Post-translational integration and oligomerization of connexin 26 in plasma membranes and evidence of formation of membrane pores: implications for the assembly of gap junctions

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Gap-junction channels provide a widespread intercellular signalling mechanism. They are constructed of a family of connexin membrane proteins that thread across the membrane four times and oligomerize to generate hexameric gap-junction hemichannels. Using an *in vitro* cell-free transcription/translation system, we demonstrate that connexin (Cx) 26, one of the smallest connexins, is integrated directly in a post-translational manner into plasma membranes. Protein-cleavage studies of Cx26 integrated into plasma membranes indicate a similar native transmembrane topography to that of Cx26 integrated co-translationally into microsomes. Cx26 integrated post-translationally into plasma membranes

oligomerizes and, when incorporated into liposomes, provides permeability to ascorbic acid, suggesting that gap-junction hemichannels are generated. The results provide the basis of a novel alternative mechanism for spontaneous assembly in plasma membranes of Cx26 gap-junction hemichannels that occurs independently of the conventional biogenesis of gap junctions involving connexin trafficking and oligomerization via membrane components of the secretory pathway.

Key words: channel assembly, membrane integration, protein trafficking.

INTRODUCTION

Gap junctions consist of closely packed clusters of intercellular communication channels that allow diffusion of ions, metabolites and second messengers [1]. They are constructed of a family of about 20 connexin proteins that traverse the membrane four times with cytoplasmic N- and C-termini, two disulphide-linked extracellular loops and a single cytoplasmic loop. Size variation in connexins (25–62 kDa) is due mainly to the variable length of the multivariant cytoplasmic tail [1,2]. Connexins oligomerize into hexameric channels, and the alignment and docking of two such connexon channels in the plasma membrane leads to the construction of a gap junction.

Connexin (Cx) 26 differs from other connexins in many respects. Intracellular trafficking of Cx26 and its assembly into hexameric connexon hemichannels is relatively unaffected by brefeldin A, a drug that disassembles the Golgi and interrupts membrane trafficking along the secretory pathway [3]. Connexins are not glycosylated, and Cx26 differs from larger connexins in not being phosphorylated on its relatively short cytoplasmic carboxyl tail. Gap junctions constructed of Cx26 have been observed to appear rapidly and independently of gap junctions constructed of other connexins, e.g. in liver, breast and brain tissues [4–7], and Cx26 can be induced in a post-translational manner by PMA in neuroblastoma cells [8]. Unlike Cx32, Cx26 shows the unusual property of both co- and post-translational integration into microsomes [9,10], a property governed not by the cytoplasmic tail [9,10] but by an amino acid located in the first transmembrane domain proximal to the cytoplasmic N-terminus [3]. Subcellular fractionation studies in guinea-pig liver have also suggested that Cx26, unlike Cx32, partially follows a different Golgi-evading intracellular pathway to gap junctions [11]. Other studies in cells expressing recombinant chimaeric connexins in which the C-terminus was fused to aequorin [12,13] or green

fluorescent protein variants [3] to trace their intracellular trafficking routes have established that the biogenesis of gap junctions constructed of Cx32 proceeds via the classical secretory pathway, but that Cx26 gap junctions are also assembled in a non-classical manner featuring a Golgi-independent, microtubule-dependent pathway [3,12].

In the present work, the assembly of Cx26 into gap-junction hemichannels was studied using a cell-free transcription/translation system [9,10]. The results show that Cx26 but not Cx32 is integrated into plasma membranes, where it assumes a native topography and forms oligomers. Using two different techniques, we detect channel properties in plasma membranes containing the integrated Cx26 after transfer into liposomes. Thus Cx26 membrane channels can be generated in a similar manner to the combinatorial assembly into channels by several bacterial toxin proteins [14,15]. The present results also parallel the insertion of proteins into thylakoid and mitochondrial inner membranes [16,17]. The autonomous generation of Cx26 hemichannels in plasma membranes suggests the retention in mammalian systems of an evolutionarily conserved membrane-integration mechanism.

