membrane patches (*a*) in the absence of and (*b*) in the presence of E2a peptide. The potential is stepped alternatingly to ±100 mV (*a* and *b*). The I-V curve of excised oocyte membrane patches without peptide (○) or with E2a peptide (●) is shown in (*c*). All recordings on the membrane patches were done in symmetric solutions (OR2), and the peptide, when applied, was present in the patch pipet.

160 pS, with a clear trend toward larger conductances with time. The difference in conductance state probably reflects various oligomerization states of the peptide.

Mapping of the extracellular loop 2 by introduction of artificial glycosylation sites

The connexins are among the best physically mapped membrane channel proteins. Several laboratories, using antipeptide antibodies and limited proteolysis, have established the existence of two extracellular and three intracellular domains. Hydropathy plots have been interpreted to predict four transmembrane segments. This mapping has been done extensively on connexin32 (Gilula, 1987; Goodenough et al., 1988; Hertzberg et al., 1988) and connexin43 (Laird and Revel, 1990). While the general transmembrane topology of

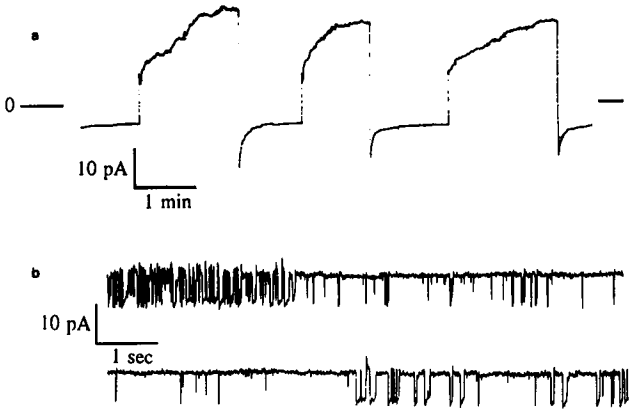

FIGURE 5 Currents in the lipid bilayer induced by peptide E2a applied unilaterally. (*a*) The potential of the artificial membrane is stepped alternatingly to ±50 mV. (*b*) Single-channel currents in a bilayer held at 100 mV.

connexin is undisputed, the resolution of the methods employed does not allow accurate localization of the transition points (membrane-extracellular, membrane-cytoplasmic), nor does it give information about the surface accessibility of amino acids of the extracellular domains.

For interpreting the data obtained with the peptides a determination of the fine structure of the extracellular loops is required. Fine mapping at the resolution of three amino acids should be possible with the creation of *N*-glycosylation sites. The consensus sequence for *N*-glycosylation is *N*-X-S/T (where X can be any amino acid) (Lis and Sharon, 1993). This sequence is used by glycosylation enzymes if it is both extracellular (intramicrosomal) and accessible (not buried). The addition of sugar moieties to such sites can be determined by the retardation of the protein on SDS-PAGE (Zhang and Ling, 1991). Eight such artificial glycosylation sites were introduced into extracellular loop 2 (E2), seven into wildtype connexin32, and one into a fully functional deletion mutant lacking 64 carboxyterminal amino acids (Table 2).

These mutants were transcribed in vitro and subsequently translated in vitro in the presence of dog pancreas microsomes. To label the newly synthesized proteins, [^{35}S]methionine was included in the translation mixture. Microsomes were collected by centrifugation so that only membrane-associated translation products would be analyzed on SDS-PAGE.

Only two of these mutants were glycosylated as indicated by a size shift on SDS-PAGE (Fig. 6). Note that the glycosylation was incomplete; almost one half of the translation product ran at the position of wildtype connexin32 (or the deletion mutant). Incubation of the translation product after solubilization of microsomes with endoglycosidase H resulted in the conversion of the slow migrating protein to a form that migrated like wildtype connexin32, indicating that the observed slow-moving band was indeed due to the glycosylation of connexin32 (Rabadan-Diehl, 1993).

These results confirm that positions 165–167 and 175–177 are extracellular and show that these sites are exposed. All

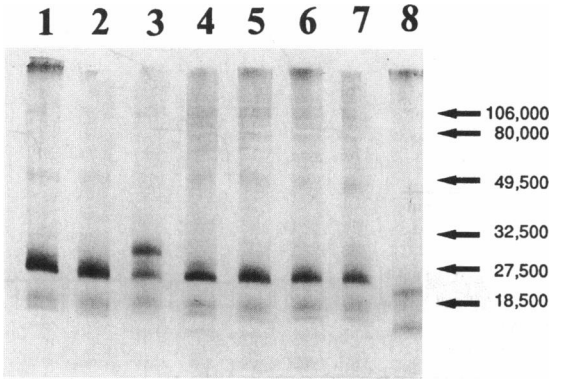

FIGURE 6 SDS-PAGE analysis of in vitro-translated wildtype connexin32 and its glycosylation site mutants (identified by the amino acid positions containing the consensus sequence for *N*-linked glycosylation) shown in Table 2. Lane 1, 159–161; lane 2, 162–164; lane 3, 165–167; lane 4, 169- 171; lane 5, 183–185; lane 6, 186–188; lane 7, 189–191: lane 8, 175–177. Arrows indicate the positions of molecular weight markers. (Note that the mutant shown in lane 8 has a different migration pattern. In this mutant the glycosylation site had been inserted into a truncated connexin lacking 64 carboxyterminal amino acids. The mutant was previously shown to be functionally indistinguishable from wildtype connexin32 (Werner et al., 1991).)

other positions that were tested are either extracellular but buried or they are not extracellular at all. Because our analysis was done with microsomes, this conclusion can only apply to channel precursors, connexins that are not yet assembled into complete gap junction channels. It also should be noted that a more comprehensive mapping is needed to determine the transition points between exposed and buried regions and their exact number.

