Targeting motifs and functional parameters governing the assembly of connexins into gap junctions

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To study the assembly of gap junctions, connexin–green-fluorescent-protein (Cx–GFP) chimeras were expressed in COS-7 and HeLa cells. Cx26– and Cx32–GFP were targeted to gap junctions where they formed functional channels that transferred Lucifer Yellow. A series of Cx32–GFP chimeras, truncated from the C-terminal cytoplasmic tail, were studied to identify amino acid sequences governing targeting from intracellular assembly sites to the gap junction. Extensive truncation of Cx32 resulted in failure to integrate into membranes. Truncation of Cx32 to residue 207, corresponding to removal of most of the 78 amino acids on the cytoplasmic C-terminal tail, led to arrest in the endoplasmic reticulum and incomplete oligomerization. However, truncation to amino acid 219 did not impair Cx oligomerization and connexon hemichannels were targeted to the

plasma membrane. It was concluded that a crucial gap-junction targeting sequence resides between amino acid residues 207 and 219 on the cytoplasmic C-terminal tail of Cx32. Studies of a Cx32E208K mutation identified this as one of the key amino acids dictating targeting to the gap junction, although oligomerization of this site-specific mutation into hexameric hemichannels was relatively unimpaired. The studies show that expression of these Cx–GFP constructs in mammalian cells allowed an analysis of amino acid residues involved in gap-junction assembly.

Key words: chimera, connexin assembly, green fluorescent protein, targeting motifs.

INTRODUCTION

Gap-junction channels are conduits for the direct exchange of ions and small molecules (< 1 kDa) between neighbouring cells and provide a potential mechanism for the intercellular propagation of signalling molecules and metabolites. Gap junctions are constructed of connexins (Cxs), a highly conserved family of proteins with 15 different isoforms identified in rodents to date. Cxs are integral membrane proteins spanning the membrane four times with two extracellular-gap-facing loops, one intracellular loop and cytoplasmically located N- and C-termini [1].

The formation of gap junctions involves a stepwise series of assembly processes. Cxs are inserted into the endoplasmic reticulum (ER) membrane where they must fold correctly prior to oligomerizing into hexameric hemichannels (connexons), an event occurring as they traffic from the ER to the Golgi via an intermediate compartment [2]. Connexons delivered to the plasma membrane then align and dock with partners in neighbouring cells to generate the gap-junction channel [3]. Gap-junction operation is regulated by transmembrane voltage [4] and chemically via cytoplasmic pH and calcium ions [5,6].

The amino acid motifs involved in assembly and targeting of Cxs to gap junctions are poorly understood. Also, channel-gating mechanisms are subject to speculation and a number of Cx mutations result in failure of gap-junction channels to operate normally [7]. Analysis of the functional consequences of these mutations is now possible owing to the increasing number of DNA mutations encoding Cxs shown to be associated with

genetically inherited diseases. For example, mutations in Cx32 are associated with Charcot–Marie–Tooth X-linked disease (CMT-X disease), a peripheral neuropathy [8], mutations in Cx26 are associated with non-syndromic sensorineural deafness [9], mutations in Cx31.1 are associated with keratoderma [10] and Cx50 and Cx46 are implicated in cataract formation [11]. These missense, nonsense and frame-shift deletions are scattered throughout the Cx sequences with some also occurring in non-coding regions of the DNA. Cx mutations may radically influence hemichannel assembly processes occurring during intracellular trafficking or gap-junction channel gating, and thus can provide a biochemical explanation of the pathophysiology underlying these diseases [7]. Defects in gap-junction channel operation resulting from Cx mutations have been studied by exogenous expression and electrophysiological techniques in *Xenopus* oocytes [12,13] and by intercellular transfer in cultured mammalian cells of the fluorescent dye Lucifer Yellow [14–16]. Several Cx32 mutations associated with CMT-X disease result in aberrant protein trafficking in mammalian cells [16–18]. In-depth analysis of selected mutations should allow definition of the roles that specific amino acids and protein domains have in the biogenesis of gap junctions.