EXPERIMENTAL

In vitro synthesis and membrane integration

Plasmids, pBSKXG Cx26 pCR-3 Cx32, pCR-3 Cx43 [12] and pCR3-Aeq [13] containing cDNA encoding Cx26, Cx32, Cx43 and aequorin respectively were synthesized *in vitro* in a reticulocyte lysate cell-free coupled transcription/translation system (TNT Quick; Promega) as described previously [10]. Briefly, 50 µl reactions were set up in which 40 µl of reticulocyte lysate was mixed with 1.0 µg of relevant plasmid DNA and 20 µCi of ³⁵S-labelled methionine and cysteine (Promix; Amersham). The reaction mixtures were incubated at 30 °C

Abbreviations used: Cx, connexin; DTT, dithiothreitol; TEA, triethanolamine.

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for 90 min for mRNA and protein synthesis. Reactions were stopped by addition of Laemmli sample buffer [18] and products analysed by SDS/PAGE and autoradiography.

To study co-translational membrane integration, the transcription/translation reactions containing endoplasmic reticulum-derived canine pancreatic microsomes (50 μ l of reaction mixture from Promega; see the Promega technical manual for further details) were set up. Glutathione (5 mM) was added to compensate for the small amounts of dithiothreitol (DTT) present in ³⁵S-Promix and microsomes. For post-translational membrane integration, the first step was performed in the absence of membranes. On completion of protein synthesis, DNase 1 (4 μ g/50 μ l; Boehringer–Mannheim) and RNase 1 (9 units/50 μ l; Promega) were added to the mixture and reactions were allowed to continue at 30 °C for 30 min to degrade plasmid cDNA and its transcript, thus preventing any co-translational translocation into membranes. Canine pancreatic microsomes (5 μ l) or lateral plasma membranes consisting of membrane strips and vesicles (50 μ g) prepared from guinea-pig livers [11,21] were then added, and reactions continued for another 90 min at 30 °C to allow pre-synthesized connexins to insert into the added membranes. In some experiments, lateral plasma membranes were resuspended in buffer (1 mM NaHCO₃, pH 9.0) using a loose-fitting Dounce homogenizer and washed three times by centrifugation at 10000 *g* for 30 min to minimize microsomal contamination. To separate membrane-integrated connexins from free connexins, the translation mixture was layered on to a 0.5 M sucrose cushion (500 μ l) prepared in PBS, pH 7.4, and centrifuged at 150000 *g* for 90 min at 4 °C to strip loosely attached or occluded proteins. Membranes were pelleted by centrifugation at 150000 *g* for 1 h at 4 °C. The supernatants were discarded and the pellets were dispersed and washed again in 0.1 M Na₂CO₃, pH 11.0, and centrifuged as before. Pellets were finally resuspended in Laemmli sample buffer and analysed by SDS/PAGE and autoradiography or solubilized in the relevant buffer for analysis by velocity sedimentation in sucrose/detergent gradients or reconstitution into liposomes as described above.

Protease assays

The topology of Cx26 integrated into the membranes was determined as described previously with Cx26 [19] or Cx32 [20,21]. After *in vitro* translation, samples (25 μ l) were chilled on ice and diluted to 125 μ l in proteolysis buffer containing 150 mM potassium acetate, 2.5 mM magnesium acetate and 50 mM triethanolamine (TEA), adjusted to pH 7.0 with acetic acid. Microsomes were stabilized by addition of CaCl₂ to a final concentration of 10 μ M and incubated on ice for 10 min. Microsomal or plasma membranes containing co- or post-translationally inserted Cx26 were separated by centrifugation through a sucrose cushion prepared in proteolysis buffer and washed in the same buffer as described above. Membrane pellets were resuspended in 100 μ l of proteolysis buffer and incubated on ice for 1 h with proteinase K (0.25 μ g/ μ l, final concentration; Sigma). Proteolysis reactions were stopped by addition of a protease-inhibitor cocktail (Sigma; diluted 1:100, v/v) and 5 mM PMSF (Sigma). Membranes containing protected fragments were separated by centrifugation through a sucrose cushion as described above. The pellets were resuspended in Laemmli sample buffer with or without β -mercaptoethanol (5%, v/v). Products were analysed by SDS/PAGE and autoradiography.