Every mutant was also tested for its ability to form cell-cell channels. The mutants that are glycosylated do not show channel activity. Considering that a bulky mass has been added to the extracellular part of the protein, the homophilic binding between hemichannels is likely to be interfered with. Furthermore, mutations within the extracellular loops of connexin32 often result in loss of function (Dahl et al., 1991, 1992; Werner et al., 1993). It is therefore surprising that three mutants, two involving changes of amino acids that are highly conserved among different connexins, exhibit full channel-forming activity.

DISCUSSION

Mapping of binding sites

Formation of cell-cell channels contained in gap junctions involves the homophilic (and sometimes heterophilic) binding of subunit assemblies in apposing membranes and the opening of the channels. Here we show that a series of synthetic peptides representing extracellular loop sequences of connexin32 inhibit cell-cell channel formation. The properties of the inhibition indicate that the docking between hemichannels involves a complex binding site that comprises large fractions of both extracellular loop sequences of connexin32. Five of the six short peptides tested inhibited channel formation, suggesting that all five different regions contribute to the binding site. A complex binding site is not unexpected because subunits can be separated from organized gap junctions. This rules out covalent bonds. Nevertheless, the bonds break only under extremely harsh conditions, e. g., 8 M urea (Manjunath and Page, 1986) as if gap junctions were held together by multiple weak noncovalent interactions. The idea of an extended docking site is corroborated by the observation that the extracellular loops are very sensitive to mutation, both to replacements of single amino acids (Dahl et al., 1991, 1992; Werner et al., 1993) and to exchange of the loops between different connexins (unpublished results).

The extracellular segments of connexins, in addition to providing the docking sites, have to form the extracellular portion of the channel and the surface toward the gap between the apposed membranes. Thus only portions of the extracellular sequences can be involved in the docking. The glycosylation experiments suggest that the extracellular loop E2 contains both surface-accessible and surface-inaccessible stretches of amino acids. Some of the sites, which according to the glycosylation results presumably are buried, can be mutated without functional consequences to the channels that are formed from such mutated connexins.

Channels made from a connexin-specific peptide

A surprising result of this study was the observation that one peptide, representing only 12 amino acids of the presumed second extracellular loop, was inactive in the inhibition assay but was capable of forming channels. To our knowledge this is the shortest peptide that has been shown to form transmembrane channels. Its length is insufficient to span a membrane in the form of an α-helix but sufficient to do so if the peptide oligomerized in the membrane to form a β barrel structure. Because of lack of constraints that would be present if it were part of a protein, the peptide might form various oligomers resulting in channels with different unit conductances. Alternatively, another conformation could exist if the peptides were to align head-to-head or head-to-tail thereby crossing the membrane as dimers.

The ability of the peptide to form channels raises the question whether this activity reflects the normal function of this sequence within the intact connexin32 protein or whether the peptide once removed from the constraining influence of the remainder of the protein acquires by chance an activity unrelated to its physiological role. Several other peptides representing segments of other ion channel proteins have been reported to form channels. Recent mutagenesis studies, however, indicate that the pore lining of those ion channels is actually formed by other segments. For example, it is generally accepted now that in voltage-gated ion channels the pore is provided by the P-segment located between S5 and S6, whereas the peptides shown to induce channel activity in bilayers were derived from S4 of Na channels (Tosteson et al., 1989) or S3 of Ca channels (Montal, 1990). On the other hand, the channel-forming capability of an M2 peptide

of ligand-operated channels is in agreement with the localization of the pore-lining in that protein (Akebas et al., 1992; Montal, 1990).

Another argument against the E2a sequence representing the channel pore is the following: the carboxyterminal lysine of the channel-forming peptide E2a had previously been suggested to be one of the determinants of the specificity of interactions between connexin hemichannels (Werner et al., 1993) and, therefore, should be located on the extracellular surface. However, this lysine could be located at the extracellular boundary of the pore wall and thus not contribute to the inner pore lining.

In addition to the caveat that a peptide sequence taken out of a protein is likely to behave differently than when part of the protein, one is cautioned by the lack of homology of different connexins in this particular region. One would presume that basic functions usually are conserved (and a pore lining of a channel has to be considered a basic function). However, the fact that connexin distribution is highly tissue-specific and that different connexins have distinct unit conductances (Bennett et al., 1991) would suggest that connexins have quite specific different transport functions involving different selectivities (Veenstra et al., 1993). If that is the case, one would expect the determinant of such selectivity to be located in the pore lining and therefore to vary between connexins. For example, in voltage-gated sodium channels a selectivity switch from sodium to calcium was achieved by a mutation within the P-segment (Heinemann et al., 1992).