Green fluorescent protein (GFP) is used widely to study intracellular trafficking routes since its properties allow rapid visualization of cellular localization with high sensitivity [19–21]. Although chimeric proteins are likely to exhibit modified behaviour, it was shown that fusion of the reporter protein aequorin (Aeq) to the C-terminus of Cx43 or Cx32 led to the assembly of

Abbreviations used: Cx, connexin; wtCx, wild-type Cx; ER, endoplasmic reticulum; CMT-X disease, Charcot–Marie–Tooth X-linked disease; GFP, green fluorescent protein; eGFP, enhanced GFP; TNT, coupled *in vitro* transcription/translation.

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functional chimeric gap-junction channels [22]. In the present work, we used Cx–GFP chimeras to study the assembly, intracellular trafficking and functionality of gap junctions and to define a region on the cytoplasmic C-terminal tail of Cx32 that is crucial for targeting to the gap junction.

EXPERIMENTAL

Construction of chimeric Cx–GFP cDNA

Cx cDNA was fused in-frame to the N-terminus of enhanced GFP (eGFP) in the vector pe-GFP-N1 (Clontech; Figure 1). For wild-type (wt) Cx26 [23] and Cx32 [24] the open reading frame was amplified by PCR from plasmids containing the relevant full-length cDNA [22] using appropriate oligonucleotide primers introducing *Bgl*II (for Cx26) and *Hind*III restriction-enzyme sites (for Cx32 constructs) at the 5' and 3' ends of the cDNA respectively. The primers used were as follows, with restriction-enzyme sites underlined and Cx sequences in bold. Cx32 forward primer, Cx32GF, 5'-CTC GAG CTC AAGCTT ATG AAC TGG ACA GGT-3'; Cx32 reverse primer, Cx32GR, 5'-CAG AAT TCG AAGCTT GCA GGC TGA GCA TCG-3'; Cx26 forward primer, Cx26GF, 5'-CTA CCG GAC TCAGATCTC ATG GAT TGG GGC ACC-3'; and Cx26 reverse primer, Cx26GR, 5'-CTT GAG CTC GAGATCTGA GAC TGG TCT TTT GGA-3'.

To generate truncated Cx32 proteins, Cx32Δ19, Cx32Δ93, Cx32Δ207 and Cx32Δ219, reverse primers incorporating the relevant 3' cDNA position and a *Hind*III site were used. The primer sequences were as follows: Cx32Δ19 reverse primer, Δ19R, 5'-CAG AAT TCG AAGCTT AAT GGC TGT AGA ATG-3'; Cx32Δ93 reverse primer, Δ93R, 5'-CAG AAT TCG AAGCTT TGC CAC GAG GAG AGC-3'; Cx32Δ207 reverse primer, Δ207R, 5'-CAG AAT TCG AAGCTT CGC CAC GTT GAG GAT-3'; and Cx32Δ219 reverse primer, Δ219R, 5'-CAG AAT TCG AAGCTT GCG GGC ACA GGC CCG GAT-3'.

The 5' primers were the same as the wild-type counterparts shown above. To generate the chimeric constructs the resulting

PCR products were digested with *Bgl*II or *Hind*III and ligated into the dephosphorylated *Bgl*II or *Hind*III sites of pe-GFP-N1 respectively [25]. The truncated mutant Cx32Δ215 was engineered by PCR amplification of wtCx32 using the forward primer Cx32P1 [22] and a reverse primer introducing a premature stop codon at amino acid position 215. These primers contained *Bam*HI sites to enable subcloning of the PCR products into the vector pCR3 (Invitrogen) as described above.

All ligation reactions were transformed into *Escherichia coli* DH5α (Gibco-BRL) and positive clones identified by mini-plasmid preparation and restriction-enzyme analysis. Selected constructs were sequenced using the dRhodamine Dye Termination Cycle Sequencing kit (Perkin-Elmer).

