J. Membrane Biol. 162, 247–257 (1998)

The Journal of
Membrane
Biology

Phosphorylated Carboxy Terminal Serine Residues Stabilize the Mouse Gap Junction Protein Connexin45 Against Degradation

B. Hertlein, A. Butterweck, S. Haubrich, K. Willecke, O. Traub
Institut fuer Genetik, Abt. Molekulargenetik, Universitaet Bonn, Roemerstr. 164, 53117 Bonn, Germany

Received: 26 September 1997/Revised: 5 January 1997

Abstract. Phosphoamino acid analysis of mouse connexin45 (Cx45) expressed in human HeLa cells revealed that phosphorylation occurred mainly at serine residues, but also on tyrosine and threonine residues. To characterize the role of Cx45 phosphorylation, different serine residues of the serine-rich carboxy terminal region were deleted or exchanged for other amino acids residues. Human HeLa cells deficient in gap junctional intercellular communication were stably transfected with appropriate constructs and analyzed for expression, localization, phosphorylation, formation of functional gap junction channels and degradation of mutant Cx45.

After exchange or deletion of nine carboxy terminal serine residues, phosphorylation was decreased by 90%, indicating that these serine residues represented main phosphorylation sites of mouse Cx45. The various serine residues of this region contributed differently to the phosphorylation of Cx45 suggesting a cooperative mechanism for phosphorylation. Substitution of different serine residues for other amino acids did not interfere with correct intracellular trafficking and assembly of functional gap junction channels, as shown by localization of mutant Cx45 at the plasma membrane and by dye transfer to neighboring cells. Truncated Cx45 was also weakly phosphorylated but was trapped in perinuclear locations. Dye transfer of these transfectants was similar as in nontransfected HeLa cells. The half-life of mouse Cx45 protein in HeLa cells was determined as 4.2 hr. Pulse-chase experiments with the different transfectants revealed an increased turnover of Cx45, when one or both of the serine residues at positions 381 and 382 or 384 and 385 were exchanged for other amino acids. The half-life of these mutants was diminished by 50% compared to wild type Cx45.

Correspondence to: O. Traub

Key words: Intercellular communication — Site-specific mutagenesis

Introduction

Gap junctions consist of aqueous channels that directly connect the cytoplasms of adjacent cells. These intercellular channels are formed by transmembrane proteins, the connexins (Cx) (for reviews *see* Paul, 1995; Goodenough, Goliger & Paul, 1996; Kumar & Gilula, 1996). Different connexins are expressed in a cell type specific manner. Connexin45 (Cx45) is one of thirteen murine connexin proteins that have been described so far. It is expressed in many tissues, e.g., lung, heart, kidney, brain and skin (Beyer, Paul & Goodenough, 1990; Hennemann, Schwarz & Willecke, 1992; Butterweck et al., 1994) and in different mammalian cell lines including WB, SKHep1, BHK and BWEM cells (Laing et al., 1994b; Koval et al., 1995).

Connexin polypeptides are inserted into membranes of endoplasmic reticulum and intramolecular disulfide bonds are formed (Evans, 1994). Connexin proteins are transported to the plasma membrane as hexamers. Oligomerization seems to be a prerequisite for transport to the plasma membrane. Musil & Goodenough (1993) reported post-ER assembly of Cx43 hexamers in the trans Golgi network, whereas Kumar et al. (1995) detected oligomerized hemichannels already in the membranes of ER of Cx32 transfectants. The mechanisms by which connexin hexamers are directed to the plasma membrane are not known, although vesicular transport seems very likely to be involved. Upon cell-to-cell contact, formation of gap junction channels can occur within seconds or minutes (Rook et al., 1990). In the plasma membrane of contacting cells, gap junction channels were found as large aggregates, i.e., the gap junction plaques (Loewenstein, 1981). Previously it was assumed

that gap junctions were removed from the plasma membrane by endocytosis of the double membranous channel complex, that is guided to the lysosomes for degradation (Larsen & Tung, 1978; Larsen et al., 1979; Vaughan & Lasater, 1990). Recent results indicate that Cx43 is degraded via the ubiquitin mediated proteosomal pathway (Laing & Beyer, 1995). Gap junctions are very dynamic structures. The half life of connexin proteins in different cells was determined as 1–5 hr (Fallon & Goodenough, 1981; Traub et al., 1987 and 1989; Laird et al., 1991).

Most connexins (Cx31, 32, 37, 40, 43, 45 and 46) are phosphorylated proteins (Traub et al., 1987; Crow et al., 1990; Musil et al., 1990*a*; Jiang et al., 1993; Butterweck et al., 1994; Laing et al., 1994b; Traub et al., 1994, 1995). Cx43 is phosphorylated in the ER or cis Golgi compartment, displaying the Cx43-P1 isoform, whereas phosphorylation to the P2 isoform occurs in the plasma membrane (Musil & Goodenough, 1991). Oligomerization and transport of Cx43 to the plasma membrane are independent of the processing of Cx43 to the P2 isoform, but the existence of P2 isoform correlates with the aggregation of gap junction plaques and is necessary for formation of functional Cx43 gap junction channels (Musil et al., 1990b; Musil & Goodenough, 1991, 1993; Puranam et al., 1993; Laird et al., 1995).

Phosphorylation also seems to be involved in degradation of connexins. The loss of higher phosphorylated isoforms of Cx43 correlated with a fast turnover of gap junction plaques and with degradation of Cx43 (Crow et al., 1990; Laird et al., 1995). In analogy, activation of protein kinase A increased phosphorylation of Cx32 and slowed down uncoupling of hepatocytes, possibly due to a diminished withdrawal of connexins from the plasma membrane (Saez et al., 1989). Phosphorylation of Cx32 via activation of protein kinase C abolished Cx32 proteolysis by calpain (Elvira et al., 1993, 1994).

Phosphorylation of connexins occurs most frequently at serine residues (Cx43: Crow et al., 1990; Filson et al., 1990; Musil et al., 1990*a*; Cx32: Saez et al., 1986; Traub et al., 1987). If Cx43 is phosphorylated at tyrosine residues, e.g., after infection of cells with Rous sarcoma virus or transformation with middle t-antigen of Polyoma virus, intercellular coupling is inhibited (Filson et al., 1990). This indicates that communication via gap junctions might be regulated by different kinases. Indeed many kinases have been shown to influence gap junction mediated intercellular communication (Bennett et al., 1991). So far it is unclear whether phosphorylation affects the gating of gap junctions directly or via regulation of the turnover of different connexins. Possibly different sites of connexin phosphorylation fulfill different functions in the metabolism and regulation of gap junctions.

To evaluate the role of phosphorylation of mouse Cx45, we replaced or deleted nine serine residues of the carboxy terminal rich region of Cx45, that are potential sites of phosphorylation by multiple kinases. The mutant Cx45 constructs were expressed in HeLa cells and analyzed. Our results showed that these serine residues are major sites of Cx45 phosphorylation. When the C-terminus of Cx45 was truncated by 26 amino acid residues the remaining protein was not transported to the plasma membrane. Exchange of different serine residues did not interfere with correct intracellular trafficking or formation of functional gap junction channels. Instead, phosphorylation of the double serine motif near the carboxy terminus of Cx45 prevented accelerated degradation of mouse Cx45 protein.

