benzodiazepine photoaffinity labelling site on the GABA<sub>A</sub> receptor α1 subunit [12]. Further, it should be pointed out that an alternative approach to synthetic peptides as antigens for directed antibody production is the use of bacterially produced subunit fragments, and indeed this strategy has been adopted for the GABA<sub>A</sub> receptor [13]. In general these antigens, although probably less expensive to produce, are larger and they do not possess the predetermined exquisite specificity of the synthetic peptide.

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Gap junction communication channel: peptides and anti-peptide antibodies as structural probes

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Introduction

The co-ordinating role of intercellular communication channels in ensuring homeostasis of macro-assemblies of cells in tissues and organs is now well established. The membrane channels that permit direct transfer of small molecules and ions are assembled at intercellular adhesive regions of the plasma membrane, termed gap junctions, constructed predominantly of a multigene family of conserved proteins, the connexins. Connexins are assembled at gap junctions into hexameric units, the connexons, and these align and adhere to connexons contributed by the neighbouring cell to create a continuous regulatable channel linking the cytoplasm. In general terms, the provision of direct pathways for the controlled exchange of molecules smaller than 1.5 kDa between adherent cells facilitates metabolic integration, regulated development, growth and cell division. More specific examples are provided by studies pointing to the involvement of gap junction mediated communication in uterine myometrial contraction at birth [1], the co-ordination of ciliary beating in tracheal cells [2], the synchronized contraction of heart cells [3], and the impairment of embryonic developmental [4] and regenerative [5] processes ensuing after the plugging of gap junction channels by antibodies to the major liver gap junctional protein.

Antibodies generated to short peptides corresponding to sequences exposed from the membrane at the extracellular ‘gap’ and the cytoplasmic regions of connexins have proven extremely useful in revealing structural features of these channel proteins involved in facilitating direct intercellular communication. A summary is now presented of how these peptides and polyclonal antibodies, generated to the peptide conjugates that also identify the linear amino acid sequences in the parent connexins, are providing information on their two-dimensional structure, the nature of putative calmodulin-binding domains, and the membrane trafficking routes followed in the cell by the constituent proteins of gap junctions.

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**Connexin topography deduced using anti-peptide antibodies**

The molecular cloning of the major liver gap junction protein connexin 32 [6, 7], has led to the production of antibodies to an increasing profile of amino acid sequences deduced from the cDNA, and the proposal of two-dimensional models of the arrangement of the connexins in the plasma membrane. Initially, an antibody generated to an amino-terminal peptide (GAP 3; see Table 1 for amino acid sequence) was used in conjunction with antibodies to the complete gap junction protein (M, 32) to show that the amino- and carboxy-termini were likely to be located at the cytoplasmic aspect of the membrane [8]. These observations, taken in conjunction with hydropathy analyses, and the sequencing of proteolytic fragments prepared from intact and peeled-apart gap junctions, indicated that connexin 32 of liver traversed the lipid bilayer four times thereby creating two extracellular peptide loops and a single intracellular loop. Subsequently, antibodies have been generated to a number of other peptides, thereby strengthening this proposed topographical model of connexin 32 in the membrane [9–12].

The synthesis of the peptides (Table 1) extending from 11 to 37 residues in length were prepared by FMOC-polyamide solid-phase chemistry, and purity was assessed routinely by h.p.l.c. and amino acid analysis. To generate antibodies in rabbits, peptides were conjugated to Keyhole limpet haemocyanin via free amino and sulphydryl groups with glutaraldehyde and m-maleimidobenzoyl-N-hydroxysuccinimide ester respectively as cross-linkers. The specificity of the antisera to the various peptides was determined first, and the identification of those antisera that recognized specifically the native protein was assessed by Western blotting and by immunofluorescence of thin liver sections [12]. In many instances, the antibodies were purified by affinity chromatography on peptide-conjugate columns.

The establishment of the arrangement of connexin 32 in gap junctions has been accomplished by applying a number of approaches, in which the development of a number of anti-peptide antibodies to specific regions of the protein (see Fig. 1) has proved pivotal. Detailed studies, involving the binding of the characterized site-specific antibody reagents to intact and split apart junctions using immuno-gold electron and fluorescent microscopy and the Western blotting of proteolytic fragments of connexin 32 produced after exposure of junctions to trypsin, chymotrypsin, proteinase K and endo-L-lys C, were carried out to deduce the overall amino acid arrangement in the junctional membrane [12]. The presence of intramolecular disulphide bonds linking the two extracellular loops of connexin 32 was also established by using the anti-peptide antibodies. Exposure of liver gap junctions to trypsin removes a large portion of the carboxy-terminal tail and a sequence in the intracellular loop of connexin 32 yielding 10 kDa and 13 kDa peptides that can be identified by Western blotting using the relevant anti-peptide antibodies. In SDS-PAGE under non-reducing conditions, the two major tryptic peptide products remain connected by the disulphide linkages and migrate as a 23 kDa product [12].

