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Turnover and phosphorylation dynamics of connexin43 gap junction protein in cultured cardiac myocytes

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Cultured cardiomyocytes were used to study the turnover and post-translational modification of connexin43 (Cx43), a major gap junction protein in neonatal cardiac myocytes. Immunoprecipitation of [^{35}S]Met-labelled lysates with anti-Cx43 antibodies followed by analysis using SDS/PAGE and fluorography revealed two bands, one at 40 kDa and the other at 42 kDa. Alkaline phosphatase treatment of [^{35}S]Met-labelled Cx43 eliminated the band at 42 kDa, suggesting that it represented a phosphorylated form of the protein. This was confirmed by [^{32}P]P_i incorporation into the 42 kDa band, but not into the band at 40 kDa. In addition, another alkaline phosphatase-sensitive phosphorylated form of Cx43 was identified at 44 kDa. In pulse–chase experiments, the half-life of Cx43 in cardiomyocytes was determined to be 1–2 h. Furthermore, the turnover rate of phosphate groups on Cx43 was found to be experimentally defined by the half-life of the protein. The observation that phosphate groups can remain with the protein throughout its life is consistent with the finding that in isolated adult rat heart gap junction plaques, Cx43 is primarily phosphorylated. We postulate that the rapid turnover of Cx43 and its multiple sites of phosphorylation play important roles in the regulation of cell–cell communication via gap junctions.

INTRODUCTION

Gap junction channels consist of hexameric aggregates of individual proteins called connexins which form a group of related gap junction proteins. Hepatocyte junctions have been studied extensively and found to contain both connexin32 (Cx32) and Cx26, but Cx43 is the major gap junction protein in cardiac myocytes. The sequences of the Cx proteins are known (Paul, 1986; Beyer *et al.*, 1987; Zhang & Nicholson, 1989) and their molecular organization in the membrane has been characterized in detail using immunocytochemical and biochemical techniques (Hertzberg *et al.*, 1985; Manjunath *et al.*, 1987; Zimmer *et al.*, 1987; Goodenough *et al.*, 1988; Milks *et al.*, 1988; Beyer *et al.*, 1989; Evans & Rahman, 1989; Yancey *et al.*, 1989; Laird & Revel, 1990).

Earlier studies on the turnover of liver gap junction proteins indicate that, compared with other membrane proteins, liver connexins have relatively rapid turnover rates (Fallon & Goodenough, 1981; Yancey *et al.*, 1981; Traub *et al.*, 1987). Until now, however, the half-life of Cx43 in cardiac myocytes has not been documented. Although there is no direct evidence that gap junction turnover plays a vital role in regulating cell–cell communication, the rapid assembly and disassembly of gap junction structures may be thought, *a priori*, to play an important role in regulating such processes as embryonic development and terminal differentiation (Ginzberg & Gilula, 1979; Dermietzel *et al.*, 1987; Larsen & Wert, 1988).

It has been known for some time that the liver gap junction protein (Cx32) is a substrate for protein kinase C (Takeda *et al.*, 1987) and cyclic AMP-dependent protein kinase phosphorylation (Takeda *et al.*, 1987; Traub *et al.*, 1987; Saez *et al.*, 1986, 1989). In a previous study we demonstrated that Cx43 in isolated junctional plaques from rat heart is also phosphorylated (Laird & Revel, 1990). Recently, a Cx43-like protein in vole fibroblasts was shown to be a phosphoprotein capable of rapid dephosphorylation (Crow *et al.*, 1990). We show here that Cx43 in cardiac myocytes is multiply phosphorylated and that, in this instance, the net turnover of the [^{32}P]P_i label is similar to the half-life of the protein.

MATERIALS AND METHODS

Culture of neonatal cardiac myocytes

Primary cultures of rat neonatal cardiac myocytes were prepared according to the procedure described by Laird & Revel (1990). Approx. (2–5) × 10^5 cells/ml were plated in 60 mm tissue culture dishes (Falcon, Lincoln Park, NJ, U.S.A.) and maintained at 37 °C in an environment of air/CO_2 (19:1). The cultured cells were 90–100% confluent after 2 days.

