A tyrosine-based sorting signal is involved in connexin43 stability and gap junction turnover

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Summary
The gap junction protein connexin43 is known to have a rapid turnover, involving degradation by both the proteasomal and lysosomal systems, but the structural features of connexin43 that govern these actions are not known. The connexin43 C-terminal sequence contains a proline-rich region corresponding to the consensus of a protein-protein interaction PY-motif (xPPxY), and an overlapping putative tyrosine-based sorting signal (Yxxφ; φ=hydrophobic), known to play a role in the intracellular trafficking of many membrane proteins. As both motifs may control turnover of connexin43, we used a combination of metabolic radiolabelling, immuno-precipitation and functional assays to determine the possible role of these motifs in controlling degradation of human connexin43 expressed in SKHep1 cells. Mutation V289D in the tyrosine-based sorting motif increased the steady-state pool of connexin43 by approximately 3.5-fold, while mutation P283L in the PY-motif produced a comparatively modest augmentation (1.7-fold). No additive effect was observed when the overlapping tyrosine was mutated. In pulse-chase experiments, the Y286A substitution increased the half-life of connexin43 from 2 to 6 hours, indicating that the increased steady-state levels reflected reduced protein degradation. Moreover, expression at the junctional membrane, as well as gap junction-mediated intercellular communication (GJC), were nearly abolished by lysosomal inhibitors and Brefeldin A in cells expressing wild-type connexin43, but were unaffected in the tyrosine mutant. These results provide strong evidence that the tyrosine-based motif of human connexin43 is a prime determinant controlling connexin43 stability, and consequently GJC, by targeting connexin43 for degradation in the endocytic/lysosomal compartment.

Key words: Connexins; Gap junctional communication; Trafficking; Degradation; Endocytosis

Introduction
Gap junctions are complexes of transmembrane proteins, called connexins, that directly link the cytoplasm of adjacent cells. These intercellular channels act as molecular sieves, allowing the passage of low-molecular-weight substances such as amino acids, ions and second messengers (Goodenough et al., 1996; Kumar and Gilula, 1996). Gap junctions play important roles in a variety of cellular processes such as embryonic development, synchronous contraction of cardiac and smooth muscle cells, regulation of exocytosis and control of cell growth. Mutations in connexins or defective production so far, undergoes several types of post-translational modifications, including phosphorylation and ubiquitination. Numerous studies have established that the conversion of unphosphorylated Cx43 to slower migrating species on SDS-polyacrylamide gel is caused by phosphorylation of Cx43, an event that facilitates gap junction channel formation and gating (Kwak et al., 1995; Laird et al., 1991; Lampe, 1994; Moreno et al., 1994; Musil et al., 1990b). The phosphorylation state of Cx43 has also been proposed to control Cx43 degradation in rat mammary tumour cells and in intact rat heart (Beardslee et
The degradation of Cx43 has been shown to occur by the lysosomal (Laing et al., 1997; Larsen and Hai, 1978; Musil et al., 2000; Naus et al., 1993; Vaughan and Lasater, 1990) and the ubiquitin-proteasomal (Laing et al., 1997; Musil et al., 2000; Laing and Beyer, 1995; Rutz and Hulser, 2001) pathways, the relative contribution of which appears to be largely cell-type specific. It is likely that some of the proteasomal degradation occurs at the level of the endoplasmic reticulum (ER), as a quality control step, to remove poorly folded or oligomerised connexin polypeptides (Musil et al., 2000; VanSlyke et al., 2000).

Despite the large body of knowledge on the rapid turnover and degradation of Cx43, and the proteolytic systems involved, virtually nothing is known about the signals and motifs that control sorting to the lysosome or promote degradation by the ubiquitin-proteasome system. Cx43 does not have an amino terminus (basic or hydrophobic, bulky amino acids) that would be recognised by the N-end rule (Varshavsky, 1992). Many rapidly degrading proteins contain PEST sequences (rich in proline, glutamic acid, serine and threonine), which have been suggested to be signals for rapid degradation (Rogers et al., 1986). Indeed, low consensus PEST sequences were described for Cx43 (Darrow et al., 1986); however, their role in Cx43 turnover has not been shown experimentally. Interestingly, Cx43 contains a proline-rich motif in its C-terminus, which conforms to the consensus of a PY motif (xPPxY, P=proline, Y=tyrosine, x=amino acid) (Fig. 1A). Such PY motifs have been shown to act as ligands for WW domain-containing proteins (Chen and Sudol, 1995). More importantly, several different ion channels interact with members of the Nedd4/Nedd4-like family of ubiquitin-protein ligases, via PY motif/WW domain interactions, leading to their degradation (Rogers et al., 1986). Indeed, low consensus PEST sequences were described for Cx43 (Darrow et al., 1995; Laird et al., 1991); however, their role in Cx43 turnover has not been shown experimentally. Interestingly, Cx43 contains a proline-rich motif in its C-terminus, which conforms to the consensus of a PY motif (xPPxY, P=proline, Y=tyrosine, x=amino acid) (Fig. 1A). Such PY motifs have been shown to act as ligands for WW domain-containing proteins (Chen and Sudol, 1995). More importantly, several different ion channels interact with members of the Nedd4/Nedd4-like family of ubiquitin-protein ligases, via PY motif/WW domain interactions, leading to their ubiquitination-dependent downregulation at the plasma membrane (Abriel et al., 2000; Abriel et al., 1999; Schwake et al., 2001; Staub et al., 1996). Hence, it is possible that this motif directs the ubiquitination of Cx43 and plays a role in its targeting for endocytosis and destruction. Overlapping the PY-motif, however, is a tyrosine-based sorting signal conforming to the consensus YxxØ (where Y is a tyrosine, x is any amino acid and Ø is an amino acid with a bulky hydrophobic side chain). Signals of this nature, contained in the cytosolic domains of many plasma membrane proteins, are also known to mediate internalisation and lysosomal targeting for degradation. In addition, some YxxØ motifs can direct traffic within the endosomal and late secretory pathways (Bonifacino and Dell’Angelica, 1999; Kirchhausen et al., 1997; Owen and Evans, 1998). The specificity of these processes is believed to be achieved through the interaction of these signals with alternative adapter complex molecules that associate with different protein-sorting machineries (Bonifacino and Dell’Angelica, 1999).

