Trafficking pathways of Cx49-GFP in living mammalian cells

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Abstract

In the present study we examined the trafficking pathways of connexin49 (Cx49) fused to green fluorescent protein (GFP) in polar and non-polar cell lines. The *Cx49* gene was isolated from ovine lens by RT-PCR. Cx49 cDNA was fused to GFP and the hybrid cDNA was transfected into several cell lines. After transfection of Cx49-GFP cDNA into HeLa cells, it was shown using the double whole-cell patch-clamp technique that the expressed fusion protein was still able to form conducting gap junction channels. Synthesis, assembly, and turnover of the Cx49-GFP hybrid protein were investigated using a pulse-chase protocol. A major 78-kDa protein band corresponding to Cx49-GFP could be detected with a turnover of 16–20 h and a half-life time of 10 h. The trafficking pathways of Cx49-GFP were monitored by confocal laser microscopy. Fusion proteins were localized in subcellular compartments, including the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment, the Golgi apparatus, and the trans-Golgi network, as well as vesicles traveling towards the plasma membrane. Time-dependent sequential localization of Cx49-GFP in the ER and then the Golgi apparatus supports the notion of a slow turnover of Cx49-GFP compared to other connexins analyzed so far. Gap junction plaques resembling the usual punctuate distribution pattern could be demonstrated for COS-1 and MDCK cells. Basolateral distribution of Cx49-GFP was observed in polar MDCK cells, indicating specific sorting behavior of Cx49 in polarized cells. Together, this report describes the first characterization of biosynthesis and trafficking of lens Cx49.

Keywords: connexin49; green fluorescent protein (GFP); mammalian cells; protein assembly; protein transport; pulse-chase experiments.

Introduction

Cells of multicellular organisms communicate via membrane channels generally referred to as gap junction channels. Gap junction channels are assembled by two connexons (hemichannels) each of which is formed by six smaller subunits, the connexins. Generation of gap junctions is initiated when connexons in membranes of two adjacent cells are aligned. Interaction of connexons in adjacent cells leads to the formation of a cell-to-cell channel, forming a direct connection of cytoplasm. Multiple gap junction channels aggregate to form gap junction plaques (White and Bruzzone, 1996). It is known from previous studies that the mammalian lens system, e.g., the ovine lens, comprises three different connexins: Cx49 and Cx44 expressed exclusively by lens fibers, and Cx43, which is expressed by lens epithelial cells (Yang and Louis, 1996, 2000; Gong et al., 1998). Due to the lack of blood vessels, the exchange of ions and molecules between the epithelium and fibers in the lens relays mainly on gap junction channels between the lens fibers (Goodenough, 1992; Eckert et al., 1999; Kistler et al., 1999; Donaldson et al., 2001).

To analyze the formation of gap junction channels between lens fibers, the trafficking of ovine Cx49, the analogous form to human Cx50, was determined by attaching the cloned Cx49 sequence to GFP. Recombinant proteins fused to GFP can be traced throughout the cellular compartments by confocal microscopy. It has been observed that the assembly and trafficking of proteins to their final destination was not disturbed by the GFP label (Hampton et al., 1996). This is in accordance with the results obtained for many other connexin proteins. In fact, several studies have demonstrated that the transport competence of connexins fused at the C-terminal end to the GFP reporter gene was not altered compared to the wild-type counterparts (Paemeleire et al., 2000). Recent studies on the biosynthesis and processing of connexins (Jordan et al., 1999; Paemeleire et al., 2000) have demonstrated that connexins are translocated into the endoplasmic reticulum membrane and transported through the Golgi apparatus on their way to the plasma membrane. In the plasma membrane they form an array of tightly packed individual channels and are arranged in gap junction plaques. The assembly or oligomerization of the individual subunits of connexins into connexons is a complex process that occurs in almost all cellular compartments along the secretion pathway, including the endoplasmic reticulum (ER) (Kumar et al., 1995; George et al., 1999), the intermediate compartment between the ER and the Golgi apparatus (ERGIC) (Musil and Goodenough, 1993; George et al., 1998b, 1999), the Golgi apparatus and the trans-Golgi network (Koval et al., 1997; Evans et al., 1999; Falk, 2000; Ahmad et al., 2001; Das Sarma et al., 2001). So far, there have been no studies on the targeting of ovine Cx49 to the plasma membrane and the subsequent arrangement of gap junction plaques in polar and non-polar cells. Polar cells, in contrast to non-polar cells, are characterized by the existence of two structurally and functionally different