Radiolabelling of Cx43 gap junction protein *in vitro*

[^{35}S]Met labelling. Confluent cultures of neonatal cardiac myocytes were rinsed four times in methionine-free Dulbecco's modified essential medium (Gibco, Grand Island, NY, U.S.A.) containing 10% (v/v) fetal calf serum (Hyclone, Logan, UT, U.S.A.) prior to methionine starvation for 45 min in the same medium. Methionine-deficient cells were labelled with [^{35}S]Met (100 μCi/ml, specific radioactivity 1000–1200 Ci/mmol; ICN Biomedicals, Irvine, CA, U.S.A.) for 2 h. To initiate the chase, the cells were rinsed three times in complete myocyte culture medium containing 1 mM-Met (Laird & Revel, 1990) and then incubated in the same medium for the specified period of chase times in phosphate-buffered saline (140 mM-NaCl/2.6 mM-KCl/1.4 mM-KH_2PO_4/8.1 mM-Na_2HPO_4, pH 7.4). The myocytes were lysed and the gap junctions were solubilized by incubating the cells in 1.2–1.5 ml of RIPA buffer [10 mM-NaH_2PO_4, 150 mM-NaCl, 2 mM-EDTA, 1% (v/v) Triton X-100, 0.25% SDS, 1% sodium deoxycholate and 2 mM-phenylmethanesulphonyl fluoride, pH 7.2] for 20 min at 4 °C. The lysates were centrifuged at 40000 *g* for 50 min and the supernatants were immunoprecipitated.

[^{32}P]P_i labelling. Cultured 2-day-old cardiac myocytes were rinsed three times and starved in P_i-deficient Dulbecco's modified essential medium (Sigma Chemical Co., St. Louis, MO, U.S.A.) containing 10% (v/v) fetal calf serum for 15 min. P_i-starved cells were labelled with [^{32}P]P_i (100–175 μCi/ml, specific radioactivity 8500–9120 Ci/mmol; New England Nuclear, Wilmington, DE, U.S.A.) for 5 h at 37 °C. In pulse–chase studies the labelled cells

Abbreviation used: Cx, connexin.

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were rinsed three times and incubated for various time intervals in culture medium supplemented with 1.4 mM-NaH_2PO_4. The cells were solubilized and prepared for immunoprecipitation as described above.

Isolation of cardiac gap junction plaques

Gap junction plaques from adult Sprague–Dawley rat hearts were isolated according to the method of Manjunath & Page (1986).

Protein assay

The concentration of protein in the cell lysates was determined using the bicinchoninic acid protein assay reagent (Pierce Chemical Co., Rockford, IL, U.S.A.) with BSA as a standard.

Immunoprecipitation and PAGE

Two site-directed anti-Cx43 antibodies were used for immunoprecipitation studies. One of these was raised against the *C*-terminus of Cx43 (designated CT-360) (Laird & Revel, 1990) and the other against the *N*-terminus (designated AT-2)(Yancey *et al.*, 1989). The cell lysates were immunoprecipitated with 5–10 μl of either preimmune or immune serum for 2–3 h at 4 °C with gentle agitation. Immunoprecipitates were collected by the addition of 100 μl of immobilized Protein A (Pierce) and incubation for 1 h with rocking at 4 °C. The Protein A beads were collected by gentle centrifugation for 3 min and washed twice with buffer containing 0.5% Tween-20, 50 mM-Tris/HCl, pH 7.5, 150 mM-NaCl and 0.1 mM-EDTA and twice with buffer containing 0.5% Tween-20, 100 mM-Tris/HCl, pH 7.5, 200 mM-NaCl and 2 M-urea. After a final wash with water, the beads were solubilized in 2% SDS/5% 2-mercaptoethanol/0.005% Bromophenol Blue/30 mM-Tris/HCl, pH 6.8.

The immunoprecipitates and isolated gap junction membrane preparations were analysed by SDS/PAGE on a 10% (w/v) acrylamide, 0.13–0.27% (w/v) bisacrylamide system (Laemmli, 1970). The gels were stained with Coomassie Blue and destained in 45% methanol/10% acetic acid for a minimum of 15 min. Gels with [^{35}S]Met-labelled samples were treated with Autofluor (National Diagnostics, Manville, NJ, U.S.A.) for 10–15 min before drying. All radiolabelled gels were dried and exposed to X-OMAT AR film (Eastman Kodak Co., Rochester, NY, U.S.A.) which was preflashed for fluorography. For quantification of the Cx43 bands, exposed films or Coomassie Blue-stained gels were scanned on a LKB Ultroscan XL Laser Densitometer and the area units were normalized to an arbitrary scale.