In vivo expression and cellular localization of Cxs

COS-7 or HeLa cells (European Collection of Animal Cell Cultures, Salisbury, Wiltshire, U.K.) were transfected with the relevant Cx cDNA and harvested for Western-blot analysis [22]. Proteins were characterized using a primary rabbit polyclonal anti-GFP antibody, generated against a GFP–calreticulin fusion protein [26] and a secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (Bio-Rad). Blots were developed using the enhanced chemiluminescence (ECL®) system (Pierce).

For cellular localization, 5 × 10⁵ cells were transfected with 1 µg of relevant cDNA. Post-transfection (24 h) the cells were split on to four 16-mm² coverslips placed in 12-well dishes followed the next day by fixation in 4% formaldehyde, and if necessary, processed for immunocytochemical analysis [22]. Co-localization of Cx and GFP was confirmed by staining the cells with the relevant primary anti-Cx antibodies: for Cx26 Gap 28H against the intracellular loop of Cx26 [27], for Cx32 a monoclonal antibody to an intracellular loop sequence was used (Chemicon) and a polyclonal rabbit antibody against calreticulin was used as an ER marker [26]. The secondary antibody was goat anti-rabbit or goat anti-mouse conjugated to Cy3 (Sigma) as required. Cells were viewed under a laser confocal microscope (Leitz) and GFP

Figure 1 Diagrammatic representation of the Cx–GFP chimeras and Cx mutants studied

Cxs were fused in-frame to the N-terminus of GFP. The Cx transmembrane domains are depicted by grey shading. The ability to target to gap junctions, whether Cxs oligomerize to connexons and whether the cells were functional according to transfer of Lucifer Yellow are indicated. ND, not determined.

expression visualized in cells using excitation (488 nm) and emission (515 nm) wavelengths. Images were processed using Adobe Photoshop 5.

Analysis of the oligomeric state of chimeric and mutated connexons

The oligomeric status of the chimeric and mutated connexons was determined by expression of the proteins in transiently transfected COS-7 cells or by cell-free synthesis using a coupled *in vitro* transcription/translation (TNT) system (Promega).

To determine the oligomeric status in cells expressing the chimeric Cx–GFP proteins, 1×10^7 cells were seeded on to 10-cm dishes and transfected with 8 μ g of the relevant Cx cDNA [22]. Ten plates (1×10^8 cells) were used for each assay. Post-transfection (48 h), cells were washed in PBS, harvested in 1 ml of PBS in the presence of protease inhibitors [22], and the cells pelleted by centrifugation at 1000 g for 5 min.

For determination of the oligomeric status using the cell-free system the relevant cDNA was synthesized using the TNT system (Promega) supplemented with canine pancreatic microsomes and 35 S promix (Amersham) as described in [27,28]. Membrane-integrated Cxs were separated from free Cxs by centrifugation as described in [28]. In both cases, connexons were extracted from the cell pellets or the membranes in an orbital rotator at 4 °C for 1 h in 500 μ l of a buffer containing 20 mM triethanolamine, pH 9.2, 20 mM EDTA, 2% (w/v) dodecyl maltoside and 10 mM dithiothreitol, a procedure that maintains the connexons intact [29]. As a control, extraction used 20 mM triethanolamine, pH 9.2, 20 mM EDTA, 2% (w/v) SDS and 20 mM dithiothreitol, which dissociates connexons into Cxs. Sucrose–detergent gradients were calibrated with carbonic anhydrase (29 kDa) and myosin (209 kDa) marker proteins to determine the approximate positions in the gradients where monomeric and hexameric Cxs sedimented, corresponding to 5 and 9 S, respectively [16,30–32]. Under the conditions used [10–40% (w/v) sucrose, 20 h, 150000 g], the sucrose gradient was calibrated as < 18% (w/v; Cxs), 18–28% (Cx oligomeric intermediates) and 28–40% (connexons) [27,31,32]. The positions of Cxs, intermediate oligomers and connexons present in cell extracts resolved by the gradients into 11–12 fractions were analysed by SDS/PAGE and ECL[®] Western blotting using an antibody to GFP as described above. With Cxs synthesized *in vitro* by the TNT system [28,32a] Cx, oligomeric Cx and connexon analysis was by autoradiography of each of the gradient fractions resolved by SDS/PAGE. Densitometric analysis of the ECL[®] Western blots or autoradiographs was carried out using the Bio-Rad 700 densitometer and software [27,28].