To better understand the molecular mechanisms that control connexin retrieval from the plasma membrane and degradation, we studied the possible contribution of the PY-motif and its overlapping tyrosine-based motif on Cx43 degradation. To this end, we studied the effects of amino acid substitutions within this region on protein stability and sensitivity to proteasome and lysosome inhibitors, as well as on functional expression. We report here that the tyrosine-based sorting signal is a primary element in this region controlling Cx43 turnover.

**Materials and Methods**

**Constructs and plasmids**

The full-length human Cx43 cDNA, supplied as a gift from D. C. Spray (Dept of Neuroscience, Albert Einstein College of Medicine, NY), was used to develop Cx43 mutant constructs. Site-directed mutagenesis was performed using a PCR-based technique (Nelson and Long, 1989) to substitute tyrosine 286 with alanine (Cx43-Y286A) or phenylalanine (Cx43-Y286F), proline 283 with leucine (Cx43-P283L), glycine 285 with alanine (Cx43-G285A), and valine 289 with aspartate (Cx43-V289D) either alone or in combination with the proline 283 to leucine mutation (Cx43-P283L/V289D), which were used for transfection studies. The cDNA clones were cloned into the pRc/CMV plasmid (Invitrogen, Carlsbad, CA). Correct mutagenesis was assessed by DNA sequencing (Microsynth, Balgach, Switzerland).

**Cell culture and transfection**

SKHeP1 cells were purchased from the American Type Culture Collection (Rockville, MD) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM), supplemented with 10% FCS and 0.6% penicillin/streptomycin (Invitrogen) in an atmosphere of humidified air (5% CO₂) at 37°C. For the development of the stable cell lines Cx43-WT (wildtype) and Cx43-Y286A, plasmids were transfected into SKHeP1 cells using the Effectene transfection reagent (Qiagen, Hilden, Germany), and transformants were selected for neomycin resistance using 400 μg/ml of G418. Likewise, transient transfections were carried out using the Effectene transfection kit, and plasmids were allowed to express Cx43 for 48 hours before SDS-PAGE and western blot analysis.

**Western blot analysis**

SKHeP1 cells, stably or transiently transfected with Cx43 constructs, were washed twice in cold PBS, lysed in radioimmunoprecipitation (RIPA) buffer pH 8.0 (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml aprotinin) and the cells harvested on ice by scraping. After centrifugation at 4°C for 5 minutes at 20,000 g, the supernatants were recovered and samples denatured by heating at 95°C for 5 minutes in protein sample buffer (295 mM sucrose, 2% SDS, 2.5 mM EDTA, 62.5 mM Tris-Cl pH 8.8, 0.05% bromophenol blue, 26 mM dithiothreitol (DTT)). Dephosphorylation experiments were performed by treating the cellular lysates with 50 units of calf intestinal phosphatase (New England Biolabs, Beverly, MA) for 3 hours at 37°C before denaturing in protein sample buffer. Cellular lysates were then electrophoresed on a 10% polyacrylamide gel (SDS-PAGE), before being transferred onto a nitrocellulose membrane. Membranes were then probed with a polyclonal antibody directed against the C-terminus of Cx43 (Zymed, San Francisco, CA) or β-Actin (Sigma). For experiments with protease inhibitors, the stable cell lines Cx43-WT and Cx43-Y286A were treated for 3 hours with either lactacystin (10 μM), leupeptin (10 μM) or NH₄Cl (10 mM) before cell harvesting and western blotting. Quantitation of recognised levels was performed on fluorograms, using a molecular imager FX (Biorad, Hercules, CA), and the results were normalised to the controls and expressed as means±s.e.m. Statistical analyses were performed using the unpaired two-tailed Student’s t test.

**Pulse-chase analysis**

Cx43-WT and Cx43-Y286A cells were grown to 80% confluency and then starved in depletion medium (DMEM without methionine) for 30 minutes at 37°C. Cells were then labelled for 60 minutes in depletion medium containing 0.1 μCi/ml [35S]-methionine. After labelling, cells were placed on ice and washed three times in ice-cold wash medium (DMEM, 10% FCS, 0.6% penicillin/streptomycin, 10
mM methionine) before being chased, for various periods of time, in pre-warmed wash medium (alone or supplemented with either 10 μM lactacystin or 10 mM NH₄Cl). At the end of the chase, cells were transferred onto ice, washed three times with ice-cold PBS, then lysed in RIPA buffer. Cells were harvested by scraping, centrifuged for 5 minutes at 20,000 g (4°C) and the supernatants immunoprecipitated overnight (4°C with rotation) using an anti-Cx43 antibody (Zymed) together with protein A sepharose beads. After immunoprecipitation, the beads were washed four times in RIPA buffer and then the immunoprecipitated proteins eluted with protein sample buffer and boiling for 5 minutes at 95°C. Radiolabelled proteins were then visualised by SDS-PAGE and autoradiography. Quantitation of [35S]-labelled Cx43 was performed on autoradiographs using a molecular imager FX (Biorad). The results were normalised to the control (t=0) and expressed as mean±s.e.m. Mono-exponential curves for each independent experiment were fitted through the values measured at each time point according to the formula y=100*exp(–kt), using Kaleidagraph v. 3.52 (Synergy Software, Reading, PA). The decay rate constants (k), which are representative of the rates of protein degradation, were determined for each curve and the mean values calculated. For statistical analyses, two-way ANOVA was performed using GraphPad, San Diego, CA).