Materials and Methods

Cells and Culture Conditions

Human cervix carcinoma HeLa cells (ATCC CCL2) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a moist atmosphere of 10% CO_2.

Construction of Mutated Cx45

Site specific mutagenesis was performed using the gapped-duplex method (Kramer & Fritz, 1988). In mouse Cx45 cDNA (Hennemann et al., 1992) serine residues at positions 374, 376, 378, 381, 382, 384, 385, 387 and 393 were exchanged for other amino acids (Fig. 2 and Table). To delete the last 26 amino acids of Cx45, including the serine rich region, a stop codon was introduced at amino acid position 371. This resulted in a truncated carboxy terminal region with only 122 amino acids left after the presumed forth transmembrane domain (construct C122). For the different constructs the following synthetic oligonucleotides were used (sequence differences to mouse Cx45 are underlined):
3Ser (= Ser374,376,378):
5′-GTG GGG *GCC* AAA *GCT* GGG *CCC* AAC AAA GGC GGT A-3′
Ser381,382:
5′-GGG TCC AAC AAA *GGC GCT* ATT AGT-3′
Ser384,385:
5′-AGC AGT ATT *GGT TTA* AAA TCA GGG-3′
9Ser (= Ser374,376,378,381,382,384,385,387,393):
5′-GTG GGG *GCC* AAA *GCT* GGG *CCC* AAC AAA *GGC GGT* A-3′ and
5′-ATT *GGT GGC* AAA *GCA* GGG GAT GGG AAG ACC *GCC* GGC-3′
C122:
5′-CGG GAA AAG AAG GCC *TAA* GTG GGA TCC-3′

Transfection of HeLa Cells

All mutant Cx45 gene constructs were ligated into the vector pBEHpac18 (Horst et al., 1991) containing the SV40 early promotor, a polyadenylation signal and a gene for puromycin resistance. DNA (2.5 μg) of the linearized construct and 20 μg of genomic DNA of HeLa cells were precipitated by calcium phosphate and added to the medium of 60% confluent HeLa cells for 16 to 20 hr. Forty-eight hours after transfection, the medium was replaced by fresh medium containing 1

μg/ml puromycin. Individual clones were picked and cultivated in selective medium for further analyses.

Northern Blot Analysis

Cells were homogenized using QIAshredder™ (Qiagen, Hilden, Germany) and total RNA was isolated using the RNeasy Total RNA Kit (Qiagen, Hilden, Germany) according to instructions of the company. Electrophoresis, Northern blotting, hybridization at high stringency (55% formamide, 42°C, 5× SSC), filter washing and autoradiography were performed as described (Willecke et al., 1991). A BamH1 restriction fragment (1.2 kb) of mouse Cx45 cDNA, corresponding to the region from nucleotide position 863 to position 2074, was purified, denatured and used as probe for hybridization analysis.

Immunochemical Analysis

Cells were grown to 80–90% confluence and harvested in lysis buffer (0.06 M Tris-HCl, pH 7.4, 3% SDS). Lysates were subjected to sonification, SDS-PAGE and immunoblot analysis as described (Traub et al., 1994). Polyclonal rabbit antibodies, directed to the carboxy terminal part of mouse Cx45 (Butterweck et al., 1994), were used for detection. Concentration of protein was determined using the Bicinchoninic Acid Protein Determination Kit (Sigma, Deisenhofen, Germany) according to the company's instructions. Immunochemical detection of Cx45 protein and its mutants by indirect immunofluorescence was performed as described (Traub et al., 1994). FITC conjugated goat anti rabbit IgG (Sigma, Deisenhofen, Germany) were used as secondary antibodies.

Crude Subcellular Fractionation

Cells were grown to 80–90% confluence and harvested in homogenization buffer (10 mM EDTA, 10 mM Tris-HCl, pH 7.4, 320 mM sucrose, 1× Complete™ protease inhibitor cocktail (Boehringer, Mannheim, Germany). Homogenization was performed by aspiration with a 1 ml syringe through a 22-gauge needle. Homogenates were microscopically controlled for intact nuclei. Fractionation was obtained by successive centrifugation steps of 10 min at 400× *g* (pelleting nuclei and bigger fragments of the cells), 20 min at 15,000× *g* (pelleting plasma membranes) and 20 min at 45,000× *g* (pelleting most cytoplasmic proteins). Samples were resuspended in lysis buffer and used for immunoblot analysis.

Microinjection and Dye Transfer

Microinjection and detection of Lucifer Yellow (Sigma, Deisenhofen, Germany) or neurobiotin (Vector Laboratories, Burlingame, CA) in HeLa wild type cells and transfectants were performed as described (Elfgang et al., 1995). For each cell line and tracer at least 20 injections were carried out.

Metabolic Labeling and Immunoprecipitation

Cells at 80–90% confluence were metabolically labeled with ^{32}P-orthophosphate and ^{35}S-methionine as described (Traub et al., 1994). However, Na-ortho-vanadate (10 mM) and protease inhibitor cocktail (Boehringer, Mannheim, Germany) were added to RIPA lysis buffer instead of phenylmethylsulfonylfluoride (PMSF) and Trasylol. ^{32}P- and ^{35}S-labeled lysates were subjected to immunoprecipitation as described (Traub et al., 1994) using polyclonal rabbit antibodies directed to the carboxy terminal part of mouse Cx45 (Butterweck et al., 1994). After SDS-PAGE, gels were fixed, dried and used for autoradiography. Labeling with ^{32}P-phosphate was performed for 4 hr. Autoradiographs were scanned and the signals of ^{32}P-phosphate labeled Cx45 compared with the total amount of protein in the lysate. Since the antibodies used for immunoprecipitation were directed to the carboxy terminal part of Cx45 protein, mutations in this region could have influenced binding of the antibodies. For correct interpretation of phosphorylation signals, the precipitable amount of Cx45 in each transfectant was estimated by immunoprecipitation of ^{35}S-methionine labelled cells (*data not shown*). From the intensity of the signals obtained, the extent of phosphorylation of mutant Cx45 was calculated.

Analysis of Phosphoamino Acids

To analyze the phosphorylated amino acids of Cx45, ^{32}P-phosphate labeled proteins were immunoprecipitated and separated by electrophoresis as described above. The separated proteins were blotted onto a PVDF membrane (Immobilon, Millipore, Eschborn, Germany) and used for autoradiography. The band representing ^{32}P-labeled Cx45 was identified on the membrane and cut out. After rehydration (incubation in methanol, followed by H_2O, each bathing for 0.5 min), acid hydrolysis, two-dimensional separation of phosphoamino acids and autoradiography were performed as described (Traub et al., 1989).