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**Table 1**

<table>
<thead>
<tr>
<th>Peptides</th>
<th>Connexin</th>
<th>Residue number</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAP 3</td>
<td>32</td>
<td>7–21</td>
<td>YTLLSGVNHRHSTAIG</td>
</tr>
<tr>
<td>GAP 5</td>
<td>32</td>
<td>182–192</td>
<td>SRPTEKTVFTV</td>
</tr>
<tr>
<td>GAP 6</td>
<td>32</td>
<td>162–176</td>
<td>MVRVKCEAFPCPN</td>
</tr>
<tr>
<td>GAP 7M</td>
<td>32</td>
<td>43–59</td>
<td>VWGDEKSSFICNTLQY</td>
</tr>
<tr>
<td>GAP 8M</td>
<td>32</td>
<td>216–230</td>
<td>ACRARRQRRSNPPSR</td>
</tr>
<tr>
<td>GAP 9</td>
<td>32</td>
<td>264–283</td>
<td>RRSPGTAZGKLAKSSDCSAC</td>
</tr>
<tr>
<td>GAP 10</td>
<td>32</td>
<td>1–21</td>
<td>MNWTLGLYTLLSGVNHRHSTAIG</td>
</tr>
<tr>
<td>GAP 11</td>
<td>32</td>
<td>151–187</td>
<td>YVFYYLPYAMVRLVKCEAFPCPNTVDCFSRPTEK</td>
</tr>
<tr>
<td>GAP 13</td>
<td>43</td>
<td>123–136</td>
<td>KVEHI-LKQIEIKKFC</td>
</tr>
<tr>
<td>GAP 14</td>
<td>43</td>
<td>314–325</td>
<td>SAEQNRMTGQQAAGSC</td>
</tr>
<tr>
<td>DES 1</td>
<td>32</td>
<td>(102–112) + (116–124)</td>
<td>EKKMRLEHGHGHLVKRHK</td>
</tr>
<tr>
<td>DES 3</td>
<td>26</td>
<td>106–119</td>
<td>FMKGEIKNEFKDIEAG</td>
</tr>
</tbody>
</table>
Topographical arrangement of the three characterized connexins

The amino acid sequences to which antibodies have been generated are indicated according to their connexin-type specificity. Detailed topography studies on connexin 26 have not been reported.

Connexins and their structurally related family of channel-constructing proteins

As the number of connexins identified by molecular cloning techniques from various sources increases, the amino acid sequences deduced from the cDNA make it clear that they are likely to have a similar topographical arrangement in the lipid bilayer [13, 14]. The establishment of membrane topography has involved the rigorous application of a variety of biochemical and immunocytochemical techniques as applied first to connexin 32 in liver and subsequently to connexin 43 (M, 43) found in heart and uterus [15-17]. Connexin 26 (M, 26) is a further connexin expressed in liver, skin, kidney and pinealocytes that shows a high degree of sequence identity to connexin 32 but possesses a shortened cytoplasmic tail [18]; it has been identified recently as a product of a tumour suppression gene [19]. A comparison of the membrane arrangements of three connexins is shown in Fig. 1, and the amino acid sequences to which antibodies have been generated are shown. Intramolecular disulphides linking the two extracellular loops are also detected in connexin 43 [20] and in view of the conservation of the three cysteine residues and many of the flanking amino acids in sequence alignments of the extracellular loops of an increasing number of connexins [12, 20], it is likely that this fundamental feature of...
**Fig. 2**

Structure of gap junctions (a) showing how the protein monomers, the connexins, are arranged into hexameric structures, the connexon or hemi-channel (b) and the topography in the membrane of connexin 32 (c).

Connexons align with connexons contributed by the neighbouring cell to construct an intercytoplasmic channel (a) and the arrangement of opposed connexins in the plasma membrane, with the position of the functional domains identified, is shown in (c). Connexin 32 traverses the membrane four times, with the extracellular loops connected by an intramolecular disulphide bridge [see ref 12]. The barrel showing the third membrane traverse (shaded) contributes to the channel wall.