Functionality of chimeric constructs

HeLa cells were transfected by calcium phosphate transfection with cDNA encoding the various Cx–GFP chimeras [22]. Stable cell populations expressing the chimeric proteins were selected by growing the transfected HeLa cells for 3 weeks in medium supplemented with Geneticin (G418 sulphate, 4 mg/ml) and GFP-positive cells were sorted using a fluorescence-activated cell sorter (Becton and Dickinson). Selected populations of cells were grown to confluency and functionality of the cell lines determined according to the ability of cells microinjected with Lucifer Yellow [5% (w/v) in 0.3 M LiCl] to transfer dye to neighbouring cells. Prior to injection (18 h) of cells, protein expression was enhanced by addition of 5 mM sodium butyrate to the media [22].

RESULTS

Properties of the Cx–GFP constructs

The intracellular trafficking of wtCx26– and wtCx32–GFP and a series of truncated Cx32–GFP cDNAs expressed in COS-7 and HeLa cells and the functionality of gap junctions formed were examined. Cx cDNAs were fused in-frame to the N-terminus of eGFP. The Cx32–GFP chimeras examined included two constructs, encoding the N-terminal 19 and 93 amino acids, but low expression and difficulties in membrane insertion precluded further studies. Therefore, two cytoplasmic tail deletions, terminating at amino acids 207 and 219, were constructed (Figure 1). Also, the assembly, trafficking and functionality of two site-specific mutations of wtCx32, Cx32E208K and Cx32 Δ 215, were included as non-chimeric mutants to validate the behaviour shown by the truncated Cx–GFP chimeric proteins.

Expression of Cx–GFP chimeras in mammalian cells

Western-blot analysis of cell lysates from cells transfected with the various Cx32–GFP and Cx26–GFP constructs resulted in the detection of immunolabelled protein using an anti-GFP antibody and anti-Cx antibodies. The predicted size pattern of the expressed protein was observed, i.e. Cx32–GFP (59 kDa) Cx32 Δ 219–GFP (51 kDa) Cx32 Δ 207–GFP (50 kDa) eGFP (28 kDa; Figure 2). A further protein product, possibly a proteolytic product, migrated ahead of the full-length protein in the gels. A similar phenomenon was reported recently for Cx43–GFP [21]. A Cx26–GFP protein product of the predicted molecular mass (54 kDa) was also expressed (Figure 2).

Cellular localization of Cx–GFP chimeras

The cellular location of the recombinant chimeric proteins was determined by virtue of the inherent fluorescence of GFP (Figure 1). Analysis by confocal microscopy showed that wtCx26 and the Cx26–GFP chimera were targeted to the plasma membrane of COS-7 cells giving characteristic punctate gap-junction staining

Figure 2 Western-blot analysis of Cx32–GFP chimeric proteins

COS-7 cells, transfected with the relevant cDNA, were harvested 48 h post-transfection and prepared for SDS/PAGE and Western-blot analysis as described in the Experimental section. Proteins were detected using a primary antibody against GFP. Lane 1, Cx32–GFP; lane 2, Cx32 Δ 219–GFP; lane 3, Cx32 Δ 207–GFP; lane 4, eGFP; lane 5, Cx26–GFP.