Alkaline phosphatase treatment

The cell lysates were dialysed against two changes of buffer containing 100 mM-Tris/HCl, pH 8.0, 40 mM-NaCl and 0.1% SDS, and a final change against the same buffer containing 0.05% SDS. After dialysis, 0.5 ml of the cell extract was treated with 10 units of alkaline phosphatase from calf intestine (3406 units/mg; Boehringer Mannheim, Indianapolis, IN, U.S.A.). The reaction was carried out at 37 °C for 3 h while being dialysed against the buffer mentioned above containing 0.05% SDS, 1 mM-$MgCl_2$ and 1 mM-$ZnCl_2$.

RESULTS

Immunoprecipitation of [^{35}S]Met-labelled Cx43

In previous studies, several anti-Cx43 site-directed antibodies were characterized and shown to be effective immunoreagents for labelling gap junctional plaques in primary cultures of neonatal cardiomyocytes (Laird & Revel, 1990) and in cardiac tissue sections (Yancey *et al.*, 1989). In order to study the dynamics of Cx43 in cardiac myocytes, lysates of [^{35}S]Met-labelled cells were prepared, subjected to immunoprecipitation with an antibody (CT-360) specific for the *C*-terminal tip of Cx43 and analysed by SDS/PAGE in conjunction with fluorography (Fig. 1*a*). A comparison of the immune (Fig. 1*a*, lane a) and preimmune (Fig. 1*a*, lane b) precipitated cell lysates revealed two intense ^{35}S-labelled bands at 40 and 42 kDa in the anti-Cx43-immunoprecipitated lysate. An additional band at 45 kDa was judged not to be derived from Cx43, as it was also immunoprecipitated by the preimmune serum (Fig. 1*a*, lane b). Most of this 45 kDa protein could be successfully removed from the cell lysate by immunoprecipitation with an anti-actin monoclonal antibody (results not shown). However, some Cx43 signal was lost during this procedure, presumably due to the aggregation of actin and Cx43 during cell lysis. Thus routine preclearing of the cell lysates with either the preimmune serum or the anti-actin antibody was avoided.

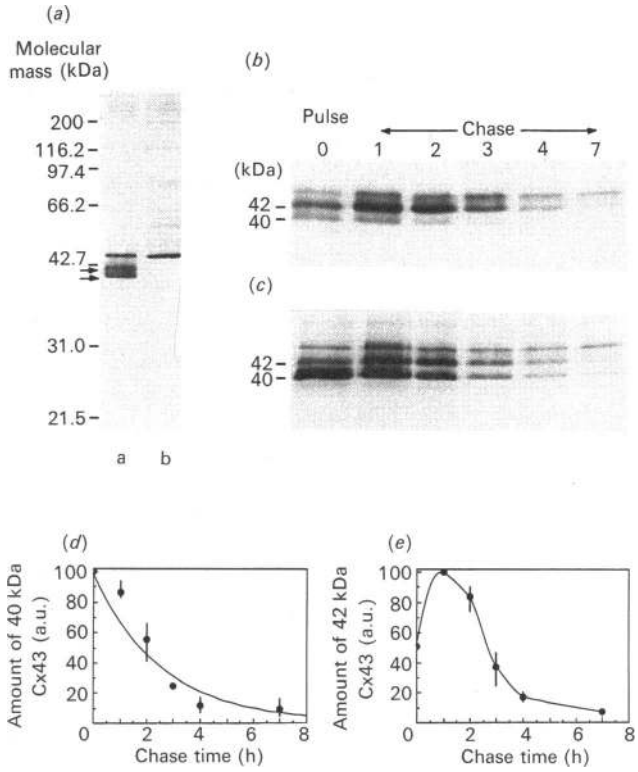

Fig. 1. Turnover of Cx43 in neonatal cardiac myocytes

(*a*) Methionine-starved cardiac myocytes were pulsed with [^{35}S]Met for 2 h, lysed and subjected to immuoprecipitation with CT-360 anti-Cx43 antibody (lane a) or preimmune serum (lane b). The immunoprecipitates were resolved on an SDS/10%-polyacrylamide gel and identified by fluorography. The arrows indicate the 40 and 42 kDa bands. The intense band at 45 kDa found in the immune- and preimmune-precipitated cell lysates represents an actin contaminant, as shown by reactivity against an anti-actin monoclonal antibody. Methionine-starved myocytes were pulsed for 2 h with [^{35}S]Met, washed and chased for 1, 2, 3, 4 or 7 h with culture medium supplemented with 1.0 mM-methionine. The cell lysates were immunoprecipitated with either the CT-360 (*b*) or AT-2 (*c*) antibody before being subjected to electrophoresis on a 10% polyacrylamide gel containing 0.13% bisacrylamide. The turnover times for the CT-360-immunoprecipitated 40 kDa (*d*) and 42 kDa (*e*) forms of Cx43 in cardiac myocytes were determined from densitometric scans of fluorographs of shorter exposure obtained from three separate pulse–chase experiments. The bars represent the range of normalized area unit (a.u.) values for each time point.