(a)

(b)

(c)
Charting connexin trafficking routes and connexon assembly using anti-peptide antibodies

The structure of connexins has been studied mainly after they have assembled into gap junctions at specific plasma membrane domains. The antibodies to the various peptides described above, in conjunction with methods that allow subcellular dissection of the hepatocyte’s organelles and membrane systems [24], have been used to assess the distribution of connexins in the cell, and to provide information on the trafficking routes followed from the endoplasmic reticulum to the lateral surface domain where gap junctions are located. Analysis of purified liver subcellular fractions using the anti-peptide antibodies have confirmed the high relative level of connexin 32 in lateral plasma membranes, and also indicate that approximately half the connexins in the hepatocyte are located in non-surface membranes. The intracellular locations identified include the Golgi apparatus, the endoplasmic reticulum and lysosomes. Despite the high relative levels recorded in the Golgi apparatus, connexin 32 is not N- or O-glycosylated. The antibodies to the peptides were also used to demonstrate by Western blotting that the intramolecular disulphide linkage is formed in the endoplasmic reticulum, possibly co-translationally. During trafficking in the cell, and when positioned in the plasma membrane, the extracellular loops of connexin 32 were highly resistant to trypsic action. However, the action of trypsin on liver microsomal fractions has revealed the presence of incompletely folded connexin intermediates. The accretion of connexins to form hexameric connexons, by analogy with studies on the assembly of virus encoded proteins in the cell, e.g. influenza haemagglutinins [25], probably occurs at an early stage in the intracellular trafficking itinerary. However, if a trans-membrane pore is created by the connexin oligomerization process in the endoplasmic reticulum, it must be closed to prevent the equilibration of the cytoplasmic/lumenal environments when located inside the cell, and leakiness to the outside environment when positioned in non-junctional plasma membrane regions. The subsequent accretion of connexons to form functional gap junction plaques containing multiple channels occurs at the lateral plasma membrane region.

Peptides as probes to identify putative calmodulin-binding sites in connexins

Intercellular communication across gap junctions is controlled by many factors, especially intracellular Ca\(^{2+}\) levels, pH and phosphorylation by protein kinases [21]. In the context of the regulation of gap junction channels by intracellular Ca\(^{2+}\) levels, the possible roles of calmodulin have been addressed [26]. Although calmodulin binds to connexin 32 in liver gap junctions examined by SDS-PAGE [8], it is necessary to demonstrate that calmodulin binds to connexins or gap junctions in a Ca\(^{2+}\)-dependent manner. The availability of peptides accounting for
most of the extracellular and cytoplasmic sequences of connexins (Table 1) has now allowed the identification of putative calmodulin-binding regions. By taking advantage of a newly developed and characterized fluorescent calmodulin derivative, a survey of the binding properties of the peptides to this calmodulin derivative under non-denaturing conditions was carried out. These studies have identified two peptides located to the amino terminus (GAP 10) and the carboxy-terminus (GAP 8M) (Table 1) that bound to the fluorescently labelled calmodulin derivative in a Ca^{2+}-dependent manner; none of the other peptides tested (Table 1) exhibited this property. Figure 2 shows the localization of these putative calmodulin binding domains in the topographical map of connxin 32. Both of these are well conserved regions within the connxin family.

**Conclusion**

This paper summarizes some of the applications of synthetic peptides, and their corresponding antibodies as site-specific biological tools, to probe the structure of the gap junction. These site-specific antibody reagents have allowed the traffic routes along intracellular pathways in liver followed by connxin 32 to be studied as well as aspects of their assembly into gap junctions at the lateral plasma membrane. The utility of the peptides to determine the putative calmodulin-binding domains in gap junctions illustrates further the potential of this analytical approach for unravelling the structure of gap junction channels. Although the inputs into connxin/gap junctional biochemistry thus far have been predominantly structural, the range of peptides that can be synthesized by automated methods, combined with the proven facility of generating antibodies that recognize both the denatured and non-denatured forms of connexins before and after assembly into junctions will allow further analysis of the functioning of these ubiquitous intercellular pathways that ensure the co-ordination and harmonization of metabolism in cell assemblies constituting tissues and organs. Of high priority is the need to analyse the functional reasons for connxin diversity, for example in the context of the contribution of various connxin subtypes to channel construction and operation in various tissues, and to deduce the amino acid sequences in the junction that regulate gap junctions in various physiological situations.


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