Oligomerization assays

Plasma membranes containing post-translationally integrated Cx26 or Cx32 co-translationally integrated into microsomes

were centrifuged through a sucrose cushion as described above and solubilized in dodecyl maltoside (2%, w/v; Sigma), 20 mM EDTA, 10 mM DTT and 20 mM TEA, pH 9.2, for 2 h [10]. Samples were centrifuged at 12000 *g* for 15 min at 4 °C and the supernatants were layered on to 15 ml preformed 10–40% (w/v) linear sucrose gradients containing 0.2% dodecyl maltoside, 20 mM EDTA, 10 mM DTT and 20 mM TEA, pH 9.2. After centrifugation at 150000 *g* for 22 h at 4 °C, fractions (1 ml) were collected, dialysed against distilled water and concentrated *in vacuo*. Concentrated samples were suspended in Laemmli sample buffer and analysed by SDS/PAGE and autoradiography [10,11].

Reconstitution into liposomes

Plasma membranes containing post-translationally inserted Cx26 were separated through a sucrose cushion and suspended in a buffer containing 50 mM KCl, 10 mg/ml L- α -phosphatidylcholine type II-S (Sigma), 20 mg/ml *n*-octyl β -D-glucopyranoside and 20 mM Tris/HCl, pH 7.4. The mixture was solubilized by sonication on ice using a 3 mm microprobe at 15 μ m amplitude (10 times for 3 s each). Cytochrome *c* (Sigma; 4 mg/ml) was added, and reconstituted liposomes were formed after detergent removal by extensive dialysis against a buffer containing 50 mM KCl and 20 mM Tris/HCl, pH 7.4 [22]. Liposomes without any protein (plain liposomes) were prepared in parallel under the same conditions. The liposome suspension was then layered on to a 10–40% (w/v) linear sucrose gradient prepared in 50 mM KCl and 20 mM Tris/HCl, pH 7.4, and centrifuged at 150000 *g* for 4 h at 4 °C [22,23]. The centrifugation tubes showed single or multiple bands containing either plain liposomes or proteoliposomes containing non-permeable and permeable channels. The relative amounts and densities in the gradient of the bands were estimated using Quantity One software (Bio-Rad, Hemel Hempstead, Herts., U.K.). Fractions (0.5 ml) containing liposome populations sedimenting at different positions were collected and assayed for their permeability to ascorbate as described below. Liposome populations were also analysed to confirm the presence of *in vitro*-synthesized radiolabelled Cx26. Liposomes collected from the gradient were dialysed against distilled water, concentrated to approx. 20 μ l and mixed with an equal volume of SDS sample buffer followed by SDS/PAGE and autoradiography.

Cx26 integration and permeability of liposomes

Prior to assay of channel activity, suspensions of cytochrome *c*-loaded liposomes were incubated at room temperature for 1 h. The absorbance of liposomes (0.5 ml) was monitored at 417 nm, for 1 min [22]. Ascorbate (0.3 mM) was then added, and changes in absorbance due to reduction of entrapped cytochrome *c* were monitored for a further 2 min. The permeability rate was determined from the slope values of a plot of absorbance against time using CricketGraph software (Microsoft).

RESULTS

Cx26 and Cx32 and a control protein, aequorin, synthesized in the reticulocyte lysate cell-free transcription/translation system produced radioactive products of the correct size (Figure 1, lanes 1, 3 and 5). Since Cx32 was co-translationally integrated into microsomes, whereas Cx26 was also integrated post-translationally [9,10], we replaced microsomes with a plasma-membrane fraction of high purity prepared from guinea-pig liver [11]. Addition of plasma membranes to the cell-free synthesis system

Figure 1 Post-translational insertion of proteins into liver plasma membranes

Cx26 (lanes 1 and 2) and Cx32 (lanes 3 and 4) were synthesized *in vitro* (—) and analysed for their post-translational insertion into lateral plasma membranes (+) isolated from guinea-pig liver. A soluble cytosolic protein, aequorin (lanes 5 and 6), was used as a control that did not associate with membrane.

resulted in Cx26 becoming integrated in a post-translational manner into these membranes (Figure 1, lane 2). Of the three connexins analysed, Cx43 was also found to integrate into plasma membranes, but in a less efficient manner than Cx26 (results not shown). With Cx32, the *in vitro* membrane-integration properties were similar to those already shown for this connexin in microsomal membranes [9,10], and it did not integrate post-translationally into plasma membranes (Figure 1, lane 4). Aequorin, a soluble protein used as a control, also did not integrate into plasma membranes (Figure 1, lane 6).