Figure 3 Localization of CxGFP chimeras

COS-7 cells were transfected with the relevant cDNA. Post-transfection (48 h), cells were fixed and either viewed for GFP autofluorescence or stained with a relevant antibody against Cx26 or Cx32 as described in the Experimental section. (A) wtCx26 immunostained with an antibody against the intracellular loop of Cx26; (B) wtCx32 stained with an antibody against the intracellular loop of Cx32; (C) eGFP; (D) Cx26–GFP viewed by GFP autofluorescence; (E) Cx26–GFP immunostained with the antibody Gap28H and a goat anti-rabbit Cy3-conjugated secondary antibody; (F) co-localization of (D) and (E); (G–H) Cx32–GFP and Cx32Δ219–GFP viewed by GFP autofluorescence; (I) Cx32Δ215 immunostained with an antibody against the intracellular loop of Cx32; (J) Cx32Δ207–GFP viewed by GFP autofluorescence; (K) cells transfected with Cx32Δ207–GFP immunostained with an antibody against calreticulin and a goat anti-rabbit Cy3-conjugated secondary antibody; (L) co-localization of (J) and (K). Arrows indicate targeting to the gap junction. Scale bars, 10 μ m.

(Figures 3A and 3D, respectively). Similarly, wtCx32 and Cx32–GFP were also targeted to areas of cell contact (Figures 3B and 3G, respectively). Immunolocalization studies using a Cx26 antibody confirmed that GFP and Cx26 expression were coincident (Figures 3D–3F), showing that the proteins expressed were chimeric. In contrast, eGFP was spread diffusely throughout the cell with some cells showing intense nuclear staining (Figure 3C). A similar cellular localization was observed for the short N-terminal segments Cx32Δ19–GFP and Cx32Δ93–GFP (results

not shown). Cx32Δ219–GFP was targeted to the plasma membrane (Figure 3H). However, cells expressing Cx32Δ207–GFP showed no punctate plasma membrane staining, but extensive staining of the ER was observed (Figure 3J).

Immunolocalization studies with relevant anti-Cx antibodies confirmed that the Cx component of each mutated chimera co-localized with GFP, thus confirming that the proteins were expressed together (results not shown). Immunostaining of cells expressing Cx32Δ207–GFP with an antibody to calreticulin,

Figure 4 Analysis by rate-sedimentation of the oligomeric status of Cx32-GFP chimeras and Cx32 mutated proteins

Connexons were extracted with detergents from COS-7 cells expressing the Cx32-GFP chimeric proteins 48 h post-transfection as described in the Experimental section. After rate sedimentation in 10–40% (w/v) linear sucrose–detergent gradients, the proteins in each fraction were concentrated and prepared for SDS/PAGE and Western-blot analysis as described in the text. (A–D) Representative sucrose-gradient profiles of connexons extracted from cells with dodecyl maltoside: (A) wtCx32 detected with an antibody against the C-terminal tail of Cx32; (B–D) Cx32-GFP, Cx32Δ219-GFP and Cx32Δ207-GFP, respectively, detected with an antibody against GFP. For quantification, see Table 1. The positions of standard proteins that co-sediment with Cxs or connexons are indicated (29 and 209 kDa, respectively) and the positions in the gradient of monomeric (M), intermediate (O) and hexameric (H) Cxs are indicated.

a resident ER protein, confirmed the predominant ER location of this mutant (Figures 3J–3L). Similar intracellular staining was also observed when the site-specific mutation Cx32E208K was expressed in COS-7 cells [16,17]. A non-chimeric protein, truncated at position 215, i.e. Cx32Δ215, was examined by immunostaining using an antibody against the intracellular loop of Cx32, and the results also showed intracellular retention (Figure 3I).