Turnover of Cx43 gap junction protein in cardiac myocytes

Primary cultures of myocytes were pulsed with [^{35}S]Met and

then chased with unlabelled methionine as detailed in the Materials and methods section. When the resulting cell lysates were immunoprecipitated with the CT-360 antibody, densitometric scans of the fluorograph revealed two distinct turnover patterns for the 40 kDa and 42 kDa bands (Figs. 1*b*, 1*d* and 1*e*). The turnover of the 40 kDa band was curve-fitted to a single exponential decay with a measured half-life of approx. 2 h (Fig. 1*d*). On the other hand, the 42 kDa band increased in intensity during the first 1 h of chase before decreasing over an additional 6 h chase period, resulting in a half-life of approx. 1–2 h (Figs. 1*b* and 1*e*). A pulse–chase experiment done in the presence of cycloheximide revealed no increase in the intensity of the 42 kDa band at the 1 h time point (results not shown). Consequently the transient increase in the intensity of the 42 kDa band in the absence of cycloheximide is believed to be due to the continuing synthesis of radiolabelled protein for the first 1 h of the chase period.

When a second anti-Cx43 antibody (AT-2) specific for the *N*-terminus of Cx43 was used to immunoprecipitate the Cx43 protein from [^{35}S]Met-labelled cell lysates, similar 40 and 42 kDa bands were identified, as was the actin band at 45 kDa (Fig. 1*c*). In comparison with the CT-360 antibody, the AT-2 antibody preferentially immunoprecipitated more of the 40 kDa band than the 42 kDa band (Fig. 1*c*). However, irrespective of the antibody used for immunoprecipitation (CT-360 or AT-2), densitometric scans of pulse–chase experiments showed that the half-lives for both the 40 and 42 kDa forms of Cx43 were similar within the range of experimental error.

[^{32}P]P_i labelling of Cx43

The constant occurrence of at least two forms of Cx43 in cultured neonatal cardiac myocytes was thought to be the result of post-translation modifications. Since Cx43 is phosphorylated in adult cardiac gap junction plaques (Laird & Revel, 1990), we sought to establish if phosphorylation could account for the multiple bands that were consistently observed when Cx43 was immunoprecipitated from ^{35}S-labelled neonatal cardiac myocytes. [^{32}P]P_i-labelled cell lysates were immunoprecipitated with the CT-360 anti-Cx43 antibody (Fig. 2*a*, lane a) as well as with preimmune serum (Fig. 2*a*, lane b). Cx43 was found to be multiply phosphorylated, with two major radiolabelled bands resolved at 42 and 44 kDa (Fig. 2*a*, lane a). Furthermore, the same two bands were specifically immunoprecipitated with an antibody (AT-2) raised against the *N*-terminus of Cx43 (Fig. 2*b*, lane a) but not by its corresponding preimmune serum (Fig. 2*b*, lane b). Thus proteolysis was not responsible for these two forms of Cx43, as both the 42 and 44 kDa bands had intact *N*- and *C*-termini. The data clearly demonstrated that Cx43 is a phosphoprotein with multiple sites of phosphorylation.

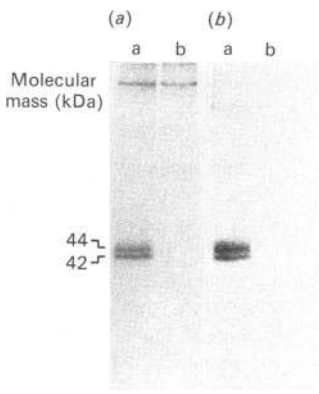

Fig. 2. Metabolic incorporation of [^{32}P]P_i into Cx43

Phosphate-starved cardiac myocytes were exposed to [^{32}P]P_i-containing medium for 5 h before being lysed in RIPA buffer. Cell lysates were immunoprecipitated with (*a*) CT-360 or (*b*) AT-2 immune (lane a) or preimmune (lane b) serum.