membrane domains that are separated by tight junctions, the apical and basolateral membranes. The distinct protein and lipid constituents of each of these two domains are subject to a sophisticated sorting process, which is crucial for the maintenance of cell polarity and intact and efficient cellular function. The sorting of membrane and secretion proteins to the apical or basolateral compartment is thought to occur in the trans-Golgi network and is mediated by diverse sorting elements localized in the sorted protein. In this study ovine Cx49 fused to GFP (Cx49-GFP) was expressed in polar and non-polar cells and analyzed by confocal laser microscopy. In polar cells Cx49-GFP proteins were sorted to the basolateral membrane and revealed a punctuate distribution pattern over this domain, reminiscent of an assembly into gap junctions. By contrast, non-polar cells showed no distinct arrangement of gap junction plaques at the cell surface, which is compatible with unspecific sorting behavior.

Results

Cx49-GFP fusion protein forms gap junction channels

The cDNA generated from Cx49-GFP was transfected in HeLa cells and the electrical conductance (G_j) of cells expressing the fusion proteins and those that did not (control) was measured using the double whole-cell patch-clamp technique (Ngezahayo et al., 2003). As shown in Figure 1, cells that did not express Cx49-GFP had a low G_j value of 0.1 ± 0.3 nS (n=4). This result corresponds to the well-known low expression of endogenous connexins in HeLa cells (Elfgang et al., 1995; Hülser et al., 1997). With the expression of Cx49-GFP, G_j in HeLa cells dramatically increased to 5.4 ± 1.3 nS (n=4). This result indicates that the GFP label did not alter the capacity of Cx49 to form functional gap junction channels.

Biosynthesis and processing of Cx49

COS-1 cells were transiently transfected with cDNA encoding ovine Cx49-GFP. The biosynthetic and structural features of the recombinant protein were assessed utilizing a pulse-chase protocol. The tagged connexins were isolated by immunoprecipitation using anti-GFP antibodies. Figure 2 shows that after 30 min of pulse labeling a 78-kDa protein band resembling the molecular weight of Cx49-GFP could be detected. The control immunoprecipitation of non-transfected cells was devoid of electrophoretically resolved bands. With increasing chase time the band intensity of Cx49-GFP decreased slowly up to 20 h of chasing, indicating that the turnover rate of Cx49 in these cells is substantially slower than that described for other connexins with rates ranging between 1 and 5 h (Musil and Goodenough 1991; Laird 1996).

Trafficking of Cx49-GFP in living mammalian cells

We further analyzed the trafficking of Cx49-GFP in living cells and chose for this purpose non-polarized COS-1
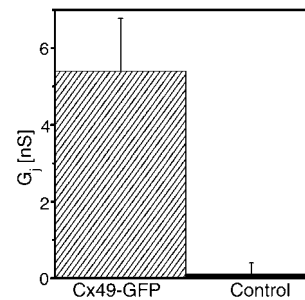

Figure 1 Gap junction coupling of Cx49-GFP in the double whole-cell patch-configuration.
Gap junction conductance G_j of Cx49-GFP expressing HeLa cells compared to control cells, which did not express the Cx49-GFP fusion protein. The results determined by the double whole-cell patch-clamp technique are given as mean±standard deviation (SD) for n=4 experiments.

and HeLa cells, as well as polarized MDCK cells. The results obtained with each cell line are individually presented.