Pulse-chase Analysis and Determination of Half-life

For pulse-chase analysis, cells were labeled with ^{35}S-methionine as described above. After 1 hr the medium was replaced by non-radioactive medium, supplemented with additional 15 mg/l methionine (final concentration: 45 mg/l). Cells were harvested at different time points of chase (0, 1, 2, 3, 4, 6 and 8 hr) and used for immunoprecipitation analysis. Autoradiographs of ^{35}S-methionine labeled Cx45 protein were scanned (Scantec system, Biometra, Goettingen, Germany). The densitrometric values of the signals were correlated to the amount of protein in the lysates. Using the software''Table curve 2D'' (Jandel Scientific Europe, Erkrath, Germany) the mathematical function $\ln y = a + bx$ was used for approximation of the results obtained. From these curves the half-life was calculated. Pulse-chase experiments were repeated twice with each transfectant. From each experiment, autoradiographs were used after three different exposure times to determine the half-life. Then the average half-life of the Cx45 protein in each transfectant was determined.

Results

Phosphoamino acid analysis of Cx45

Stable transfection of HeLa cells, defective in intercellular transfer of Lucifer Yellow, with mouse Cx45 cDNA led to expression of the 45 kDa protein that was phosphorylated (Butterweck et al., 1994). Analysis of the phosphoamino acids of Cx45 revealed phosphorylated serine, threonine and tyrosine residues (Fig. 1). A strong signal was detected for phosphoserine. With prolonged time of acid hydrolysis, the intensity of this signal increased, whereas the signal for phosphotyrosine de-

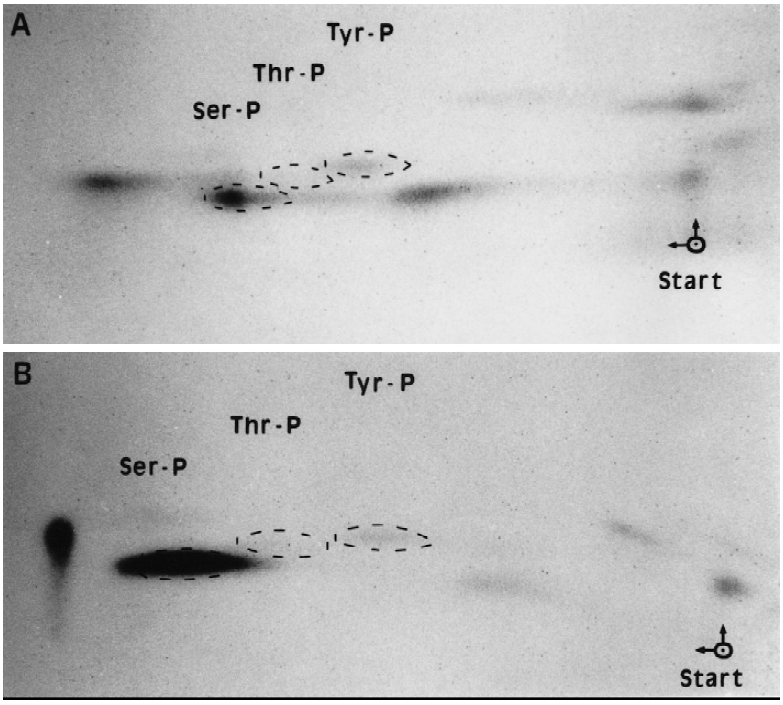

Fig. 1. Phosphoamino acid analysis of Cx45. HeLa cells transfected with mouse Cx45 cDNA were metabolically labeled with $^{32}PO_4$ and protein extracts were subjected to immunoprecipitation with anti-Cx45 as described in Materials and Methods. Acid hydrolysis of immunoprecipitates was performed at 110°C for 30 min (*A*) and 2 hr (*B*). Lysates were separated two-dimensionally by electrophoresis and chromatography, followed by autoradiography (exposure time: 6 weeks). The positions of phosphoserine (Ser-P), phosphothreonine (Thr-P) and phosphotyrosine (Tyr-P) were determined by nonradioactive phosphoamino acids.

creased. Phosphothreonine was detected only after two hours of acid hydrolysis as a weak signal. However, the predominant phosphoamino acid in mouse Cx45 expressed in HeLa cells is phosphoserine.

Exchange of Carboxy Terminal Serine Residues

In the carboxy terminal part of Cx45 there is a region rich in serine residues. The amino acids at position 374 to 396 include nine serine residues, four of which are arranged in a double serine motif. In a variety of constructs (Fig. 2) we deleted all these serine residues (construct C122) or exchanged them for other amino acids (constructs 3Ser, Ser381,382, Ser384,385 and 9Ser). After transfection of HeLa cells, expression of the mutated mouse Cx45 cDNA was determined by Northern blot analysis (data not shown). Transfectants showing the highest level of Cx45 mRNA (2.2 kb) were selected for further examination. In immunoblot analysis (Fig. 3), we obtained signals for wild type as well as mutant Cx45 protein in which different serine residues were exchanged. Deletion of the last 26 amino acids of Cx45 led to a faster migrating protein, observed at a position corresponding to 42 kDa.

Cellular Localization of Mutated Cx45

Indirect immunofluorescence analysis of the different HeLa transfectants revealed the typical punctuate staining pattern of gap junctions at the plasma membrane (Fig. 4). Except for the deletion mutant C122, all other transfectants showed immuno signals at apposed plasma membranes. With C122, fluorescent signals could only be detected in perinuclear location. To confirm these results we used a simple protocol to prepare subcellular fractions from different transfectants. The distribution of mutant Cx45 in these fractions is shown in Fig. 5. Truncated Cx45 was found predominantly in the cytoplasm, whereas with all other mutants the strongest signals were obtained in the fraction of plasma membranes.

Phosphorylation

In all mutants the level of phosphorylation of Cx45 was decreased (Fig. 6*A*). Compared to wild-type Cx45, exchange (construct 9Ser) or deletion (construct C122) of all nine carboxy terminal serine residues led to a 89% decrease of phosphorylation signals (Fig. 6*B*). Since both mutants still showed weak signals, other phosphorylated sites were present besides the serine-rich carboxy terminal region. These findings correlated with the results of phosphoamino acids analysis, showing that in addition to serine residues other amino acids became also phosphorylated.

If distinct serine residues of the carboxy terminal region were missing, phosphorylation was decreased by 67% with mutant 3Ser, by 78% with mutant Ser381,382 and by 32% with mutant Ser384,385 compared to wild-type Cx45. This means that serine residues at positions 381 and 382 contributed much more to the extent of

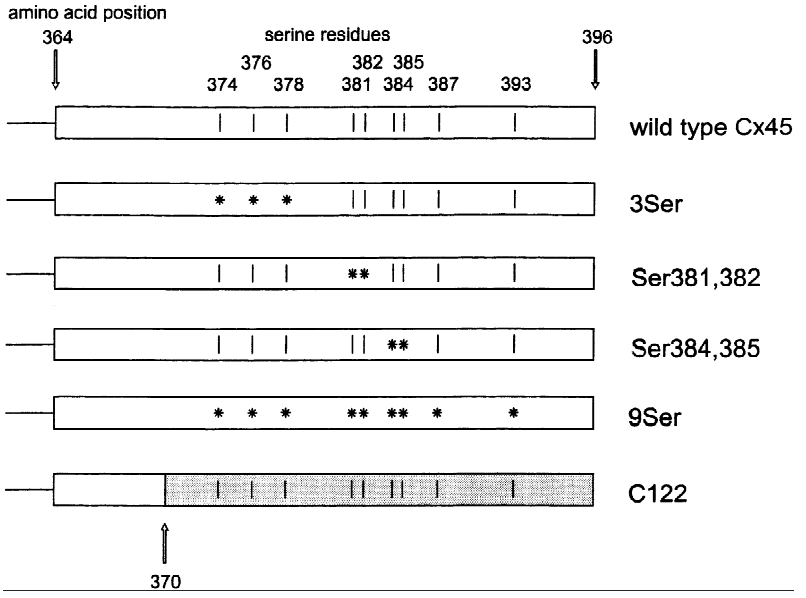

Fig. 2. Location of Cx45 carboxy terminal mutations. Schematic representation of the carboxy terminal region of mouse Cx45 (amino acids 364-396) and the different constructs with serine residues exchanged or deleted. I, serine residue; ✳, substituted serine residue; , deleted region.