Other observations also point to the E2a segment of connexin32 as the pore lining in gap junction channels. Properties of E2a-peptide channels and properties of hemichannels made from connexin46 in single oocytes (Paul et al., 1991) are very similar. The E2a segment of connexin32 may interact with other connexin regions not considered extracellular. In unpublished experiments from this laboratory chimeras were generated in which domains of different connexins were interchanged. In all these mutants, the switch-over from one type of connexin to the other was placed into a stretch of amino acid sequence identity. Whereas an exchange of the E1 domain was mostly tolerated with no loss of channel-forming ability, no chimeric connexins in which the E2 region was exchanged between connexin32, connexin43, and connexin38 formed channels. Even when both extracellular loops were derived from the same connexin the chimeras did not form channels. These results suggested that the E2a segment of connexin32 interacts with a different nonextracellular segment, most likely a transmembrane segment. If the E2a sequence is part of a pore-forming β structure it likely interacts with other transmembrane segments of connexin32. Consequently, mutations in these other domains could affect the properties of the pore indirectly.

β structures as the lining of the pore in membrane channels are emerging as a general theme. X-ray crystallographic analysis of porin has revealed β structures (Walian and Jap, 1990). Furthermore, in voltage-gated ion channels the pore includes a segment that, based on hydrophobicity plots, was not predicted to be transmembranous (Durell and Guy, 1992). These P-segments are thought to form a β structure. Also, for ligand-operated channels evidence has been presented that appears to exclude α-helical structures as pore lining (Akabas et al., 1992). Interestingly, early x-ray diffraction studies on isolated gap junctions had been interpreted as containing β structures in the membrane (Makowski et al., 1977). Thus, even if E2a is not part of the channel pore, other segments of the connexin molecule should be reexamined for their potential as β-pore structures.

A tentative model for the pore

If the channel forming of the E2a peptide indeed reflects a role in the intact protein, the following model would appear attractive. With the basic orientation of the connexin protein in the membrane unchanged (Fig. 7 *a*), relocation of the boundary between M3 and E2 (which were undefined anyway) would place the E2a peptide sequence into, rather than outside of, the membrane. E2a would be part of a β structure comprising amino acids from approximately residue 147 to 167. This entire segment would be analogous to the P-segment of voltage-gated ion channels. The sequence amino terminal to this segment M3, previously proposed to form the channel lining in the form of an amphipathic α-helix (Bennet et al., 1991), may be α-helical or contributing to the β structure. Mutations in this segment are likely to affect channel properties regardless of whether M3 is itself channel lining or whether it interacts with the analog of a P-segment. The sequence represented by the E2a peptide, of course, could also provide the lining of the pore within the extracellular gap.

Hemichannels are closed. As an exception to this rule, connexin46 expressed in single oocytes provides a transmembrane conductance most likely resulting from open hemichannels. No experimental procedure is known today that would open hemichannels. Even the peptides used in this study that partially mimic the docking process are unable to

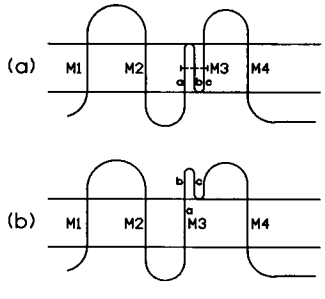

FIGURE 7 (*a*) Model of transmembrane topology of connexins if peptide E2a (indicated by dotted line) is part of the pore. In this model, M1, M2, and M3 are α-helical as shown in previous models. The general region M3 could consist of either one α-helical segment (a) and a β structure involving segments b and c, or the entire M3 segment (a, b, and c) could be in the β conformation. (*b*) Alternate model, where in the hemichannel state the pore does not exist. Instead, the pore-forming segment is located on the extracellular surface but inserts into the membrane to form the pore as part of a major conformational change induced by the docking of two hemichannels, yielding the same structure as in (*a*).

open hemichannels. Only the real docking that occurs between two hemichannels opens the channels in a process that excludes any detectable leakage to the extracellular space (Werner et al., 1993). Leakage during channel formation or conduction through hemichannels would result in cell death as demonstrated with connexin46 expression in single oocytes (Paul et al., 1991).

A possible explanation for the absence of conductance in hemichannels is that in hemichannels the P-segment is not located in the membrane. At the hemichannel state, the P-segment may be extracellular but becomes inserted into the membrane as part of a major conformational change induced by the docking (Fig. 7). The results obtained with introduction of glycosylation sites in E2 are consistent with such a model. In a variant of this model, the P-analogs of hemichannels reciprocally insert into the membrane of the apposing cell and form the pore there. This variant, however, is less likely because of the conduction observed in connexin46 hemichannels in single oocytes.

Considering that β structures as pore linings are so widely used it is certainly worthwhile to test these models. For example, interchanging the pore lining between connexins would be the most critical test because it should result in the transfer of channel properties from one connexin to another.

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