Immunofluorescence microscopy
For immunofluorescence labelling, cell lines were cultured on glass coverslips and incubated for 3 hours in new medium alone (control) or supplemented with 10 μM lactacystin, 10 mM NH₄Cl or 2 μg/ml Brefeldin A before fixation for 2-3 minutes in methanol at –20°C. The coverslips were then rinsed in PBS and incubated successively with 0.2% Triton X-100 for 60 minutes, 0.5 M NH₄Cl for 15 minutes and PBS supplemented with 2% bovine serum albumin for an additional 30 minutes. Cells were then rinsed and incubated overnight with polyclonal antibodies (diluted 1:30) against Cx43 (Alpha Diagnostics, San Antonio, TX). After washing in PBS, the coverslips were incubated with secondary antibodies, conjugated to FITC for 3 hours and then examined using fluorescent microscopy. Images were acquired with a high-sensitivity CCD Visicam (Visitron systems GmbH, Germany) camera connected to a personal computer. Images were captured using the software Metafluor 4.01 (Universal Imaging, West Chester, PA) and processed using Adobe Photoshop 5.5 (Adobe Systems Inc.).

Cell-coupling measurements
Dye coupling studies were performed on subconfluent monolayers of cells incubated in a solution (external solution) containing (in mM): 136 NaCl, 4 KCl, 1 CaCl₂, 1 MgCl₂, and 2.5 glucose, and was buffered to pH 7.2 with 10 mM HEPES-NaOH. Single cells were impaled with microelectrodes backfilled with a 4% lucifer yellow solution prepared in 150 mM LiCl (buffered to pH 7.4 with 10 mM HEPES). The fluorescent tracer was allowed to fill the cells by simple diffusion for 3 minutes. After the injection period, the electrode was removed and the number of fluorescent cells was counted. Cells were visualised using epifluorescent illumination provided by a 100 W mercury lamp and the appropriate set of filters. The results were expressed as mean±s.e.m. To examine the effects of proteasome/lysosome inhibitors on dye coupling, subconfluent monolayers of cells were incubated for 3 hours in the external solution supplemented with either 10 μM lactacystin, 10 mM NH₄Cl or 2 μg/ml Brefeldin A.

For electrical coupling studies, the dual whole-cell patch-clamp approach was applied to pairs of cells incubated in the external solution. Both cells of a pair were voltage clamped at a common holding potential of 0 mV. To measure gap junctional conductance (gj), transjunctional potential differences (Vj) were elicited by changing the holding potential of one member of a cell pair. Vj was defined as the current recorded in the cell kept at 0 mV. Junctional conductance (gj) was then calculated by gj=Ij/Vj and the results displayed as a scattered plot displaying the individual gj values, including the mean±s.e.m. Series resistance was not compensated for and was less than 2% of the combined junctional and cell input resistance. Patch electrodes were filled with a pCa 7 solution containing (in mM): 138 KCl, 1 NaCl, 2.9 CaCl₂, 5.5 EGTA, 2 MgCl₂, and buffered to pH 7.2 with 10 mM HEPES-KOH. Statistical analyses were performed using the two-tailed Student’s t test for unpaired data.

Results
Tyrosine 286 is involved in the turnover of connexin43
Inspection of the C-terminus of Cx43 reveals the existence of a PY-motif (xPPxY), known to serve as a ligand for WW domain-containing proteins such as the members of the Nedd4/Nedd4-like family of ubiquitin-protein ligases (Rotin et al., 2000). Adjacent to this PY-motif lies a consensus tyrosine-based sorting signal (Yxxφ), which may contribute to protein degradation by directing traffic to the endosomal/lysosomal compartment (Fig. 1A). To assess whether gap junction stability is mediated by these motifs, we substituted the tyrosine 286 to alanine (Y286A), and investigated the effect of this substitution on Cx43 steady-state levels. cDNAs encoding the human Cx43 wild-type protein (Cx43-WT) and its mutant counterpart
(Cx43-Y286A) were transiently transfected into the hepatoma cell line SKHep1, which does not express Cx43 endogenously. Cx43 expression, driven by the cytomegalo virus (CMV) promoter, was then assessed by SDS-PAGE and western blot analysis using an anti-Cx43 antibody. As shown in Fig. 1B, Cx43 could be detected in three different forms, which were not present in untransfected cells. The lower, and most abundant band, corresponds to the non-phosphorylated form of Cx43 (NP), whereas the two slower migrating species, which were most apparent in the Cx43-Y286A mutant, represent the phosphorylated forms, P1 and P2, as confirmed by treatment with calf intestinal phosphatase (Fig. 1B; CIP treatment). Longer exposure also shows the presence of P forms in Cx43-WT-expressing cells (data not shown). Interestingly, the abundance of Cx43 detected in cells transfected with tyrosine-mutated Cx43 was consistently higher than in those transfected with the wildtype. Quantitation of the total pool of Cx43 revealed the increase in Cx43 protein levels to be approximately 3.5-fold (Fig. 2B). A similar result was also observed when the tyrosine was substituted with a phenylalanine (data not shown). This suggested that one of the two overlapping motifs, or both, may control Cx43 steady-state levels. To determine whether the tyrosine residue was important in the context of the PY-motif or the adjacent Y-based motif, we generated a series of substitution mutations around Y286. These included substitution of proline 283 with leucine (Cx43-P283L), glycine 285 with alanine (Cx43-G285A), and valine 289 with aspartate (Cx43-V289D). These constructs were then transiently transfected into SKHep1 cells and Cx43 expression assayed by SDS-PAGE and western blot analysis. As shown in Fig. 2A, the mutation of valine 289, an essential hydrophobic residue within the tyrosine-based sorting signal, mimicked the Y286A mutation. Indeed, quantitative analysis confirmed the increased steady-state expression of Cx43 to be at levels comparable to Cx43-Y286A (Fig. 2B). But mutating the proline residue, a crucial amino acid in the PY-motif, had a less pronounced effect that resulted in a 1.7-fold increase in Cx43 levels. The mutation of glycine 285, which is not required for the sorting signal nor the PY-motif, had no observable effect. We note that mutation of the tyrosine, being part of both the PY-motif and the Y-based motif, showed no additive effect when compared with the valine mutant. Similarly, no additive effect was observed in a P283L/V289D double mutant (data not shown). These results therefore suggest that the tyrosine-based motif is the major element in this region controlling Cx43 protein levels.