COS-1 cell lines   The trafficking pathways of ovine Cx49-GFP transfected into COS-1 cells were examined by confocal microscopy. Fluorescence labeling corresponding to Cx49-GFP was revealed in the ER, the Golgi apparatus and the trans-Golgi network, clearly indicating that the chimeric protein is transport-competent. The pattern of distribution observed was time-dependent. At 24 h after transfection, ovine Cx49-GFP was localized predominantly in the ER and to a lesser extent in the Golgi apparatus (Figure 3A). At 48 h post-transfection, Cx49-GFP was located predominantly in the Golgi apparatus and the trans-Golgi network (Figure 3B). This time-dependent sequential localization of Cx49-GFP in one organelle and not in the other, as shown for the ER and the Golgi apparatus, is suggestive of slow trafficking and subsequent low turnover, and supports the biochemical analyses. Between pairs of neighboring cells a punctuate pattern along the plasma membrane was observed that indicates the association of Cx49-GFP with gap junctional plaques (Figure 3C). The direct movement of transport vesicles to the plasma membrane is indicated in a time-lapse series in Figure 3D.

HeLa cell line   Cx49-GFP was expressed transiently in HeLa cells which are known to express endogenous connexins at a very low level (Elfgang et al., 1995; Hülser et al., 1997). The expression level, however, obviously
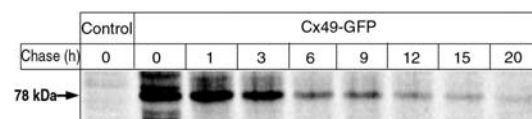

Figure 2 Pulse-chase analysis of Cx49-GFP in COS-1 cells. Transiently transfected COS-1 cells were pulsed with [35S]-methionine for 30 min and chased for different time periods. The cells were lysed, followed by immunoprecipitation of Cx49-GFP and SDS-PAGE analysis. In mock-transfected cells (control) no protein bands could be detected. A major band of approximately 78 kDa was apparent after transfection with Cx49-GFP. It is interesting to note the slow decline in the intensity of Cx49-GFP.
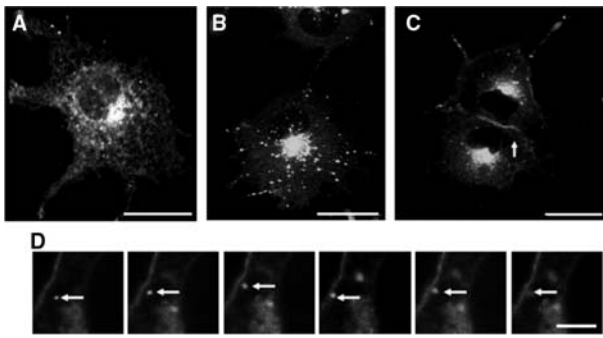
Figure 3   Distribution of Cx49-GFP proteins in transiently trans-fected COS-1 cells.
COS-1 cells were transiently transfected with Cx49-GFP and analyzed (A) 24 or (B) 48 h post-transfection by confocal micro-scopy. In (A) ER- and Golgi-specific staining of the cells can be observed. (B) At 48 h after transfection the Golgi apparatus was predominantly labeled by Cx49-GFP. In (C) two aligned COS-1 cells depict Cx49-GFP labeling in cell-to-cell contact areas (arrow). (D) Time lapse series of vesicular movement in living cells. Images were taken in 4-s intervals and show the move-ment of a Cx49-GFP transport vesicle (arrow) to the plasma membrane. Scale bars: (A–C) 10 μm; (D) 2 μm.

has no influence on the function of exogenously added connexins, as previously shown for other connexins (Elf-gang et al., 1995, George et al., 1998a). In HeLa cells, ovine Cx49-GFP was transported throughout the cellular compartments towards the plasma membrane (Figure 4A). The tagged connexin proteins were located in the ER compartment, the Golgi apparatus and the trans-Gol-gi network. Transport vesicles carrying Cx49-GFP con-structs were monitored moving through the cytoplasm of the cell. Smaller vesicles were located underneath the cell membrane, where Cx49-GFP proteins seemed to be already integrated into the plasma membrane as dem-onstrated by the fluorescent appearance of the mem-brane (Figure 4B).