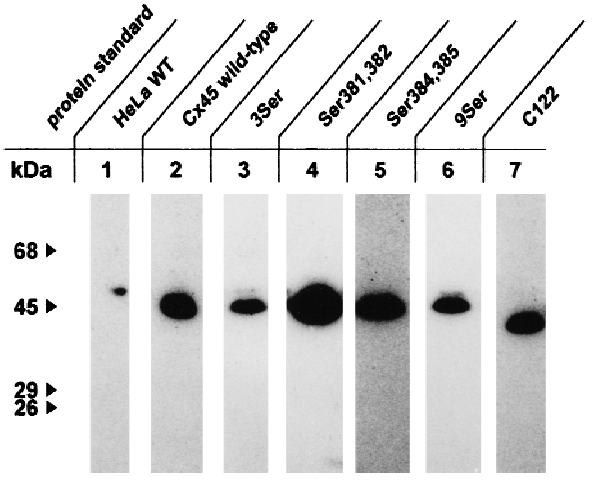

Fig. 3. Expression of mutant Cx45 in HeLa transfectants. Immunoblot of electrophorezed protein lysates of different HeLa transfectants after incubation with polyclonal rabbit Cx45 antibodies as well as ^{125}I-labeled protein A and autoradiography. In each lane 50 μg of protein were applied onto the gel.

phosphorylation of Cx45 than the other serine residues. Summation of the residual phosphorylation signals of the mutants 3Ser, Ser381,382 and Ser384,385 exceeded the 100% level of phosphorylation of wild-type Cx45. Therefore we conclude that the different events of serine phosphorylation are dependent of each other.

Intercellular Communication

To analyze whether gap junction channels made of mutant Cx45 protein were functional, the fluorescent dye Lucifer Yellow was microinjected into different transfectants. Dye transfer was observed in all transfectants expressing mutated Cx45 protein in which serine residues were substituted. Figure 7 shows the results of transfectants with wild-type Cx45 and mutant 9Ser. The deletion mutant C122 as well as nontransfected HeLa cells did not transfer any Lucifer Yellow to neighboring cells (*data not shown*). Compared to Lucifer Yellow (M_r 443, two negative charges) injection of neurobiotin, (M_r 287, one positive charge) into HeLa cells transfected with wild-type Cx45 caused more extensive spreading of the microinjected dye. Therefore gap junctional communication was also analyzed by transfer of neurobiotin (Table 1). Again the deletion mutant C122 showed less than 10% transfer of microinjected neurobiotin to neighboring cells compared to wild-type Cx45 transfectants. This low level of dye transfer did not exceed the one observed with nontransfected HeLa cells. Thus, transfection of HeLa cells with construct C122 did not lead to any additional gap junction channels between adjacent cells. The other HeLa transfectants, expressing mutant Cx45, showed increased coupling compared to nontransfected cells, indicating that exchange of serine residues did not interfere with formation of functional gap junction channels. Dye transfer of the transfectants Ser381,382, Ser384,385 and 9Ser was similar as in wild type Cx45 transfectants. The decreased cell to cell transfer of mutant 3Ser was most likely due to the low level of expression of this protein.

Half-life of Cx45 Mutants

Pulse-chase analyses with ^{35}S-methionine were performed in order to determine the half-life of mutant Cx45

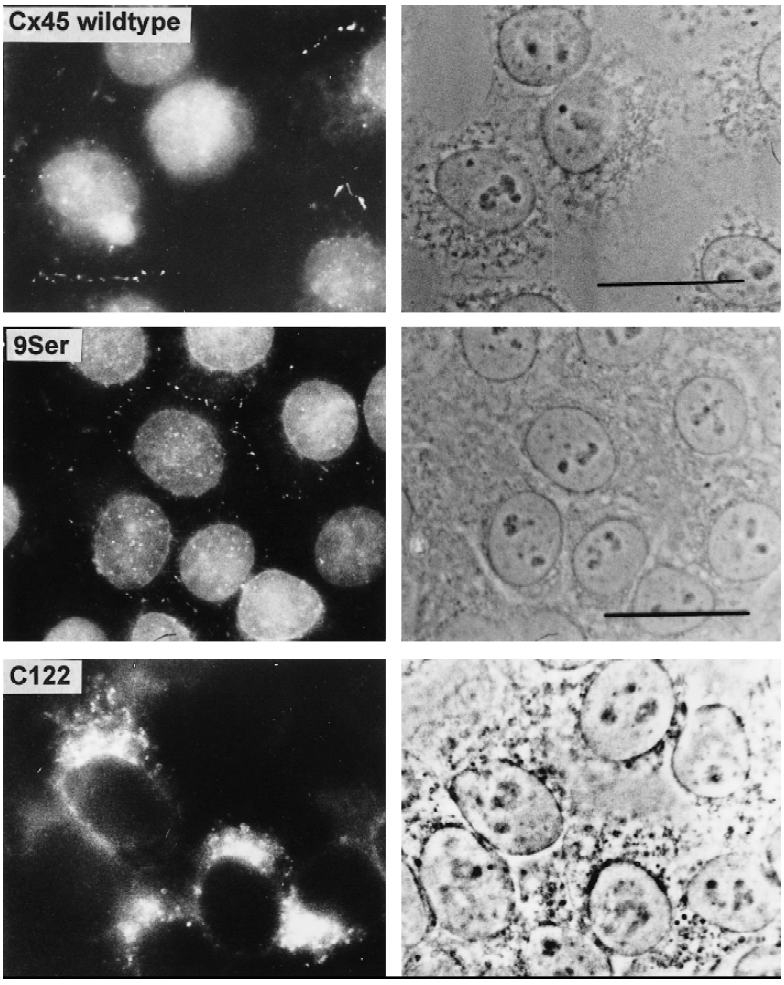

Fig. 4. Localization of mutant Cx45 protein by indirect immunofluorescence in cultured HeLa transfectants Cx45, 9Ser and C122 after incubation with polyclonal rabbit Cx45 antibodies and FITC conjugated goat anti-rabbit IgG. Micrographs shown on the left side were taken at fluorescent illumination. On the right side the corresponding phase contrast images are depicted. Bar corresponds to 20 μm.

protein. Figure 8 shows autoradiographs of different transfectants after a pulse of one hour and a chase of up to 8 hr. The signals were measured by densitometric analysis, correlated to the total amount of protein in the lysates and expressed as a mathematical function. From these values, the half-life of each Cx45 mutant was calculated. The average half-life times of the different mutants are shown in Table 1. Pulse-chase experiments revealed a half-life of 4.2 hr for Cx45 in HeLa-transfectants. After substitution of all nine serine residues of the carboxy terminal region of Cx45 (construct 9Ser), the half-life was reduced by 50%. If the serine residues at positions 381 and 382 (construct Ser381,382) or at positions 384 and 385 (construct Ser384,385) were exchanged, half-life times were decreased to 2.3 and 2.6 hr, respectively. Exchange of serine residues at position 374, 376 and 378 (construct 3Ser) or deletion of the last 26 amino acids (construct C122) had little influence on the turnover of Cx45. The half-life time of the 3Ser mutant protein was 4.1 hr, and that of the C122 protein 3.9 hr.