The increased expression level of Cx43-Y286A could be due to differences either in the rate of biosynthesis or in the degradation of mutant Cx43, as compared with Cx43-WT. To discriminate between these two possibilities, we developed stable SKHep1 cell lines expressing either Cx43-wildtype or Cx43-Y286A, which was representative of the tyrosine sorting mutations, and examined the turnover of these proteins by pulse-chase analysis. Cells were metabolically labelled for 60 minutes in the presence of [35S]-methionine and then chased for 0, 2, 4 and 6 hours with an excess of unlabelled methionine. Cx43 was then immunoprecipitated and the amount of radiolabelled protein analysed by SDS-PAGE and fluorography. The results, depicted in Fig. 3A, revealed that the degradation of Cx43, harbouring the Y286A mutation, was considerably slower than that of the wild-type Cx43. The two proteins had comparable translation rates as judged by the similar amounts of [35S]-methionine incorporation at time 0. Using this approach, however, Cx43 was predominantly detected as a single band which, according to its predicted molecular weight, corresponds to the NP-form. This suggests that either this assay is not sensitive enough to detect the less-abundant P forms or, alternatively, that immunoprecipitation could not isolate the phosphorylated species. To ensure that the disappearance of the NP form in the pulse-chase experiments represented degradation and not maturation into the phosphorylated forms, we also performed similar experiments in the presence of calf intestinal phosphatases so as to analyse the entire pool of Cx43, and found identical results (data not shown). The [35S]-labelled Cx43 species were quantified in
WT and Cx43-Y286A is highly significant (exponential decay function is shown. ANOVA statistical analysis of chase from four independent experiments fitted using a mono-
percentage of pulse-labelled Cx43 remaining after 0, 2, 4 and 6 hours
fluorographs using a molecular imager FX. A plot of the mean±s.e.m.
Having established that the tyrosine 286 was involved in Cx43
important role in Cx43 turnover.
with alanine results from a decrease in the rate of degradation
state levels brought about by the substitution of tyrosine 286
Collectively, these data show that the increase in the steady-
rate is similar between wild-type and mutant protein.
(Fig. 2B; 3.5-fold), which can be expected if the accumulation
Cx43-Y286A determined in our transient transfection assays
This approximates the increased steady-state abundance of
Effects of proteasomal and lysosomal inhibitors on
connexion turnover
Having established that the tyrosine 286 was involved in Cx43
stability, we next investigated the underlying cellular mechanism. As Cx43 has been shown to be degraded by both
the proteasome and the lysosome (Beardslee et al., 1998; Laing
and Beyer, 1995; Laing et al., 1997; Musil et al., 2000), we
used pharmacological agents to inhibit the function of these
intracellular compartments and measured Cx43 protein levels
by western blot analysis (Fig. 4A). Note that because of the
high connexin levels in Cx43-Y286A-expressing cells, reduced
fluorography has been used on this blot for comparative
analysis with the wildtype. Quantitative analysis of Cx43
expression with these treatments is shown in Fig. 4B.
Treatment of Cx43-WT-expressing cells with the proteasomal
inhibitor lactacystin (Fenteany et al., 1995) resulted in an
approximate 3-fold increase only in the phosphorylated forms
of Cx43 (Fig. 4B; right panel, grey columns). A similar effect
could be observed using MG-132 (Palombella et al., 1994), an
alternative proteasomal inhibitor (data not shown). By contrast,
treatment of wild-type cells with two different lysosomal
inhibitors, namely leupeptin and NH4Cl (Hart et al., 1983),
resulted in an increase in the nonphosphorylated form
compared with the P1 and P2 forms, in the order of 1.5- and
2-fold, respectively (Fig. 4B; left panel, grey columns). These
results are in agreement with the notion that both proteolytic
pathways control the steady-state levels of Cx43.
In contrast to wild-type Cx43, Cx43-Y286A-expressing
cells showed a very different response to proteasomal and
lysosomal inhibitors (Fig. 4A). As previously shown, the ratio
of P:NP forms of Cx43-WT (1:5.6) was already increased in
untreated cells compared with Cx43-Y286A cells (1:2.6), and
quantification further revealed that treatment with lactacystin
had a much less prominent effect on the accumulation of the
P1 and P2 forms (Fig. 4B; right panel, black columns). Most
strikingly though, leupeptin and NH4Cl no longer had an effect
on the steady-state levels of unphosphorylated Cx43 (Fig. 4B;
left panel, black columns). To further confirm this result, pulse-
chase experiments were performed in the presence of NH4Cl.
As shown in Fig. 5A,B, treatment with NH4Cl markedly
reduced Cx43-WT turnover but had only a minor effect on the
more stable mutant Cx43. Quantitative analysis of Cx43
expression levels revealed that the degradation rate of Cx43-
WT (k=0.34 h⁻¹) was significantly reduced (~fivefold) in the
presence of NH4Cl (k=0.07 h⁻¹), as indicated by ANOVA
analysis (P<0.0001). But NH4Cl treatment had a much weaker
effect on the tyrosine mutant, as seen by the calculated
degradation rates (control k=0.12 h⁻¹, NH4Cl k=0.074 h⁻¹). In
contrast to NH4Cl, lactacystin did not affect the turnover of
either Cx43-WT or Cx43-Y286A (data not shown; recall that
only the NP form of Cx43 can be detected in the pulse-chase
experiments). Taken together, these results show that the
differences in Cx43 steady-state levels reflect a differential
degradation of the wild-type and Y286A connexin primarily
by the lysosome. Inhibition of the proteasome appears to affect
mostly the ratio between NP and P forms and, to a much lesser
extent, the total pool of Cx43.