MDCK cell line   Analysis of the targeting of ovine Cx49-GFP in MDCK, a prototype of polarized cells, was carried out to elucidate the sorting behavior of proteins to the apical or basolateral membrane. For this purpose, stable MDCK cell lines expressing Cx49-GFP were gen-erated and analyzed by confocal microscopy. The pattern of intracellular transport of Cx49-GFP was essentially
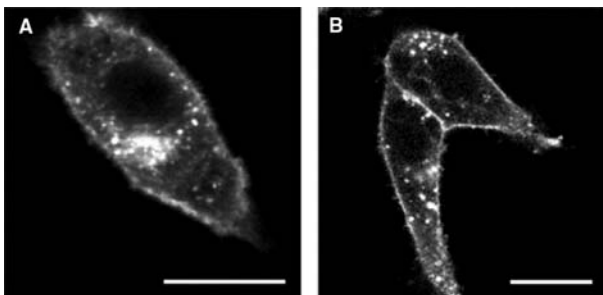

Figure 4   Distribution of Cx49-GFP in HeLa cells.
(A) In HeLa cells Cx49-GFP could be detected in the ER, Golgi apparatus and at the plasma membrane. (B) Two aligned HeLa cells expressing Cx49-GFP show intense labeling underneath the plasma membrane. Scale bars, 10 μm.

similar to that described above for COS-1 cells. Again, the assembly of gap junction plaques could be observed as small fluorescent dots adjusted along the plasma membranes between neighboring cells (Figure 5A). The Cx49-GFP fusion proteins were predominantly detected as fluorescent dots within the plasma membranes of the cells, which is probably indicative of the insertion of gap junction hemichannels into the plasma membrane prior to gap junction formation. Transversal recorded images of a number of cell gap junction channels were in general traced as long fluorescent palisade structures between two polar MDCK cells (Figure 5B) indicating a lateral as-sembly and lateral sorting of the Cx49 protein. Proteins associated with gap junction plaques were not distri-buted at the apical or basal membranes.

Discussion

Connexins are members of a superfamily of proteins that are directly implicated in the formation of gap junctions. Despite the large body of information that has accumu-lated on the structure and function of connexins, little is known about the transport and targeting of the ovine Cx49. Detailed analyses of these pathways are indis-pensable in view of the pivotal and almost exclusive role of gap junctions, and therefore of Cx49, in the commu-nication between lens fiber cells (Goodenough, 1992; Eckert et al., 1999; Kistler et al., 1999; Donaldson et al., 2001). This study focused on the analysis of expression and targeting of chimeras of ovine Cx49 fused to GFP (Cx49-GFP) in polarized and non-polarized cells. Since the function of Cx49 in forming conducting gap junction channels was not altered by fusion with GFP, we studied the structure, intracellular transport and localization of Cx49-GFP in mammalian cells. Biosynthetic analysis combined with confocal laser microscopy provides un-equivocal evidence of a transport-competent conforma-tion of Cx49-GFP. The main species of an apparent molecular mass of 78 kDa is distributed along the secre-tion pathway, in the ER, ERGIC, Golgi apparatus and the trans-Golgi network (TGN). The turnover rate of Cx49-GFP, which was determined to be approximately 16–20 h with a half-life of 10 h, is strikingly slower than that of
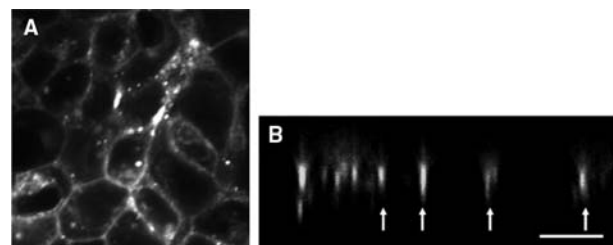

Figure 5   Distribution of Cx49-GFP in polar MDCK cells.
(A) Confocal image of MDCK$_{Cx49-GFP}$ cells with gap junction plaque assembly resembling the classical punctuate distribution pattern. (B) Transverse section (xz-view) of polar MDCK$_{Cx49-GFP}$ cells. Gap junction plaques are localized between adjacent cell membranes in a palisade-like structure (denoted by arrows) with a lateral distribution of GFP-tagged Cx49. In the apical or basal plasma membrane no Cx49-GFP could be observed. Scale bars, 10 μm.

several other known connexins. For example, the half-life for Cx43 is estimated to be 1–5 h (Musil and Goodenough, 1991; Laird, 1996) and is therefore several-fold faster than Cx49. The slow turnover of Cx49 was also suggested by confocal microscopy analysis of transfected cells at 24 and 48 h post-transfection. Most of the Cx49-GFP proteins were localized in the ER after 24 h of transfection and only discrete labeling was ascribed to the Golgi apparatus. After 48 h most of the labeled Cx49 proteins become confined to the Golgi apparatus and TGN, indicative of slow transport of the protein from the ER.