Comparison of [^{35}S]Met- and [^{32}P]P_i-labelled bands

Upon careful alignment of the immunoprecipitated [^{32}P]P_i-labelled bands (Fig. 3, lane a) and the [^{35}S]Met-labelled bands (Fig. 3, lane c), it became clear that the ^{35}S-labelled band at 40 kDa did not incorporate any P_i. Thus this band may represent the non-phosphorylated form of Cx43. The major form of Cx43 at 42 kDa was labelled with both [^{35}S]Met and [^{32}P]P_i (Fig. 3, lanes a and c). However, it is not clear if the [^{32}P]P_i-labelled Cx43 band at 44 kDa is also labelled with [^{35}S]Met due to its close proximity to the intense non-Cx43 band at 45 kDa, which we believe to be primarily actin.

Alkaline phosphatase treatment of radiolabelled Cx43

Confirmation that Cx43 is a phosphoprotein was obtained by treating radiolabelled Cx43 with alkaline phosphatase. The two [^{32}P]P_i-labelled bands at 42 and 44 kDa were completely eliminated by phosphatase treatment (Fig. 3, lane b). In addition, when [^{35}S]Met-labelled Cx43 was subjected to alkaline phosphatase, the major band at 42 kDa disappeared, with a substantial increase in the band at 40 kDa and the appearance of a minor band at 41 kDa (Fig. 3, lane d). It is likely that the 41 kDa band represents a partially dephosphorylated form of Cx43 due to the fact that this band did not appear in more complete digestions. Similar results were obtained when the AT-2 antibody was used to immunoprecipitate the Cx43 protein from alkaline phosphatase-treated [^{35}S]Met labelled cell lysates (results not shown).

Dynamics of Cx43 phosphorylation

Studies have suggested that phosphorylation and dephosphorylation may play an important role in gap junction regulation (Wiener & Loewenstein, 1983; reviewed by Murray & Gainer, 1989). Thus it was of interest to perform pulse–chase experiments in an attempt to determine the turnover rate of phosphate groups on Cx43 gap junction protein in cultured cardiac myocytes. Immunoprecipitation in conjunction with autoradiographic and densitometric techniques revealed that the half-life of the phosphate groups on both the 42 and 44 kDa

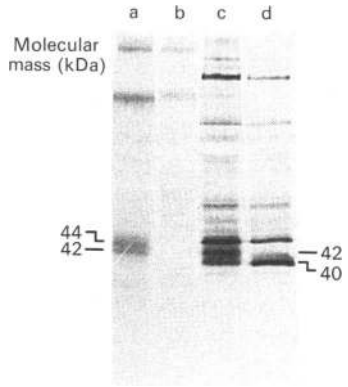

Fig. 3. Alignment of [^{32}P]P_i- and [^{35}S]Met-labelled Cx43 polypeptide bands

Lysates from [^{32}P]P_i-labelled cardiomyocytes were immunoprecipitated with CT-360 antibody (lane a) or treated with alkaline phosphatase before immunoprecipitation (lane b). Likewise, untreated (lane c) or alkaline phosphatase-treated (lane d) lysates from [^{35}S]Met-labelled myocyte cultures were immunoprecipitated with CT-360 antibody.

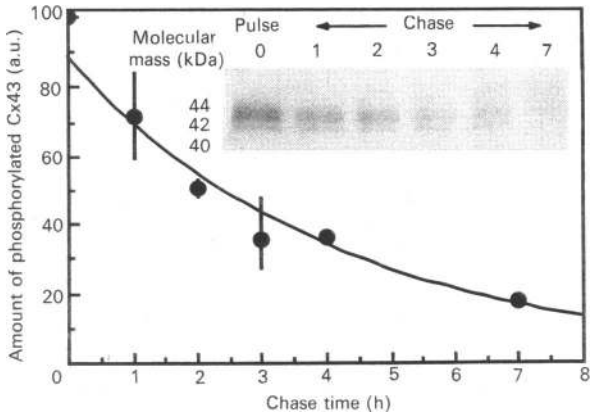

Fig. 4. Dynamics of Cx43 phosphorylation

Phosphate-starved cells were labelled with [^{32}P]P_i for 5 h and chased for 1, 2, 3, 4 or 7 h with culture medium containing 1.4 mM-NaH_2PO_4. Cell lysates were immunoprecipitated with CT-360 antibody and subjected to SDS/PAGE and autoradiography (see insert). Densitometric scans from three pulse–chase experiments were used to determine the turnover of phosphate on Cx43. The bars represent the range of normalized area units (a.u.) for each time point. The line drawn through the points represents a best-fit single-exponential decay.