Mutation of tyrosine 286 increases gap junctional
staining
As a next step, we sought to examine the localisation of the
wild-type and Y286A Cx43 within cells. Immunofluorescence
localisation studies were therefore performed on both cell lines

Fig. 3. Tyrosine 286 is involved in Cx43 stability. (A) Pulse-chase
analysis was performed on stably transfected SKHep1 cells
expressing either Cx43-WT or Cx43-Y286A, by pulsing for 60
minutes with [35S]-methionine and then chasing for 0, 2, 4 or 6 hour
periods. Cells were then lysed, Cx43 immunoprecipitated overnight,
and SDS-PAGE and fluorography performed. (B) Pulse-chase
experiments were performed as in A, and the level of
immunoprecipitated [35S]-labelled Cx43 was quantified from
fluorographs using a molecular imager FX. A plot of the mean±s.e.m.
percentage of pulse-labelled Cx43 remaining after 0, 2, 4 and 6 hours
of chase from four independent experiments fitted using a mono-
exponential decay function is shown. ANOVA statistical analysis
indicates that the difference in the degradation curves between Cx43-
WT and Cx43-Y286A is highly significant (P<0.0001).

four different experiments, and measured values were
subjected to ANOVA statistical analysis. As shown in Fig. 3B,
the degradation of Cx43-Y286A was significantly slower
(P<0.0001) compared with that of Cx43-WT, with the half-life
of Cx43 being increased from approximately 2 to 6 hours. The
calculated rate of degradation for Cx43-WT (k=0.34 h⁻¹) was
reduced 2.72-fold on mutation of Cx43-Y286A (k=0.125 h⁻¹).
This approximates the increased steady-state abundance of
Cx43-Y286A determined in our transient transfection assays
(Fig. 2B; 3.5-fold), which can be expected if the accumulation
rate is similar between wild-type and mutant protein.
Collectively, these data show that the increase in the steady-
state levels brought about by the substitution of tyrosine 286
with alanine results from a decrease in the rate of degradation
of the mutant protein, suggesting that this amino acid plays an
important role in Cx43 turnover.

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Effects of proteasomal and lysosomal inhibitors on
connexion turnover
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using an anti-Cx43 polyclonal antibody. Modest Cx43 immunoreactivity was detected in cells transfected with wild-type Cx43 (Fig. 6A; control), both intracellularly and at appositional membranes (indicated by arrows). The staining was strikingly stronger in the mutant cell line, which displayed larger and more abundant gap junctional plaques, as well as vesicular intracellular structures (Fig. 6B; control). We then examined the effects of proteasomal and lysosomal inhibitors on Cx43 localisation. Lactacystin, which specifically increased the phosphorylated forms of Cx43 (Fig. 2), markedly increased the gap junctional staining of Cx43-WT-expressing cells without any appreciable changes in intracellular labelling (Fig. 6A; lactacystin). This is in agreement with the notion that conversion of Cx43 to the P1 and P2 forms occurs after transport to the cell surface (Laird et al., 1995; Musil and Goodenough, 1991; Nagy et al., 1997). By contrast, lactacystin had no effect on the Cx43 labelling in the Y286A cell line (Fig. 6B).

Treatment of Cx43-WT-expressing cells with NH₄Cl gave a very different response than lactacystin. As can be seen in Fig. 6A, NH₄Cl, consistent with its inhibitory effect on lysosomal degradation, markedly increased Cx43 immunoreactivity within intracellular vesicles, which was probably due to the accumulation of undigested Cx43 in lysosomes. Moreover, the appositional staining was virtually abolished, suggesting that NH₄Cl may have some additional effects such as impairing the delivery or the recycling of connexins to the plasma membrane. In sharp contrast, NH₄Cl had no effect on Cx43 staining in the Cx43-Y286A mutant, with strong staining still clearly evident at cell-cell interfaces (Fig. 6B; control). The failure of NH₄Cl to disrupt the Cx43-Y286A-containing plaques may suggest that endocytosis had been affected in the connexin mutant. To further investigate this hypothesis, we used Brefeldin A (BFA), which prevents the delivery of newly synthesised proteins to the cell surface (Lippincott-Schwartz et al., 1991), to follow the fate of the Cx43 pool localised at the plasma membrane. In cells expressing wild-type Cx43, treatment with BFA resulted in a
Tyrosine-based motif of Cx43 controls turnover

Tyrosine 286 affects Cx43 gap junctional communication

To investigate whether mutation within Cx43’s tyrosine-based motif was also associated with a change in gap junctional communication (GJC), intracellular injections of lucifer yellow (LY) were carried out on both SKHep1 stable cell clones. As shown in Fig. 7A (control), both cell clones transferred LY to a similar extent, indicating that Cx43-Y286A can form functional gap junction channels. This was further confirmed by measuring junctional conductance in pairs of cells by the dual patch-clamp approach. No significant difference in electrical coupling between the wild-type and mutant cell clones was detected (Fig. 7B; \( P = 0.064 \)). We cannot exclude, however, the possibility that Cx43-Y286A has a lower permeability for LY and/or a reduced single-channel conductance that is compensated for by a larger number of channels at the cell surface.

Exposure of Cx43-WT and Cx43-Y286A cells to lactacystin

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did not significantly change the extent of dye coupling (Fig. 7A; lactacystin). By contrast, treatment with NH₄Cl had a marked effect on GJC. According to the immunohistochemistry, such a treatment should result in nearly complete loss of junctional staining in the wild-type, preventing LY transfer, while having little effect on the mutant. Indeed, under this condition, GJC in wild-type cells was significantly decreased compared with Y286A-cells, which remained LY competent (Fig. 7; NH₄Cl). Likewise, treatment with BFA had a marked effect on the ability of Cx43-WT cells to transfer LY, whereas diffusion of the fluorescent tracer remained efficient in cells expressing mutant Cx43 (Fig. 7; BFA).

**Discussion**

Data presented in this paper provide novel insights into the understanding of the process of endocytosis as an initial step in Cx43 gap junction channel degradation. Here we propose that a tyrosine-based sorting signal (YKLV) in the C-terminus of Cx43 controls gap junction turnover by affecting internalisation and targeting of Cx43 for degradation in the endosomal/lysosomal compartment.