Discussion

Phosphorylation of Cx45 in HeLa Transfectants

Analysis of phosphorylated amino acids of mouse Cx45 expressed in HeLa transfectants revealed signals for phosphoserine, phosphothreonine and phosphotyrosine, with serine residues as the major target of phosphorylation. Previously, phosphorylation on serine residues has been established for Cx43 (Crow et al., 1990; Filson et al., 1990; Musil et al., 1990*a*), Cx32 (Traub et al., 1989; Crow et al., 1990) and Cx45 (Darrow et al., 1995). In contrast to our findings, the latter publication reported serine residues as the only phosphoamino acids of Cx45 in cultured neonatal rat ventricular myocytes. However, these primary cells express Cx45 in quite lower amounts than HeLa Cx45 transfectants. The phosphorylation of tyrosine residues of Cx45 observed in our experiments did not lead to an inhibition of dye transfer between transfected HeLa cells. This is in contrast to tyrosine

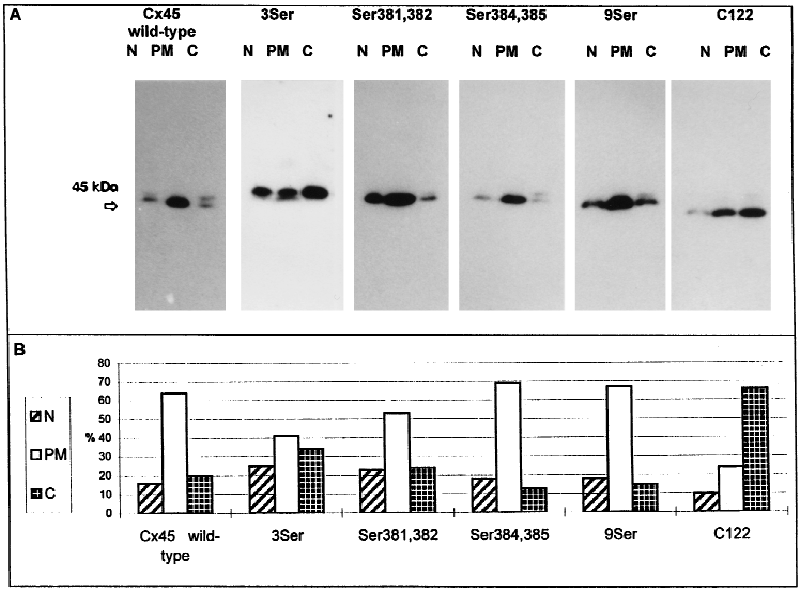

Fig. 5. Localization of mutant Cx45 proteins analyzed by subcellular fractionation. (A) Immunoblot of electrophorezed protein lysates of different HeLa transfectants that have been fractionated into nuclei (N), plasma membranes (M) and cytoplasmic proteins (C) as described. In each lane, 50 μg of protein were applied to the gel, with exception of the cytoplasmic fractions of transfectants HeLa45, 3Ser, Ser381,382 and C122, where due to smaller yields only 30 μg of protein were applied. (B) Diagrammatic representation of the results shown in A after densitometric evaluation of the autoradiographs, standardized per μg of protein applied to each lane.

phosphorylation of Cx43 by $pp60^{v\text{-}src}$ that inhibited gap junctional intercellular communication (Crow et al., 1990; Filson et al., 1990). Phosphorylation of connexins at different sites was hypothesized to fulfill different functions. It was shown that under similar phosphorylating conditions permeability and single-channel conductances of gap junction channels composed of Cx26, 43 or 45 were differentially regulated (Kwak et al., 1995).

We found that phosphorylation of mutant Cx45 in HeLa transfectants was decreased, compared to normal Cx45. Exchange (construct 9Ser) or deletion (construct C122) of all nine serine residues of the serine-rich carboxy terminal region (amino acids 374-396) caused a decrease of phosphorylation signals by about 90%, indicating that these serine residues were the main sites of Cx45 phosphorylation. Since the mutated 9Ser and C122 proteins were weakly phosphorylated, other amino acids besides these serine residues were functioning as targets of phosphorylation. Phosphorylation of these other sites was independent of the existence or phosphorylation of serine residues in the carboxy terminal region. Laing et al. (1994*a*) had reported multiple phosphorylation sites of chicken Cx45 protein in the carboxy terminal region and the cytoplasmic loop. In contrast to Cx43 (no serine residues in the cytoplasmic loop), the central cytoplasmic loop of mouse as well as chicken Cx45 contains three serine residues, representing possible candidates for phosphorylation. The various serine residues contributed differently to the phosphorylation of Cx45. Possibly, phosphorylation of serine residues at positions 381 and 382 is a prerequisite for additional phosphorylation on other amino acids. Such a cooperative mechanism for phosphorylation has been shown for the protein p53, that is only a substrate for SV40 T-antigen activated

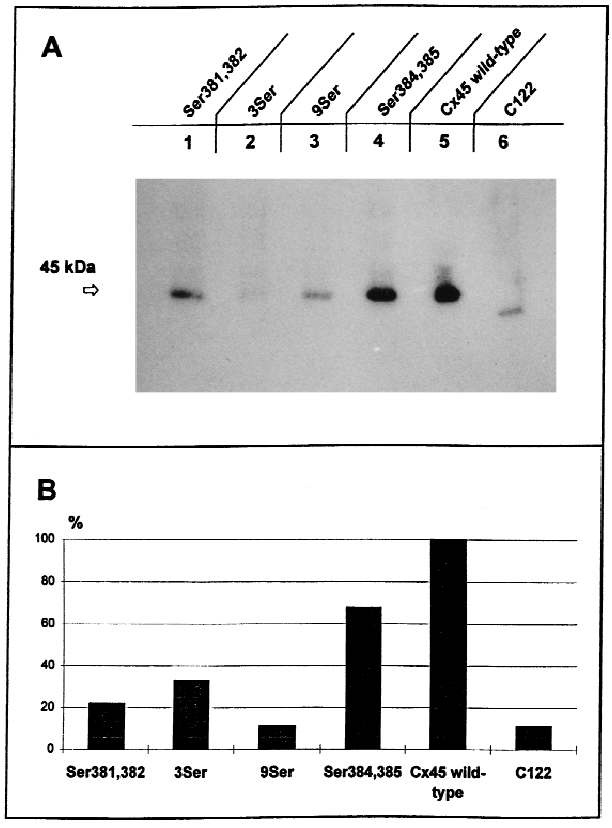

Fig. 6. Phosphorylation of mutant Cx45. (*A*) Autoradiograph of $^{32}PO_4$ labeled and immunoprecipitated Cx45 protein of different transfectants. Fifty μg of protein extract were applied to the gel. (*B*) Diagrammatic representation of the extent of phosphorylation evaluated as described. The average values of phosphorylation are shown in relation to wild type Cx45.