forms of Cx43 was approx. 2 h (Fig. 4). In essence, the turnover rate of phosphate groups on Cx43 is limited by the half-life of the protein. A similar half-life for the Cx43 phosphate groups was obtained when the cells were labelled with [^{32}P]P_i for 1 h only before chasing with excess phosphate. Under these conditions, the radiolabelled pool of intracellular phosphate was separated by t.l.c. and shown to turn over three times faster than the phosphorylated forms of Cx43 (results not shown).

Extent of Cx43 phosphorylation in adult gap junction plaques

We chose to determine the extent of Cx43 phosphorylation in heart gap junction plaques, as these structures are believed to contain the bulk of the Cx43 protein (Yancey *et al.*, 1989; Aoumari *et al.*, 1990; Laird & Revel, 1990) and represent areas of cell–cell coupling. When isolated adult heart gap junction plaques were separated on a high-resolution SDS/polyacrylamide gel, a broad band at 43 kDa, two minor bands at 41 and 40 kDa, and bands at 35, 33 and 31 kDa were resolved (Fig. 5). In a previous study we showed that several anti-Cx43 antibodies bound to the broad spectrum of bands between 40 and 43 kDa as well as to the three breakdown products at 35, 33 and 31 kDa (Laird & Revel, 1990). In addition, when gap junctions were treated with alkaline phosphatase, the bands at 43 and 41 kDa are decreased in size to 40 kDa. Thus in adult heart gap junction plaques the 40 kDa band represents the non-phosphorylated form of the protein. Based on densitometric scans, only 7.5% of the total undegraded form of the Cx43 protein in gap junction plaques is in the 40 kDa unphosphorylated form (Fig. 5). The bulk of the Cx43 protein in gap junction plaques is either moderately phosphorylated as a 41 kDa band (15.1% of the total) or extensively phosphorylated as a broad band at 43 kDa (77.2% of the total). Such a distribution of phosphorylated Cx43 cannot be confirmed in cultured cardiomyocytes, since junctional plaques have not yet been isolated from this system. The similarity of half-lives measured using [^{32}P]P_i and [^{35}S]Met labelling techniques suggests that Cx43 in junctional plaques of cardiomyocytes is also mostly in the phosphorylated form.

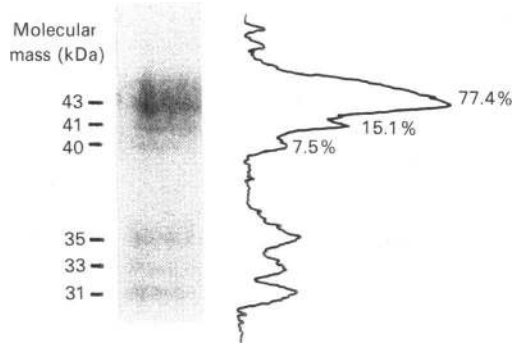

Fig. 5. Extent of Cx43 phosphorylation in adult cardiac gap junction plaques

Cx43 in isolated gap junction plaques was solubilized and separated on SDS/10%-polyacrylamide gel containing 0.4% bisacrylamide. Coomassie Blue-stained Cx43 bands were subjected to densitometric scans and the relative amount of intact Cx43 in each band was determined (%).

DISCUSSION

Turnover of Cx43

In this study we have determined the half-life of Cx43 in cultured neonatal cardiac myocytes using a pulse–chase protocol. ^{35}S-labelled Cx43 was successfully immunoprecipitated at different time intervals, and the half-life of Cx43 was determined to be 1–2 h. It had been previously speculated, on the basis of morphometric analysis of cultured adult cardiomyocytes, that the half-life of the cardiac gap junction protein would be longer than those of its counterparts in the liver (Severs *et al.*, 1989). This hypothesis is not supported by our data, as our results give a half-life similar to that determined by Traub *et al.* (1987) for the liver 26 kDa gap junction protein in cultured hepatocytes (2–3 h). Thus one can speculate that the members of the connexin family of proteins may actually all exhibit similar half-lives *in vitro*. Although only a few gap junction protein turnover studies have been performed in the whole animal, the results suggest that, at least in the liver, connexins *in vivo* also have a relatively short half-life (Fallon & Goodenough, 1981; Yancey *et al.*, 1981).