Our data show that the tyrosine residue at position 286 (Y286) is a crucial amino acid involved in Cx43 turnover. Pulse-chase analysis in stably transfected SKHep1 cells indicates that substitution of Y286 with alanine increases the half-life of Cx43 from approximately 2 to 6 hours. Consistent with this, the steady-state levels of Cx43-Y286A is also elevated by a factor of ~3.5, which can be expected if one assumes equal biosynthesis rates of wild-type and mutant Cx43, which appears to be the case (Fig. 3A; compare time 0 of Cx43-WT with Cx43-Y286A). As outlined above, Y286 is part of two putative protein-protein interaction motifs (Fig. 1A): first, a PY motif (Chen and Sudol, 1995), known to interact with WW domain-containing proteins such as the Nedd4/Nedd4-like family of ubiquitin-protein ligases (Rotin et al., 2000), and second, a tyrosine-based sorting signal (consensus: Yxxφ, where φ is a hydrophobic amino acid), which is part of a family of degenerate motifs involved in the targeting of many transmembrane proteins to different cell compartments (Bonifacio and Dell'Angelica, 1999). Our mutational analysis of the region around Y286 and the subsequent transfection experiments in SKHep1 cells supports the notion that it is primarily the tyrosine motif that determines the stability of Cx43. Mutation of valine 289 to aspartate (V289D) in the tyrosine motif had the same drastic effect on the stability of Cx43 as Y286A, whereas mutation of proline 283 to leucine (P283L), which is part of the PY motif, increased Cx43 stability to a much weaker extent. Importantly, Y286A, being part of the two putative motifs, showed no additive effect on the stability (compared with V289D), as was the case for a double mutant (P283L/V289D) (data not shown). This therefore suggests that the PY motif plays only a limited, if any, role in the control of Cx43 turnover. It is possible, considering the steric properties of proline residues, that the P283L substitution has affected the functionality of the tyrosine-based sorting motif, providing evidence for the comparably small increase in stability of this mutant. Indeed, it has been proposed recently that residues upstream of the crucial tyrosine may be important for the binding of effector molecules to tyrosine sorting motifs (Owen et al., 2001).

Alternatively, we cannot exclude that the PY motif and the tyrosine motif act on the same pathway. The mutagenesis results further revealed that the substitution mutant Y286F behaves in a similar manner to that of the Y286A construct. This indicates that it is the tyrosine itself that is of crucial importance to Cx43 turnover and not its aromatic nature. Interestingly, tyrosine-based sorting signals are remarkably similar to the phospho-tyrosine-based motifs that direct SH2 domain binding (Pawson, 1995; Songyang et al., 1993). On this basis, we cannot exclude an involvement of tyrosine phosphorylation in this effect, especially as phosphorylation of such sorting signals has been shown to control protein localisation by regulating their interaction with the transport machinery (Bradshaw et al., 1997; Schaefer et al., 2002; Shiratori et al., 1997; Stephens and Banting, 1997). There is no evidence, however, that Y286 is a site of Cx43 phosphorylation.

Tyrosine-based sorting signals have been shown to interact with the medium chain (mu) subunits of the adaptor complexes (AP), which are components of the machinery involved in either clathrin-dependent or -independent formation of membrane-bound transport intermediates (e.g. coated vesicles) (Bonifacio and Dell’Angelica, 1999; Hirst et al., 1999; Hirst and Robinson, 1998; Simpson et al., 1996). Interestingly, annular gap junctions (e.g. internalised gap junctions) have been proposed to be clathrin coated (Larsen et al., 1979). Moreover, Cx43 has been shown to colocalise with clathrin (Huang et al., 1996), and has been found in close proximity to clathrin-coated pits within the plasma membrane (Naus et al., 1993), suggesting the possible involvement of a clathrin-mediated pathway in Cx43 trafficking. Therefore, it will be interesting to examine if the YKLV motif exerts its effects by interacting with one of the different adaptor complexes.

Several lines of evidence suggest that Y286 regulates the stability of Cx43 by controlling targeting of Cx43 for lysosomal degradation. Inhibitors of endosomal/lysosomal degradation, such as NH₄Cl and leupeptin, slowed down dramatically the degradation of Cx43-WT. This was accompanied by increased levels of the NP form of Cx43-WT (Fig. 4) and by an intracellular accumulation of the protein in vesicles that probably represent endosomes/lysosomes (Fig. 6). This is consistent with previous reports implicating the lysosomal system in the degradation of Cx43 (Berthoud, 2000; Laing and Beyer, 1995; Laing et al., 1997; Musil et al., 2000; Naus et al., 1993). By contrast, NH₄Cl (Fig. 4) and leupeptin (not shown) displayed only a marginal effect on the degradation rate and did not affect the steady-state levels of Cx43-Y286A, which remained elevated in all conditions (Fig. 4). The fact that NH₄Cl can still repress marginally the decay of the Y-mutant (Fig. 5) suggests that NH₄Cl has some small effects on the degradation mechanisms of Cx43 that are independent of Y286A. Brefeldin A, an inhibitor of transport from the ER to the plasma membrane, affected the cell-surface location and GJC of Cx43-WT, but not of Cx43-Y286A, indicating that mutation of tyrosine 286 may affect the retrieval of Cx43 from the plasma membrane. Our data further show that in transfected SKHep1 cells, the proteasome inhibitors lactacystin and MG-132 have only minor effects on overall stability (Fig. 4), but that they increase the Cx43 P forms in Cx43-WT-expressing cells, and, to a lesser extent, in Cx43-Y286A cells. Consistent with this observation, they also
increase the staining of Cx43-WT at appositional membranes (Fig. 5). Possibly, proteasome inhibitors may inhibit ER-dependent degradation (ERAD), as described previously for Cx32 and Cx43 (Van Slyke et al., 2000; Van Slyke and Musil, 2002), which could lead to an increased export of Cx43 to the cell surface. Alternatively, they may interfere with a direct role of the proteasome in the internalisation of Cx43, as has been described for the growth hormone receptor (van Kerkhof et al., 2000).