Protease treatments

The topological orientation of Cx26 post-translationally integrated into membranes was analysed by probing the accessibility of extramembrane sequences to proteolytic breakdown. Figure 2(a) indicates the points of proteolytic hydrolysis anticipated when Cx26 is inserted into membranes with a topography of four transmembrane regions, two disulphide-linked extracellular loops highly resistant to hydrolysis and the

Figure 2 Analysis of protease-protected fragments to determine the topology of Cx26 in microsomal and plasma membranes

(a) A map showing the predicted proteolytic cleavage patterns of Cx26 based on its deduced sequence. Single arrows indicate the likely proteolytic cleavage sites of proteinase K and the arrow with the diamond tail shows the putative cleavage site characteristically observed *in vitro* during co-translational membrane integration. The proteolytic products (A–D) and their expected size (in kDa) based on the amino acid residues are shown in parentheses. The intramolecular disulphide bond linking two extracellular loops is also indicated. (b, c) Cx26 synthesized *in vitro* and translocated co-translationally in microsomes (ct mc) or post-translationally in microsomes (pt mc) or plasma membrane (pt pm) was analysed to examine the effects of proteolysis with proteinase K. Membrane vesicles containing connexins were treated with proteinase K and the proteolytic products were analysed by SDS/PAGE under reducing (b, lanes 4–6) or non-reducing conditions (c, lanes 1–3), where the intramolecular disulphide bonds remain intact. A gel with a higher percentage of acrylamide (15%, w/v) was used in (b) to resolve the lower-molecular-mass bands. The positions of bands (A–D) as expected (a) are indicated on the gels. See the text for further explanation.

Figure 3 Oligomeric status of Cx26 in membranes

Microsomal membranes containing *in vitro*-synthesized and co-translationally inserted Cx26 (a) or plasma membranes containing post-translationally inserted Cx26 (b) were solubilized and fractionated in linear sucrose gradients as described in the Experimental section. Connexins in the fractions collected from the sucrose gradients were analysed by SDS/PAGE and autoradiography.

protease-sensitive intracellular loop and carboxyl tail regions [20,21]. The two protease-resistant extracellular loops covalently linked by disulphide bridges facilitate analysis of the peptide fragments under reducing and non-reducing conditions, since products of different sizes are separated by electrophoresis in polyacrylamide gels, as studied in detail with Cx32 biogenetic analysis in liver plasma membrane, Golgi and microsomal fractions [21]. As observed previously [10,24], *in vitro* cell-free synthesis of Cx26 in the presence of microsomal membranes generated an N-terminally cleaved product (Figure 2b, lane 1); that is, an *in vitro* artifact caused by a signal peptidase [24,25]. This cleavage is, however, mainly a feature in co-translational integration and occurred only to a minor extent when Cx26 was integrated post-translationally into either microsomal or plasma membranes (Figure 2b, lanes 2 and 3). Radioactively labelled Cx26 was synthesized and either co- or post-translationally integrated into microsomal or plasma membranes. The membranes were then treated with proteinase K to analyse Cx26 topography in the membrane. Under non-reducing conditions a combined band of proteolytic products C+D was observed (17.8 kDa; approx. 163 amino acids; Figure 2c, lanes 2 and 3). However, during co-translational integration into microsomes, in addition to bands C+D, a lower-molecular-mass band of products B+D (13.5 kDa; approx. 123 amino acid residues) was observed (Figure 2c, lane 1) that was presumed to be produced by the action of a microsomal protease cleaving at amino acid 41 [25]. Under reducing conditions, band C (10.5 kDa; approx. 96 amino acid residues) and band D (7.3 kDa; approx. 67 amino acids) were detected (Figure 2b, lanes 4–6). In addition to bands C and D, two further bands, A (4.4 kDa; approx. 40 amino acids) and B (6.2 kDa; approx. 56 amino acids) were also detected and presumed to be the products of the endogenous microsomal protease hydrolysing at amino acid 41 of the first transmembrane/first extracellular loop junction [25] (Figure 2b, lanes 4–6). Differences in the relative intensities of products A–D were also detected. For example, in the case of co-translational membrane integration, due to cleavage by the endogenous microsomal protease, band B was present at a higher intensity and band C at a lower intensity (Figure 2b, lane 4). However, with post-translational integration into microsomal and plasma membranes, product C was the higher-intensity band (Figure 2b, lanes 5 and 6). Products A and B were also present in low amounts in post-translationally integrated Cx26, possibly due

to minor artifactual cleavage occurring in the *in vitro*-synthesis system, as is evident in Figure 2b (lanes 2 and 3). Overall, the data show that Cx26 was integrated into microsomes and plasma membranes with a transmembrane topography similar to that determined for Cx26 and Cx32 in gap junctions [19,20], as well as Cx32 in plasma membranes and intracellular membranes [21].