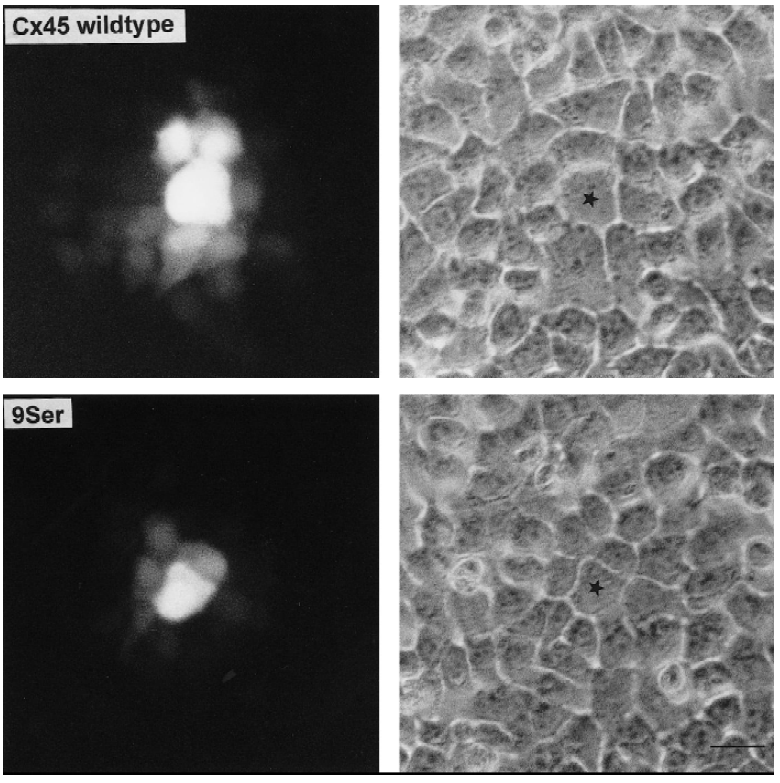

Fig. 7. Intercellular transfer of microinjected Lucifer Yellow. Fluorescence of wild type Cx45 and 9Ser HeLa transfectants after microinjection of Lucifer Yellow. Micrographs shown on the left were taken at fluorescent illumination, the corresponding phase contrast images are depicted on the right, ★, injected cell; bar, 20 μm.

kinase after it has been phosphorylated by cdk-2-kinase at an adjacent serine residue (Müller & Scheidtmann, 1995).

Phosphorylation and Intracellular Transport of Cx45

We conclude from our results that accumulation of the shortened Cx45 protein (C122 mutant) in the cytoplasm was due to truncation of the polypeptide and not to the missing serine residues. Probably the very end of the carboxy terminus of Cx45 contains signal sequences necessary for trafficking to the plasma membrane. Alternatively, the carboxy terminus may be important for the oligomerization process which is assumed to be a prerequisite for transport of connexins to the plasma membrane (Musil & Goodenough, 1993). In contrast to mouse Cx45, truncation of Cx43 or Cx32 protein did not interfere with the formation of functional gap junction channels (Spray et al., 1996). When truncated Cx45 was expressed in ROS cells, it could be detected at the plasma membrane (Koval et al., 1995). However, ROS cells endogenously express Cx43, in contrast to HeLa cells used in our experiments. Therefore, it is possible that in ROS cells truncated Cx45 was transported to the plasma membrane together with Cx43 via heteromeric hemichannels.

Although truncated Cx45 was not transported to the plasma membrane in transfected HeLa cells, it still became phosphorylated. Therefore, phosphorylation of

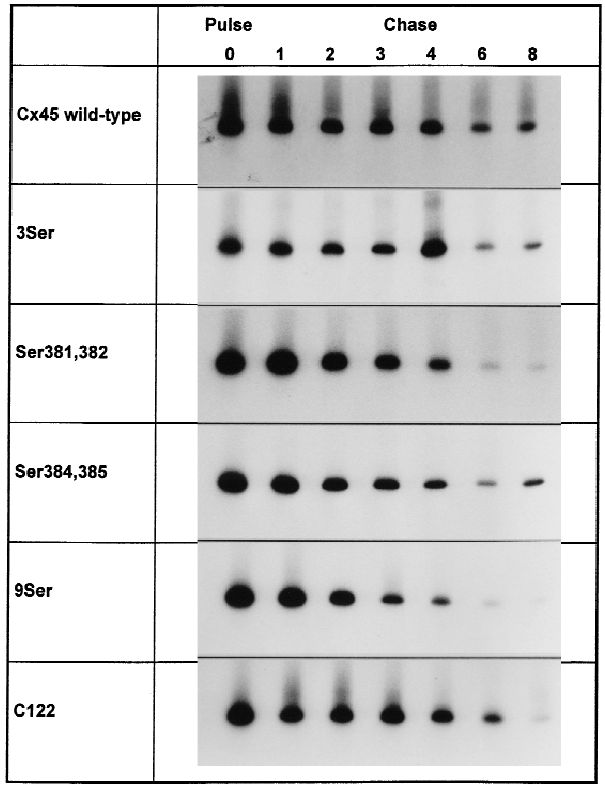

Fig. 8. Half-life of Cx45 mutants determined by pulse-chase analysis. Autoradiographs of ^{35}S-methionine labeled and immunoprecipitated Cx45 of the different transfectants are shown. Cells were labeled for 1 hr (pulse) and chased in nonradioactive medium for up to 8 hr.

Table. Results of analyses performed with the different Cx45 mutants

Name of transfectant	Mutation	Protein (kDa)	Main protein localization	Transfer of Lucifer Yellow	Transfer of Neurobiotin		Phosphorylation	Half-life of protein	
				Number of stained cells	Number of stained cells	Percentage of transfer	%	Hours	%
HeLa45wt		45	M	11	30	100	100	4.2	100
3Ser	S374A S376A S378P	45	M	3	13	43	33	4.1	98
Ser381,382	S381G S382A	45	M	3	26	87	22	2.3	55
Ser384,385	S384G S385L	45	M	4	30	100	68	2.6	62
9Ser	S374A S376A S378P S381G S382A S384G S385G S387A S393A	45	M	8	30	100	11	2.1	50
C122	K371Stop	42	C	0	3	10	11	3.9	93

M, apposed plasma membranes; C, cytoplasm.

Cx45 at other sites than the carboxy terminal serine residues must have occurred early in the process of trafficking. Phosphorylation in endoplasmic reticulum or Golgi has been shown for many other proteins, among them Cx43 (Laird et al., 1995). The functional role of connexin phosphorylation in these compartments is yet unknown. Possibly it prevents misfolding or early degradation of connexins or supports the process of oligomerization of connexins to form hexamers. Our experiments revealed that carboxy terminal serine residues of Cx45 are not involved in this process.