Surprisingly, the Y286A mutation increased the level of Cx43 immunoreactivity at cell–cell membrane contacts without affecting the extent of dye coupling. Dual patch-clamp analysis of pairs of Cx43-WT- and Cx43-Y286A-expressing cells confirmed that the two cell clones do not differ in terms of junctional conductance values. One explanation may be that the expression levels achieved in cells transfected with a CMV-driven Cx43 construct are high enough to cause maximal dye coupling that is already seen in Cx43-WT cells. Alternatively, the Cx43-Y286 mutant protein may be less efficient than Cx43-WT in transferring LY, which is compensated for by a larger number of channels at the cell surface. In our SKHep1 clones, no specific information could be obtained on the biophysical properties that may alter the normal behaviour of individual connexin components (Martinez et al., 2002). Despite these possibilities, mutation of Y286 prevented the decrease in GJC by inhibitors of the endosomal/lysosomal degradation pathways that was normally observed in cells expressing wild-type Cx43.

In conclusion, our data show that a tyrosine-based sorting signal present in the C-terminus of Cx43 controls turnover by targeting the protein for lysosomal degradation, thereby regulating the strength of gap junctional communication. The existence of such putative sequences in other connexin genes may suggest a common mechanism for the sorting of some members of the gap junction family. However, tyrosine-based signals are not the only recognised sequences to direct endocytosis and sorting of transmembrane proteins (Hu et al., 2001; Johnson and Kornfeld, 1992; Letourneau and Klausner, 1992; Stroh et al., 1999). Thus, the existence of several mechanisms for the sorting and degradation of gap junction channels made of distinct connexins may play important roles in various pathophysiological situations to maintain and/or modulate specific connexin expression and function at the junctional membrane.

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References

Abriel, H., Loffing, J., Rehun, J. F., Pratt, J. H., Horisberger, J.-D., Rotin, D. and Staub, O. (1999). Defective regulation of the epithelial Na⁺ channel (ENaC) by Nedd4 in Liddle’s syndrome. J. Clin. Invest. 103, 667-673.

Abriel, H., Kamynina, E., Horisberger, J.-D. and Staub, O. (2000). Regulation of the cardiac voltage-gated Na⁺ channel (H1) by the ubiquitin-protein ligase Nedd4. FERS Lett. 466, 377-380.

Beardslee, M. A., Laing, G., Beyer, E. C. and Saffitz, J. E. (1998). Rapid turnover of connexin43 in the adult rat heart. Circ. Res. 83, 629-635.

Berthoud, V. M., Tadros, P. N. and Beyer, E. C. (2000). Connexin and gap junction degradation. Methods 20, 180-187.

Bonifacino, J. S. and Dell’Angelica, E. C. (1999). Molecular bases for the recognition of tyrosine-based sorting signals. J. Cell Biol. 145, 923-926.

Bradshaw, J. D., Lu, P., Leytze, G., Rodgers, J., Schieven, G. L., Bennett, K. L., Linsley, P. S. and Kurtz, S. E. (1997). Interaction of the cytoplasmic tail of CTLA-4 (CD152) with a clathrin-associated protein is negatively regulated by tyrosine phosphorylation. Biochemistry 36, 15975-15982.

Chen, H. I. and Sudol, M. (1995). The WW domain of Yes-associated protein binds a novel proline-rich ligand that differs from the consensus established for SH3-binding modules. Proc. Natl. Acad. Sci. USA 92, 7819-7823.

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

Fenteany, G., Standaert, R. F., Lane, W. S., Chois, S., Corey, E. J. and Schreiber, S. L. (1995). Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification of lactacystin. Science 268, 726-731.

Galetta, G., Derinck, T. J., Adams, S. R., Bouwer, J., Tour, O., Laird, D. W., Sosinsky, G. E., Tsien, R. Y. and Ellisman, M. H. (2002). Multicolor and electron microscopic imaging of connexin trafficking. Science 296, 503-507.

Goodenough, D. A., Goliger, J. A. and Paul, D. L. (1996). Connexins, connexons, and intercellular communication. Annu. Rev. Biochem. 65, 475-502.

Hart, P. D., Young, M. R., Jordan, M. M., Perkins, W. J. and Geisow, M. J. (1983). Chemical inhibitors of phagosome-lysosome fusion in cultured macrophages also inhibit saltryt lysosomal movements. A comparative microscopic and computer study. J. Exp. Med. 158, 477-492.

Hirst, J., Bright, N. A., Roux, B. and Robinson, M. S. (1999). Characterization of a fourth adaptor-related protein complex. Mol. Biol. Cell 10, 2787-2802.

Hirst, J. and Robinson, M. S. (1998). Clathrin and adaptors. Biochim. Biophys. Acta 1404, 173-193.

Hu, W., Howard, M. and Lukaas, G. L. (2001). Multiple endocytic signals in the C-terminal tail of the cystic fibrosis transmembrane conductance regulator. Biochem. J. 354, 561-572.

Huang, X. D., Horackova, M. and Pressler, M. L. (1996). Changes in the expression and distribution of connexin 43 in isolated cultured adult guinea pig cardiomyocytes. Exp. Cell Res. 228, 254-261.

Johnson, K. F. and Kornfeld, S. (1992). A His-Leu-Leu sequence near the carboxyl terminus of the cytoplasmic domain of the cation-dependent mannose 6-phosphate receptor is necessary for the lysosomal enzyme sorting function. J. Biol. Chem. 267, 17110-17115.

Jordan, K., Chodock, R., Hand, A. R. and Laird, D. W. (2001). The origin of annular junctions: a mechanism of gap junction internalization. J. Cell Sci. 114, 763-774.

Kirchhausen, T., Bonifacio, J. S. and Riezman, H. (1997). Linking cargo to vesicle formation: receptor tail interactions with coat proteins. Curr. Opin. Cell Biol. 9, 488-495.

Kumar, N. M. and Gilula, N. B. (1996). The gap junction communication channel. Cell 84, 381-388.