Oligomeric status of Cx-GFP constructs

To investigate the biochemical parameters underlying targeting to ER or the gap junction, the oligomeric status of wtCx32-GFP, Cx32Δ219-GFP and Cx32Δ207-GFP expressed in COS-7 cells was determined by a velocity sedimentation procedure that separates monomeric and oligomeric Cxs [31,32]. The amount of each of the chimeric Cxs sedimenting as monomer [in the 5 S fraction, i.e. fractions < 18% (w/v) sucrose], incompletely oligomerized product [18–28% (w/v) sucrose] and hexameric connexons [in the 9 S fractions, i.e. > 28% (w/v) sucrose] was determined by Western-blot analysis of each fraction from the gradient and subsequent densitometric analysis. The results show that when wtCx32 was extracted from COS-7 cells using dodecyl maltoside, a non-ionic detergent that extracts connexons [29], > 70% of the expressed protein sedimented as hexamer, 18% as intermediate oligomeric products and 2% as monomers (Figure 4A and Table 1). These results show that most of the wtCx32 expressed in cells was assembled into connexons. Chemical cross-linking studies of the 9 S fraction showed previously that hexameric connexons were present in this fraction [31]. In contrast, when wtCx32 was extracted from cells with SDS, which dissociates connexons into Cxs [31], > 70% of the protein was present as monomers (Table 1A). A study of the oligomerization

Table 1 Oligomeric status of Cx32-GFP chimeras and Cx32 mutated proteins

(A) The oligomerization assay shows the distribution of Cx constructs expressed in COS-7 cells. (B) The oligomerization potency of the constructs was assessed by using an *in vitro* assay system. The relative oligomeric status of Cxs in each fraction collected from the sucrose–detergent gradients was determined by densitometric analysis of ECL[®] Western blots (A) or autoradiograms (B). The results are represented as the percentages of Cxs present in the relevant fractions of the sucrose gradient: Cx monomer fractions, < 18% (w/v) sucrose, intermediate oligomer fractions, 18–28% (w/v) sucrose, and connexon fractions, > 28% (w/v) sucrose. Data are means $\pm$ S.E.M., $n = 3$.

(A) Construct	% Cx monomer	% Intermediate Cxs	% Connexons
wtCx32	2.2 $\pm$ 1.3	18.7 $\pm$ 2.9	79.2 $\pm$ 4.1
Cx32-GFP	17.2 $\pm$ 0.5	37.4 $\pm$ 1.0	45.4 $\pm$ 1.3
Cx32Δ219-GFP	5.3 $\pm$ 1	51.8 $\pm$ 1.7	42.9 $\pm$ 1.5
Cx32Δ207-GFP	48.6 $\pm$ 0.5	49.6 $\pm$ 1.4	1.8 $\pm$ 2.7
wtCx32 (extracted with SDS)	72.4 $\pm$ 3.2	27.4 $\pm$ 1.3	0.2 $\pm$ 0.9

(B) Construct	% Monomer	% Intermediate Cxs	% Connexons
wtCx32	30 $\pm$ 2.6	42.3 $\pm$ 1.1	27.2 $\pm$ 1.5
Cx32E208K	39 $\pm$ 4	33 $\pm$ 5.5	27 $\pm$ 4.5
Cx32Δ215	87 $\pm$ 11	11 $\pm$ 7.8	2 $\pm$ 0.4
wtCx32 (extracted with SDS)	64 $\pm$ 3.5	33 $\pm$ 1.1	2.2 $\pm$ 2.4

Table 2 Functionality of Cx-GFP chimeric proteins

HeLa cells expressing the various Cx-GFP proteins were assessed for their ability to transfer Lucifer Yellow. The results are expressed as the percentages of cells transferring dye to 0–2 cells, 3–5 cells and to > 5 cells ($\pm$ S.E.M.). Experiments were performed in triplicate and at least 20 cells were injected per experiment.