Generation of connexin oligomers

Velocity sedimentation in linear sucrose gradients separated connexins sedimenting as monomers in the top region of the gradient from hexameric connexons in the bottom region of the gradient [10]. Incompletely oligomerized connexin products, especially prevalent in the *in vitro* transcription/translation system [10,11], and also observed in studies of mutated connexins detected in communicationopathies such as Charcot Marie Tooth X-linked disease [26], are assembly intermediates present in varying amounts and sedimenting in the middle of the sucrose gradients [27]. Analysis of plasma membranes containing post-translationally integrated Cx26, following solubilization and fractionation in linear sucrose gradients, showed that about 50% of the protein sedimented as connexons towards the bottom of the gradient (Figure 3b). In contrast, lower amounts of connexons were detected in the gradients after co-translational integration of Cx26 into microsomal membranes, with higher relative amounts of intermediate oligomers being evident (Figure 3a).

Permeability properties in liposomes of oligomerized Cx26

In order to demonstrate connexon channel permeability, plasma membranes containing post-translationally inserted Cx26 were reconstituted into liposomes loaded with cytochrome *c*. On sucrose-gradient centrifugation, liposomes incorporating reconstituted Cx26 sedimented as two main bands, distinct from those seen with plain liposomes or liposomes reconstituted with plasma membranes only (Figures 4x and 4y). Both bands from the Cx26-reconstituted liposomes were shown to contain Cx26 (Figure 4z). It is possible that, whereas the denser-sedimenting band contained mainly permeable connexon hemichannels, the lighter band contained mainly impermeable connexons. The liposomes containing connexons (Figure 4, treatment C) showed higher permeability to ascorbate (120 ± 14) (calculated as uptake rates [10]) than liposomes that incorporated plasma-membrane proteins (Figure 4, treatment B; 60 ± 18). Plain liposomes (Figure 4, treatment A) showed a permeability rate of 28 ± 12 . These results from five separate experiments and with a significance level of $P < 0.01$ between A and C, support the conclusion that post-translationally inserted Cx26 has assembled into functional connexon hemichannels.

DISCUSSION

The results show that Cx26, shown previously to integrate post-translationally into microsomal vesicles [9,10], also integrated directly into plasma membranes with a topographical arrangement in the membrane similar to that determined in gap junctions [19]. Membrane-integrated Cx26 also formed oligomers spontaneously in plasma membranes, and these showed channel-like properties after incorporation into liposomes, as demonstrated by two methodologies.

Post-translational integration of proteins into membranes and the generation of channels with permeability properties are

Figure 4 Separation of liposomes, liposomes reconstituted with plasma-membrane proteins and liposomes reconstituted with plasma membranes incorporating *in vitro*-synthesized Cx26

Using sucrose gradients (x), the separation of liposomes made with phospholipids (treatment A), liposomes reconstituted with plasma-membrane proteins (treatment B) and liposomes reconstituted with plasma membranes containing *in vitro*-synthesized Cx26 (treatment C) was performed. (y) The liposomes were loaded with cytochrome *c*, permitting an analysis of their distribution into peaks (arrows) in the sucrose gradients. E417, absorbance at 417 nm. (z) The incorporation of Cx26 into two higher-density bands shown in centrifuge tube C was shown by collecting these from the gradients and analysing the radiolabelled (*in vitro*-synthesized) Cx26 by SDS/PAGE and autoradiography; P may correspond to permeable channels and C to closed channels. Tn is a sample obtained prior to reconstitution of *in vitro*-synthesized products into liposomes.