The question that arises is related to the physiological significance of the prolonged turnover rate of Cx49 compared to other connexins. One plausible explanation takes into consideration the natural occurrence of Cx49 in the lens of sheep and its assembly into connexons and gap junction channels between adjacent lens fibers. Lens fibers lack organelles and are not attached to blood vessels or other structures important for nourishment or communication via exchange of small molecules or second messengers. Therefore, stable connexins, such as Cx49, would be more useful in ensuring efficient ion and molecule exchange within the cells of the ovine lens than connexins with high turnover.

It could be postulated that the elongation in the half-life of Cx49-GFP is based on the addition of GFP, which is insensitive to many proteases (Ormo et al., 1996). In this case the polypeptide chain of Cx49, representing more than half of the fusion protein, would be still accessible for proteolytic degradation and GFP-tagged degradation products should appear after longer chase periods in the lower molecular weight range, which is not the case (Figure 2). Furthermore, others have previously demonstrated that the fusion of GFP to the carboxy-terminus of another connexin variant, Cx43, does not alter the life cycle and dynamics of this polypeptide (Laird et al., 2001).

Most of the known connexins are phosphorylated on their transport route towards the plasma membrane, but the location where post-translational modifications of connexins take place is still being questioned. Studies by Lampe and Lau (2000) suggest that the phosphate groups are attached to the carboxy-terminus during packaging of the proteins into vesicles and subsequent transport to the cell surface. Analysis of the transport pathways of Cx49-GFP in COS-1 cells indicates that the protein is transported from the ER to the Golgi apparatus and TGN, and vesicular structures of varying sizes could be detected on their way to the cell surface. Similar observations have been reported for Cx43, Cx40, Cx32 and Cx26 (Jordan et al., 1999; Falk, 2000; Martin et al., 2000, 2001). However, the formation of gap junction plaques, as indicated by fluorescent dots or stretches within the plasma membrane, could not be conclusively detected. The vesicles remained at a location in close vicinity to the cell surface. In contrast, Cx49-GFP assembles into gap junction channels in the plasma membrane of adjacent polar MDCK cells. Here, fluorescent dots or stretches appear, lining adjacent cells. The chain-like pattern of these dots suggests that gap junction plaques are sequentially formed along the plasma membranes by

insertion of Cx49 connexons and connection of adjacent Cx49 connexons to gap junction channels. Presumably the formation of gap junctions requires the presence of other proteins that exhibit an auxiliary function and are not expressed in non-polar COS-1 cells. However, transversal images of stable transfected polar MDCK cells indicate basolateral distribution of the Cx49-GFP connexons, and hence gap junction channels, in the cellular membrane. Palisade-like structures between neighboring cells associated with gap junctions demonstrate the specific sorting behavior of polar MDCK cells. Evidence has been presented that cell polarity and specific vesicle transport are related (Jacob and Naim, 2001). Such an association between cell polarity and cell surface localization of connexins has been demonstrated by Guerrier et al. (1995), supporting our data on a polarized mode of delivery of Cx49-GFP to the basolateral cell surface, as observed in the MDCK cell line. Both COS and MDCK cell types are known to express endogenous connexins characteristic for these cell lines. Since the association of different connexin types within a single connexon is possible, the formation of heteromeric connexon variants between Cx49-GFP and endogenous connexin cannot be excluded. Nevertheless, this view is challenged by data on the expression of Cx49-GFP in HeLa cells. One of the exquisite characteristics of these cells is that they can be considered to be almost connexin-free due to the low level of gap junction channels, as determined by electrophysiological experiments (Elfgang et al., 1995; George et al., 1998a). As demonstrated by the fluorescent appearance of the plasma membrane and individual fluorescent dots associated with gap junction plaques, connexon assembly and gap junction formation proceed in HeLa cells as well. This has been confirmed by electrophysiological measurements of gap junctions formed by Cx49-GFP in HeLa cells.