Construct	Cells transferring Lucifer Yellow (%)		
	0–2 cells	3–5 cells	> 5 cells
Cx26-GFP	53.4 $\pm$ 5.6	18.8 $\pm$ 3.5	26.3 $\pm$ 4.3
Cx32-GFP	56.8 $\pm$ 5	19.4 $\pm$ 6.5	23.7 $\pm$ 6.9
Cx32Δ219-GFP	88 $\pm$ 4.3	11.9 $\pm$ 4.8	0
Cx32Δ207-GFP	90.7 $\pm$ 7.3	6.0 $\pm$ 1.5	3.2 $\pm$ 3.2
HeLa control	96.6 $\pm$ 3.5	2.7 $\pm$ 1.4	1.0 $\pm$ 1.0

status of the chimeric Cx32-GFP expressed proteins was therefore carried out. This showed that wtCx32-GFP and Cx32Δ219-GFP assembled into connexons, albeit at a reduced efficiency compared with wtCx32 (45% compared with 70%; Figures 4B and 4C, and Table 1A). The reduced level of connexons produced resulted in an increase in the ratio of oligomeric intermediate non-functional products, suggesting that fusion of GFP to Cxs impeded the oligomerization of Cxs into connexon hexameric hemichannels. In contrast to the oligomerization behaviour of wtCx32-GFP and Cx32Δ219-GFP, Cx32Δ207-GFP was expressed in cells mainly as monomers and intermediate oligomers with only 2% of the protein sedimenting in the 9 S fraction (Figure 4D and Table 1A). Parallel studies using a complimentary *in vitro* oligomerization assay system were also carried out to analyse two non-chimeric mutants. These studies showed that Cx32Δ215 did not oligomerize into hexameric connexons whereas a site-specific mutation, Cx32E208K, assembled into hexameric proteins with a similar efficiency to

wtCx32 in this *in vitro* assay system (Table 1B). The results identify amino acids 207–219 on the C-terminal tail of Cx32 as playing an essential role in the oligomerization of Cxs into hexameric hemichannels.

Intercellular dye transfer of cells expressing chimeric Cx–GFP

To analyse further intercellular communication by gap junctions formed by the Cx–GFP chimeric proteins, HeLa cells that do not assemble gap junctions [32] were transfected stably with the various constructs (Figure 1). The functionality of gap junctions assembled by these cell lines was analysed by their ability to transfer Lucifer Yellow to 3–5, or 5 or more, neighbouring cells. The results show that cells expressing wtCx26–GFP or wtCx32–GFP efficiently formed gap-junction channels with 18.8 and 19% of injected cells transferring dye to 3–5 cells, respectively, and 26.3 and 23.7% of the cells transferring dye to 5 or more neighbouring cells, respectively. In control (non-transfected) HeLa cells only 1% transferred dye to more than 5 neighbouring cells (Table 2). The functionality of Cx32Δ219–GFP was reduced compared with wtCx32–GFP, with 11.9% of cells transferring dye to 3–5 cells, although Student's *t*-test analysis showed this not to be significantly reduced compared with wtCx32–GFP. However, communication to more than 5 cells was severely compromised ($P < 0.05$). The functionality of Cx32Δ207–GFP was reduced further, and was similar to that of the 'null' HeLa cells ($P < 0.05$) (Table 2).

DISCUSSION

The assembly of multimeric gap-junction intercellular communication channels from the constituent Cx subunits is a complex process [2]. As two or more cells contribute to the formation of the direct cell-to-cell channel, the component reactions leading to assembly can be analysed by following the formation of gap-junction hemichannels. Following insertion and correct topological folding of the Cx subunit in the ER, oligomeric association generates hexameric structures in which Cxs are arranged around a central pore [33]. Connexons may be homomeric or heteromeric in composition, which can result in varying channel permeability properties [31,34]. The position in the secretory pathway where Cx subunit association leading to channel formation occurs is unlikely to be the trans-Golgi [30], for Cx oligomerization is a progressive and sequential process initiated in the ER, and completed in the Golgi apparatus [31,32].