unusual in eukaryotes and contrast with the well-explored biochemically complex co-translational integration mechanisms that feature in the insertion of correctly folded proteins into the endoplasmic reticulum membrane [28] and the subsequent post-translational chemical modifications that occur during transit along the secretory pathway. Nevertheless, a number of proteins have been reported to insert post-translationally into membranes, including lactose permease [29], syntaxin [30], mitochondrial porin [16,31] and the glucose transporter [32]. Many bacterial proteins, some functioning as toxins, insert

post-translationally and spontaneously by hydrophobic forces into membranes and generate pores or channels [14,15]. Despite this, the biochemical mechanisms allowing the post-translational integration of proteins into membranes are unknown. Cx26 was shown to integrate into microsomes and plasma membranes, but the biogenetic relationships between the endoplasmic reticulum and the plasma membrane are generally believed to comprise a vesicular trafficking process. However, other studies, for example in calcium signalling, pinpoint the possibility of direct functional interactions occurring between the endoplasmic reticulum and

the plasma membrane or related membranes [33]. Direct physical contact between two different membranes is believed to facilitate conformational coupling between ryanodine and store-operated calcium channels in the endoplasmic reticulum and the plasma membrane, respectively, and have been hypothesized to explain the basis of capacitative calcium entry into cells [33,34]. The post-translational properties of Cx26 now uncovered could reflect a general mechanism accounting for non-classical processes that occur in gap-junction biogenesis and that can be modulated independently of the conventional vesicle-mediated secretory pathway used by other connexins to the plasma membrane and operating via the Golgi apparatus.

Cx26 differs from Cx32 mainly in the length and sequence of the cytoplasmic carboxyl tail (16 compared with 78 amino acids) as well as amino acids in the single cytoplasmic loop. In contrast with other connexins investigated, Cx26 is not phosphorylated. The four transmembrane domains of Cx32 were shown to have a mainly α -helical conformation in liver gap junctions, and it is likely that Cx26 is similar [35]. The two disulphide-linked extracellular loops in connexins have been proposed to form a β -barrel-like structure that bridges the intercellular gap in docked junctions [35,36]. Although Cx32 was not observed to insert post-translationally into membranes [9,10], a site-specific mutation in the first transmembrane region of Cx32 conferred partial post-translational integration properties, suggesting that crucial sorting signals are located at the N-terminal domain of connexins [3].

Connexins, in common with many proteins that integrate post-translationally into membranes, especially in prokaryotes, do not contain an N-terminal signal sequence. However, with connexins synthesized *in vitro*, an endogenous microsomal protease produces a characteristic artifactual product [25]. However, only low levels of this proteolytic product were observed when plasma membranes were used as the insertion membrane vehicle in the present work, attesting to the purity of the plasma-membrane preparations used. The efficiency of oligomerization of connexins integrated into membranes studied *in vitro*, as in the present work, was found to be lower than that in cells expressing recombinant connexins [26] or in tissues such as liver [11]. However, the present results showed that replacing microsomes with plasma membranes resulted in a higher efficiency of Cx26 oligomerization, reflected in the much-reduced amounts of incompletely oligomerized connexins migrating in the middle of the sucrose gradients used to monitor oligomerization [27]. Perhaps oligomerization efficiency *in vitro* is related to the higher cholesterol content of plasma membranes, since isolated gap junctions are enriched in cholesterol [37,38].

The present observations demonstrate that different membrane-integration properties shown by two connexins can explain a number of properties that distinguish Cx26 from other connexins. These include results showing Cx26 trafficking and assembly into gap junctions via a Golgi-independent route, as indicated by its insensitivity to the Golgi-disrupting drug brefeldin A and by the virtual cessation of vesicular trafficking that occurred in cells incubated at 17 °C. Also, Cx26 trafficking is highly dependent on intact microtubules. All these properties shown by Cx26 differ from those shown by Cx32 and Cx43 [3,12,39]. Studies with mouse hepatocytes derived from Cx32-knockout mice suggest that Cx26 is expressed independently of Cx32 and is likely to be regulated in a post-translational manner [39]. Also, Cx26 gap junctions, unlike those constructed of Cx32, form smaller plaques [39] and are not associated with proteins characteristic of tight junctions in hepatocytes [40]. Assuming that the direct integration of Cx26 into plasma membranes and

the generation of gap-junction hemichannels now demonstrated *in vitro* is operational *in vivo*, the present results may also explain the independent appearance in hormonal and developmental circumstances of Cx26 gap junctions in liver breast and brain tissues [4–7].

Finally, one may speculate that the unusual membrane-integration behaviour of Cx26 and its association into the component channels of gap junctions reflect the preservation in mammalian cells of an evolutionarily conserved and relatively simple direct and rapid method for translocating and integrating proteins into the plasma membrane. This mechanism uncovered by the cell-free approaches described may have been adapted for rapidly generating Cx26-based gap-junction channels facilitating direct intercellular communication and underpinning multicellular homeostasis.

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