Phosphorylation and Formation of Functional Gap Junction Channels of Cx45

The function of gap junction channels was determined by transfer of Lucifer Yellow or neurobiotin between cells expressing mutated Cx45 protein. Compared to non-transfected HeLa cells, all transfectants with serine residues substituted showed increased dye transfer to neighbouring cells. Even when all nine carboxy terminal serine residues of Cx45 had been replaced, the mutant transfectants showed the same extent of dye transfer as wild-type Cx45 transfectants. The deletion mutant C122 led to the same low level of homotypic dye transfer as measured in non transfected HeLa cells. Since truncated Cx45 protein could not be detected at apposed plasma membranes but accumulated in the cytoplasm, the formation of additional gap junction channels was obviously abolished. In Cx43, exchange of the serine residue at position 364 led to a significant reduction of dye transfer between cells expressing this mutated protein (Britz-Cunningham et al., 1995). We did not observe a similar effects of an altered serine residue in the carboxy terminal region of Cx45. Only with construct 3Ser, dye transfer between transfected cells was lower than with other Cx45 mutants. But this decrease was most likely due to the lower expression level of this mutated Cx45 protein. Further analyses are necessary to determine whether single channel conductance of Cx45 is changed due to the exchange of different serine residues.

Phosphorylation and Degradation of Cx45

Phosphorylation of connexins has been correlated to degradation of these proteins. Laird et al. (1995) showed a correlation between the loss of higher phosphorylated isoforms of Cx43 and a rapid turnover of gap junction plaques. Phosphorylation of Cx32 slowed down internalization of gap junction channels from the plasma membrane and led to the decreased proteolysis of connexins (Saez et al., 1989; Elvira et al., 1993, 1994). For chick Cx45 it has been shown that decreased phosphorylation coincided with degradation of this protein (Laing

& Beyer, 1996). The half-life time of Cx45 in rat myocytes was demonstrated to be 2.9 hr (Darrow et al., 1995). In our analyses the half-life of wild-type Cx45 protein in HeLa cells was 4.2 hr. We found that phosphorylation of distinct serine residues in the carboxy terminal region of mouse Cx45 correlated with increased degradation. Cx45 protein of the constructs Ser381,382, Ser384,385 and 9Ser was degraded twice as fast, resulting in half-lives of 2 to 2.5 hr. Protein of the construct 3Ser showed a similar turnover as Cx45 wild type, although phosphorylation of mutant 3Ser was less than phosphorylation of Ser384,385. Only when the double serine residues at positions 381 and 382 or 384 and 385 were exchanged for other amino acids, the half-life time was decreased up to 50%. Since the deletion mutant C122 also lacked these serine residues, the turnover of C122 protein should have been less than the observed 3.9 hr. However, in contrast to the other mutants, truncated Cx45 could not be detected at the plasma membrane and, therefore, was probably degraded via a different pathway. During accumulation in perinuclear membranes, C122 protein was not subject to obvious degradation. Possibly it was not preferentially eliminated as a defective protein, but did not carry the necessary signals for correct transport to the plasma membrane.

The mechanism(s) by which Cx45 is degraded may include the ubiquitin-mediated proteolysis in proteasomes. This pathway has been shown to prevail in degradation of Cx43 (Laing & Beyer, 1995). Further analyses should provide more information about the correlation between phosphorylation and ubiquitination of Cx45. Degradation of dephosphorylated isoforms could reveal an important role of phosphatases in the regulation of intercellular communication via gap junctions.

We thank Petra Koenig and Carmen Hansen for excellent technical assistance. Birgit Hertlein received a stipend of the Graduierten Kolleg ''Funktionelle Proteindomaenen.'' This work was supported by a grant of the Deutsche Forschungsgemeinschaft through SFB 284, project C1.

References

Bennett, M.V.L., Barrio, L.C., Bargiello, T.A., Spray, D.C., Hertzberg, E., Saez, J.C. 1991. Gap junctions: new tools, new answers, new questions. *Neuron* **6:**305–320

Beyer, E.C., Paul, D.L., Goodenough, D.A. 1990. Connexin family of gap junction proteins. *J. Membrane Biol.* **116:**187–194

Britz-Cunningham, S.H., Shah, M.M., Zuppan, C.W., Fletcher, W.H. 1995. Mutations of the connexin43 gap junction gene in patients with heart malformations and defects of laterality. *N Engl. J. Med.* **332:**1323–1329

Butterweck, A., Gergs, U. Elfgang, C., Willecke, K., Traub, O. 1994. Immunochemical characterization of the gap junction protein connexin45 in mouse kidney and transfected human HeLa cells. *J. Membrane Biol.* **141:**247–256

Crow, D.S., Beyer, E.C., Paul, D.L., Kobe, S.S., Lau, A.F. 1990. Phosphorylation of connexin43 gap junction protein in uninfected and Rous Sarkoma Virus-transformed mammalian fibroblasts. *Mol. Cell. Biol.* **10:**1754–1763

Darrow, B.J., Laing, J.G., Lampe, P.D., Saffitz, J.E., Beyer, E.C. 1995. Expression of multiple connexins in cultured neonatal rat ventricular myocytes. *Circ. Res.* **76:**381–387

Elfgang, C., Eckert, R., Lichtenberg-Fraté, H., Butterweck, A., Traub, O., Klein, A.R., Hülser, D.F., Willecke, K. 1995. Specific permeability and selective formation of gap junction channels in connexin transfected HeLa cells. *J. Cell Biol.* **126:**805–817

Elvira, M., Diez, J.A., Wang, K.K.W., Villalobo, A. 1993. Phosphorylation of connexin-32 by protein kinase C prevents its proteolysis by μ-Calpain and m-Calpain. *J. Biol. Chem.* **268:**14294–14300

Elvira, M., Wang, K.K.W., Villalobo, A. 1994. Phosphorylated and non-phosphorylated connexin-32 molecules in gap junction plaques are protected against calpain proteolysis after phosphorylation by protein kinase C. *Biochem. Soc. Trans.* **22:**793–796

Evans, W.H. 1994. Assembly of gap junction intercellular communication channels. *Biochem. Soc. Trans.* **22:**788–792

Fallon, R.F., Goodenough, D.A. 1981. Five-hour half-life of mouse liver gap junction protein. *J. Cell Biol.* **90:**531–526

Filson, A.J., Azarnia, R., Beyer, E.C., Loewenstein, W.R., Brugge, J.S. 1990. Tyrosine phosphorylation of a gap junction protein correlates with inhibition of cell-to-cell communication. *Cell Growth Differ.* **1:**661–668

Goodenough, D.A., Goliger, J.A., Paul, D.L. 1996. Connexins, connexons, and intercellular communication. *Ann. Rev. Biochem.* **65:**475–502

Hennemann, H., Schwartz, H.J., Willecke, K. 1992. Characterization of gap junction genes expressed in F9 embryonic carcinoma cells: molecular cloning of mouse connexin31 and -45 cDNAs. *Eur. J. Cell Biol.* **57:**51–58

Horst, M., Harth, N., Hasilik, A. 1991. Biosynthesis of glycosylated human lysozyme mutants. *J. Biol. Chem.* **266:**13914–13919