The current work aimed to identify specific amino acid sequences involved in targeting of Cxs/connexons from intracellular stores to the plasma membrane and gap junction. The approach taken involved analysis of the expression in mammalian cells of a range of intact and truncated Cxs with GFP attached to the Cx C-terminal tail. Chimeric Cx32–GFP or Cx26–GFP were targeted to the gap junction and studies involving intercellular transfer of Lucifer Yellow showed the intercellular channels to be functional. The experiments using chimeric Cx32 showed that most of the 78 amino acids comprising the cytoplasmic C-terminal tail were not required for intracellular trafficking to the gap junction, but a short amino acid sequence leading from the fourth membrane traverse was crucial. A truncated chimera, Cx32Δ207–GFP, which inefficiently oligomerized into hemichannels, was arrested mainly in the ER. In contrast, Cx32Δ219–GFP, which oligomerized with a similar efficiency to Cx32–GFP, trafficked along the secretory pathway and was targeted to the plasma membrane. Thus a gap-junction-

targeting motif resides between amino acid residues 207 and 219 of Cx32. To analyse further this short sequence, we studied two further mutations, Cx32E208K, a point mutation, and Cx32Δ215, a truncated protein, both observed in the peripheral neuropathy, CMT-X disease [7]. The results, taken together with others [16,17], show that the Cx32E208K and Cx32Δ215 mutants accumulated primarily in intracellular membranes. Other Cx32 mutations associated with CMT-X, e.g. Y211stop, R215W, R215Q and R215stop, were shown not to form gap junctions using electrophysiological techniques in *Xenopus* oocytes [13] but their trafficking competence in mammalian cells was not studied. The Cx32E208K mutant [16] oligomerized to a similar extent as wtCx32. Although productive association of Cx subunits to generate a hexameric hemichannel is a fundamental requirement for formation of functional gap junctions, the present results suggest that targeting motifs present in this 12-amino acid region on the cytoplasmic tail can override association into hemichannels [16].

Targeting sequences of monotopic membrane proteins are invariably located at cytoplasmic areas contiguous to the membrane-traversing sequence [35], but with polytopic proteins, such as Cxs, the situation appears more complex. Further domains appear to be involved in the assembly of multimeric membrane-associated proteins. For example, both N- and C-terminal regions contribute to the assembly and functional expression of homo- and heteromeric voltage-gated K^+ channels [36]. The fifth and sixth membrane-spanning regions of the inositol 1,4,5-trisphosphate receptor have been shown recently to be key determinants in receptor oligomerization [37]. The targeting sequence now identified on the C-terminal tail of Cx32 is cytoplasmic, situated close to the membrane and coincides with one of two calmodulin-binding sites identified on Cx32 [38]. Analysis of several Cx32 mutations at the N-terminal tail suggests that targeting determinants also exist in this domain [16]. However, the truncation mutants Cx32Δ19–GFP and Cx32Δ93–GFP could not be used owing to the role of this domain in insertion into the ER [28,32a]. Furthermore, gap-junction targeting determinants may be interlinked with properties underlying association into hexamers in which Cxs are arranged around a central channel. The present data obtained in cells expressing Cx32E208K emphasize the dominant requirement for maintaining the correct amino acid sequence in this region of the protein. The accumulation mainly in the ER of Cx32E208K, where oligomerization occurred, and the limited oligomerization and intracellular location of Cx32Δ207–GFP, illustrate the complexity of the various processes that regulate intracellular trafficking and connexon hemichannel formation in the secretory pathway.

The present results illustrate the utility of GFP as a reporter protein in the study of the targeting of Cxs and their assembly into gap junctions. GFP was more resilient in the recombinant construction of Cx chimeras than other reporter proteins, such as aequorin (Aeq), used to measure the cytoplasmic calcium environment [22,40] and β -galactosidase [41]. Although a Cx26–Aeq chimera was shown not to be expressed [22], this deficiency was corrected by exchanging the short C-terminal tail of Cx26 with that of Cx43 [40]. The trafficking properties of a range of truncated and mutated Cxs in gap-junction-associated disease models such as CMT-X disease [16,27] and sensorineural deafness [27] are now open to investigation in live cells expressing Cx–GFP chimeras.

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