Kwak, B. R., Saez, J. C., Wilders, R., Chanson, M., Fishman, G. I., Hertzberg, E. L., Spray, D. C. and Jongsma, H. J. (1995). Effects of cGMP-dependent phosphorylation on rat and human connexin43 gap junction channels. Pflugers Arch. 430, 770-778.

Laing, J. G. and Beyer, E. C. (1995). The gap junction protein connexin43 is degraded via the ubiquitin proteasome pathway. J. Biol. Chem. 270, 26399-26403.

Laing, J. G., Tadros, P. N., Westphale, E. M. and Beyer, E. C. (1997). Degradation of connexin 43 gap junctions involves both the proteasome and the lysosome. Exp. Cell Res. 236, 482-492.

Laird, D. W. (1996). The life cycle of a connexin: gap junction formation, removal, and degradation. Bioenerg. Biomembr. 28, 311-318.

Laird, D. W., Puram, K. L. and Revel, J. P. (1999). Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes. Biochem. J. 273, 67-72.

Laird, D. W., Castillo, M. and 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.

Tyrosine-based motif of Cx43 controls turnover
Musil, L. S. and Goodenough, D. A. (1991). Biochemical analysis of
Musil, L. S., Le, A. C., VanSlyke, J. K. and Roberts, L. M.
Moreno, A. P., Laing, J. G., Beyer, E. C. and Spray, D. C.
Nelson, R. M. and Long, G. L.
Naus, C. C., Hearn, S., Zhu, D., Nicholson, B. J. and Shivers, R. R.
(1998). A structural explanation for the
Owen, D. J. and Evans, P. R.
Owen, D. J., Setladi, H., Evans, P. R., McEver, R. P. and Green, S. A.
(2001). A third specificity-determining site in mu 2 adaptin for sequences
upstream of Yxx phi sorting motifs.

Pawson, T. (1995). Protein modules and signalling networks. *Nature* 375, 573-578.

Rabie, R., Lopez-Bigas, N., Arbones, M. L. and Estivill, X. (2002). Connexin mutations in hearing loss, dermatological and neurological
disorders. *Trends Mol. Med.* 8, 205-212.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences commonly to rapidly degraded proteins: the PEST hypothesis. *Science* 234, 364-368.

Rotta, D., Staub, O. and Hagenauener-Tsapis, R. (2000). Ubiquitination and endocytosis of plasma membrane proteins: role of Nedd4/Rsp5p family of ubiquitin-protein ligases. *J. Membr. Biol.* 176, 1-17.

Rutz, M. L. and Hulser, D. F. (2001). Supramolecular dynamics of gap junctions. *Eur. J. Cell Biol.* 80, 20-30.

Scharf, W. K., Kamei, Y., Kamiguchi, H., Wong, E. V., Rapoport, I., Kirchhausen, T., Beach, C. M., Landreth, G., Lemmon, S. K. and Lemmon, V. (2002). L1 endocytosis is controlled by a phosphorylation-dependent endocytosis cycle stimulated by outside-in signaling by L1. *J. Cell Biol.* 157, 1223-1232.

Schwabe, M., Friedrich, T. and Jentsch, T. J. (2001). An internalization signal in CIC-5, an endosomal Cl-channel mutated in dent’s disease. *J. Biol. Chem.* 276, 12049-12054.

Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, J. S. and Saito, T. (1997). Tyrosine phosphorylation controls internalization of CTLA-4 by regulating its interaction with clathrin-associated adaptor complex AP-2. *Immunity* 6, 583-589.

Simon, A. M. and Goodenough, D. A. (1998). Diverse functions of vertebrate gap junctions. *Trends Cell Biol.* 8, 477-483.

Simpson, F., Bright, N. A., West, M. A., Newman, L. S., Darnell, R. B. and Robinson, M. S. (1996). A novel adaptor-related protein complex. *J. Cell Biol.* 133, 749-760.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Hase, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* 72, 767-778.

Spray, D. C. (1996). Molecular physiology of gap junction channels. *Clin. Exp. Pharmacol. Physiol.* 23, 1038-1046.

Staub, O., Dho, S., Henry, P. C., Correa, J., Ishikawa, T., McGlade, J. and Rotin, D. (1996). WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle’s syndrome. *EMBO J.* 15, 2371-2380.

Stephens, D. J. and Banting, G. (1997). Insulin dependent tyrosine phosphorylation of the tyrosine internalisation motif of TGN38 creates a specific SH2 domain binding site. *FEBS Lett.* 416, 27-29.

Stroh, A., Schafer, W., Berghofer, S., Eickmann, M., Teuchert, M., Burger, I., Klenk, H. D. and Garten, W. (1999). A mono phenylalanine-based motif (F790) and a leucine-dependent motif (L176) mediate internalization of furin. *Eur. J. Cell Biol.* 78, 151-160.

Traub, O., Drue, P. M. and Willecke, K. (1983). Degradation and resynthesis of gap junction protein in plasma membranes of regenerating liver after partial hepatectomy or cholestasis. *Proc. Natl. Acad. Sci. USA* 80, 755-759.

van Kerkhof, P., Govers, R., Alves dos Santos, C. M. and Strous, G. J. (2000). Endocytosis and degradation of the growth hormone receptor are proteasome-dependent. *J. Biol. Chem.* 275, 1575-1580.

VanSlyke, J. K. and Musil, L. S. (2002). Dislocation and degradation from the ER are regulated by cytosolic stress. *J. Cell Biol.* 157, 381-394.

VanSlyke, J. K., Deschenes, S. M. and Musil, L. S. (2000). Intracellular transport, assembly, and degradation of wild-type and disease-linked mutant gap junction proteins. *Mol. Biol. Cell* 11, 1933-1946.

Varshavsky, A. (1992). The N-end rule. *Cell* 69, 725-735.

Vaughan, D. K. and Lasater, E. M. (1990). Renewal of electrotonic synapses in teleost retinal horizontal cells. *J. Comp. Neurol.* 299, 364-374.

Yamasaki, H. and Naus, C. C. (1996). Role of connexin genes in growth control. *Carcinogenesis* 17, 1199-1213.