In conclusion, in these experiments we have isolated ovine Cx49 cDNA and generated a fluorescent fusion protein with the capability of forming functional gap junction channels. Furthermore, we visualized the intracellular traffic and transport pattern of ovine Cx49-GFP in non-polar and polar cells. The ability to view Cx49-GFP proteins within different cellular compartments and to examine the transport route of individual vesicles will help to further elucidate the regulation and mechanisms underlying connexin transport in mammalian cell lines. These studies may also give clues to the elements influencing the sorting behavior of connexin proteins and proteins directly involved in connexin protein folding, connexin transport towards the cell plasma membrane, gap junction formation and intercellular communication.

Materials and methods

Nucleic acid extraction from ovine lens preparations and cDNA synthesis

Total RNA was extracted from ovine lens preparations (animal age ranged between 1 and 14 days) by cesium chloride precipitation and was used for cDNA synthesis with the Amersham Pharmacia cDNA Kit (Uppsala, Sweden). cDNA served as a template for a standard RT-PCR procedure to amplify ovine Cx49

cDNA (Yang and Louis, 1996). The following primers were used: Cx49_EcoRI_{up}, 5′-CAG AAT TCG CCC TTG CAT TG-3′; and Cx49_EcoRV_{do}, 5′-GCG ATA TCC GGT GAG ATC G-3′.

Cloning procedures and generation of fusion proteins

Connexin fragments obtained by PCR were cloned into the TOPO TA pCR2.1 vector according to the manufacturer's instructions (Invitrogen, Karlsruhe, Germany). Samples were sequenced and confirmed by comparison with the ovine Cx49 sequence (Yang and Louis, 1996). Cx49 cDNA was cloned into the unique EcoRI/SmaI sites of pEGFP-N1 (Clontech, Heidelberg, Germany). The plasmid generated was denoted pCx49-GFP.

Cell culture and transfection

Confluent COS-1 cell dishes (10 cm in diameter) were split 1:6 1 day prior to transfection and incubated overnight in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal calf serum (FCS) at 37°C with 5% CO_2. Cells were transfected by the diethyl aminoethyl (DEAE)-dextran method (Sambrook and Russel, 2001). For biochemical analysis the cells were treated with 60 mg/ml chloroquine (Sigma, Deisenhofen, Germany). For stable transfection, MDCK cells were transfected according to the modified polybrene-dimethyl sulfoxide (DMSO) method of Graham and Van Der Eb (1973) and selected with neomycin. HeLa cells were transfected with the Effectene transfectant reagent according to the manufacturer's instructions (Qiagen, Hilden, Germany). The efficiency of transient infection into COS-1 or HeLa cells was in the range of 10–20% as assessed by GFP fluorescent vs. non-fluorescent cells.

Confocal microscopy

Transfected cells on cover slips were imaged either 1 day (24 h) or 2 days (48 h) after transfection using the 488-nm argon/krypton laser line on a Leica TCS SP2 inverted microscope with a 63× water immersion objective. For monitoring vesicle trafficking, time series were taken at 4-s intervals.

Biosynthetic labeling

For biosynthetic labeling, cells were rinsed with methionine-free MEM and incubated with 50 μCi [^{35}S]methionine for 30 min at 37°C, followed by different chase times in DMEM/10% FCS. Lysis and immunoprecipitation of Cx49-GFP were carried out according to Naim et al. (1988) using a GFP-specific antibody (Clontech). The samples were further analyzed by SDS-PAGE.

Measurement of gap junction coupling in the double whole-cell patch-configuration

To test whether GFP-tagged Cx49 was able to form gap junction channels, cDNA encoding for Cx49-GFP was transfected into HeLa cells. After 48 h, cell pairs with typical GFP fluorescence in the contact region were chosen and the electrical conductance (G_j) of the gap junction channels formed was determined using the double-whole cell patch-clamp technique, as described previously (Ngezahayo et al., 2003). Cell pairs that did not show typical GFP fluorescence were used as controls.

Acknowledgments

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