Jiang, J.X., Paul, D.L., Goodenough, D.A. 1993. Posttranslational phosphorylation of lens fiber connexin46: a slow occurrence. *Invest. Ophthalmol. Vis. Sci.* **34:**3558–3565

Koval, M., Geist, S.T., Westphale, E.M., Kemendy, A.E., Civitelli, R., Beyer, E.C., Steinberg, T.H. 1995. Transfected connexin45 alters gap junction permeability in cells expressing endogenous connexin43. *J. Cell Biol.* **130:**987–995

Kramer, W., Fritz, H.J. 1988. Oligonucleotide-directed construction of mutations via gapped duplex DNA. *Meth. Enzymol.* **154:**350–367

Kumar, N.M., Friend, D.S., Glilula, N.B. 1995. Synthesis and assembly of human β1 gap junctions in BHK cells by DNA transfection with the human β1 cDNA. *J. Cell Sci.* **108:**3725–3734

Kumar, N.M., Gilula, N.B. 1996. The gap junction communication channel. *Cell* **84:**381–388

Kwak, B.R., Hermans, M.M.P., de Jonge, H.R., Lohmann, S.M., Jongsma, H.J., Chanson, M. 1995. Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. *Mol. Biol. Cell* **6:**1707–1719

Laing, J.G., Lampe, P.D., Darrow, B.J., Saffitz, J.E., Beyer, E.C. 1994a. The gap junction protein connexin45 is multiply phosphorylated. *Mol. Biol. Cell Suppl.* **5:**197*a*. (*Abstr.*)

Laing, J.G., Westphale, E.M., Engelmann, G.L., Beyer, E.C. 1994b. Characterization of the gap junction protein, connexin45. *J. Membrane Biol.* **139:**31–40

Laing, J.G., Beyer, E.C. 1994. The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. *J. Biol. Chem.* **270:**26399–26403

Laing, J.G., Beyer, E.C. 1996. Turnover of the gap junction protein Cx45. *In:* Molecular Approaches to the Function of Intercellular Junctions. Keystone Symposia on Molecular and Cellular Biology 1996, Lake Tahoe, California, 313 (*Abstr.*)

Laird, D.W., Puranam, K.L., Revel, J.P. 1991. Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. *Biochem. J.* **272:**67–72

Laird, D.W., Castillo, M., Kasprzak, L. 1995. Gap junction turnover, intracellular trafficking, and phosphorylation of connexin43 in brefeldin A-treated rat mammary tumor cells. *J Cell Biol.* **131:**1193–1203

Larsen, W.J., Tung, H.N. 1978. Origin and fate of cytoplasmic gap junctional vesicles in rabbit granulosa cells. *Tissue Cell* **10:**585–598

Larsen, W.J., Tung, H.N., Murray, S.A., Swenson, C.A. 1979. Evidence for the participation of actin microfilaments and bristle coats in the internalization of gap junction membrane. *J. Cell Biol.* **83:**576–587

Loewenstein, W. 1981. Junctional intercellular communication: The cell-to-cell membrane channel. *Physiol. Rev.* **61:**829–913

Müller, E., Scheidtmann, K.H. 1995. Purification and characterization of a protein kinase which is activated by SV40 large T antigen and phosphorylates the tumor suppressor protein p53. *Oncogene* **10:**1175–1185

Musil, L.S., Beyer, E.C., Goodenough, D.A. 1990*a*. Expression of the gap junction protein connexin43 in embryonic chick lens: molecular cloning, ultrastructural localization and post-translational phosphorylation. *J. Membrane Biol.* **116:**163–175

Musil, L.S., Cunningham, B.A., Edelman, G.M., Goodenough, D.A. 1990*b*. Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* **111:**2077–2088.

Musil, L.S., Goodenough, D.A. 1991. Biochemical analysis of connexin43 intracellular transport, phosphorylation and assembly into gap junctional plaques. *J. Cell Biol.* **115:**1357–1374

Musil, L.S., Goodenough, D.A. 1993. Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* **74:**1065–1077

Paul, D.L. 1995. New functions for gap junctions. *Curr. Op. Cell Biol.* **7:**665–672

Puranam, K.L., Laird, D.W., Revel, J.P. 1993. Trapping an intermediate form of connexin43 in the Golgi. *Exp. Cell Res.* **206:**85–92

Rook, M.B., de Jonge, B., Jongsma, H.J., Masson-Pevet, M.A. 1990. Gap junction formation and functional interaction between neonatal rat cardiocytes in culture: a correlative physiological and ultrastructural study. *J. Membrane Biol.* **118:**179–192

Sáez, J.C., Spray, D.C., Naim, A.C., Hertzberg, E.L., Greengard, P., Bennett, M.V.L. 1986. cAMP increases junctional conductance and stimulates phosphorylation of the 27 kDa principal gap junction polypeptide. *Proc. Natl. Acad. Sci. USA* **83:**2473–2477

Sáez, J.C., Gregory, W.A., Watanabe, T., Dermietzel, R., Hertzberg, E.L., Reid, L., Bennett, M.V.L., Spray, D.C. 1989. cAMP delays disappearance of gap junctions between pairs of rat hepatocytes in primary culture. *Am. J. Physiol.* **257:**C1–C11

Spray, D.C., Vink, M.J., Moore, L.K., Fishman, G.I. 1996. Physiological consequences of connexin phosphorylation. *In:* Molecular Approaches to the Function of Intercellular Junctions. Keystone Symposia on Molecular and Cellular Biology 1996, Lake Tahoe, California, 26 (*Abstr.*)

Traub, O., Look, J., Paul, D., Willecke, K. 1987. Cyclic adenosine monophosphate stimulates biosynthesis and phosphorylation of the 26 kDa gap junction protein in cultured mouse hepatocytes. *Eur. J. Cell Biol.* **43:**48–54

Traub, O., Look, J., Dermietzel, R., Brümmer, F., Hülser, D., Willecke, K. 1989. Comparative characterization of the 21-kD and 26-kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* **108:**1039–1059

Traub, O., Lichtenberg-Fraté, H., Eckert, R., Bastide, B., Scheidtmann, K.H., Hülser, D., Willecke, K. 1994. Immunochemical and electrophysiological characterization of murine connexin40 and -43 expressed in transfected human cells and mouse tissues. *Eur. J. Cell Biol.* **64:**101–112

Traub, O., Butterweck, A., Elfgang, C., Hertlein, B., Balzer, K., Gergs, U., Hafemann, B., Willecke, K. 1995. Immunochemical characterization of connexin31, -37, -40, -43 and -45 in cultured primary cells, transfected cell lines and murine tissues. *In:* Progress in Cell Research, Vol. 4: Intercellular communication through gap junctions. Y Kanno, K Kataoka, Y Shiba, Y Shibata, and T Shimazu, editors. pp. 343–347. Elsevier, Amsterdam

Vaughan, D.K., Lasater, E.M. 1990. Renewal of electrotonic synapses in the teleost retinal horizontal cells. *J. Comp. Neurol.* **299:**364–374

Willecke, K., Heynkes, R., Dahl, E., Stutenkemper, R., Hennemann, H., Jungblut, S., Suchyna, T., Nicholson, B.J. 1991. Mouse connexin37: cloning and functional expression of a gap junction gene highly expressed in lung. *J. Cell Biol.* **114:**1049–1057