It has been observed that proteins that possess proline, glutamic acid, serine and threonine (PEST)-rich regions are subject to short half-lives (Rechsteiner, 1988). Upon close examination of the Cx43 polypeptide sequence, no classic PEST-rich regions were observed. However, Cx43 does possess two putative PEST-like regions (residues 272–285 and 327–340) on the *C*-terminus which may account for the rapid turnover of the protein. Although no direct evidence exists for the involvement of Cx43 biosynthesis and degradation in gap junction regulation, the rapid rate of protein turnover suggests that it could play an important part in modulating cell–cell communication in response to physiological stimuli.

Differential immunoprecipitation of [^{35}S]Met-labelled Cx43

The AT-2 antibody preferentially immunoprecipitated more of the 40 kDa as opposed to the 42 kDa form of Cx43 from [^{35}S]Met-labelled cell lysates. The CT-360 antibody, in contrast, often immunoprecipitated more of the 42 kDa than the 40 kDa form of Cx43. Nevertheless, both antibodies appear to recognize the same two forms of Cx43. This preferential binding of the two site-directed antibodies to the different forms of Cx43 may be related to the accessibility of their respective epitopes in the solubilization buffer (i.e. the *C*-terminus epitope is less exposed to the CT-360 in the 40 kDa form of the protein as compared

with the 42 kDa form). An alternative explanation for these differential immunoprecipitation results could be due to the existence of a similar 40 kDa heart gap junction protein that is recognized by the AT-2 antibody but not by the CT-360 antibody. Although a second heart gap junction protein has not been clearly identified, the mRNA for a Cx46 gap junction protein has been detected in cardiac tissue, and this protein has been shown to have an *N*-terminal sequence similar to that of Cx43 (Beyer *et al.*, 1988).

Cx43 is a phosphoprotein

(*a*) Proteolysis is not responsible for the multiple forms of Cx43. Immunoprecipitation of ^{35}S-labelled cardiac myocytes demonstrated that there are at least two forms of Cx43, one at 40 kDa and the other at 42 kDa. On the assumption that the 42 kDa form of Cx43 may represent a phosphorylated form of the protein, the radiolabelled cell lysate was digested with alkaline phosphatase and subsequently immunoprecipitated. The increase in the intensity of the 40 kDa band at the expense of the 42 kDa band is consistent with the dephosphorylation of Cx43. The intermediate 41 kDa band which is occasionally observed under these conditions reflects another phosphorylated form of Cx43 in which the phosphate-containing amino acid residues reside in molecular positions which are relatively inaccessible to the phosphatase. It is highly unlikely that the 40 kDa form of Cx43 that we observe before or after alkaline phosphatase digestion is a proteolytic degradation product of the 42 kDa protein, since site-directed antibodies against both the *N*- and *C*-termini of the Cx43 molecule still detect their epitopes. In addition, nicking by proteinases at the extreme ends of the molecules would not be likely to produce such a change in molecular mass.

(*b*) Incorporation of [^{32}P]P_i into Cx43. Confirmation that Cx43 is a phosphoprotein was provided by the incorporation of radiolabelled P_i into Cx43. Two labelled forms of Cx43, at 42 and 44 kDa, were consistently immunoprecipitated with the CT-360 antibody. It appears that the 40 kDa band seen by [^{35}S]Met labelling procedures represents the essentially non-phosphorylated form of the protein. The band at 42 kDa could be labelled with both [^{35}S]Met and [^{32}P]P_i, indicating that this band represents one phosphorylated form of the protein. A second phosphorylated form of Cx43 observed by [^{32}P]P_i labelling at 44 kDa either did not take up [^{35}S]Met or the corresponding ^{35}S-labelled band was not resolved due to its close proximity to the major non-Cx43 band at 45 kDa. One also has to consider the possibility that the 44 kDa band represents a form of Cx43 that has a slow turnover. If this were so, the 2 h period used to label Cx43 with [^{35}S]Met might be insufficient to adequately label this form of the protein. However, attempts to clearly resolve a [^{35}S]Met-labelled Cx43 band at 44 kDa from the non-Cx43 band at 45 kDa failed even when labelling periods were increased to as much as 12 h (results not shown). Yet another possibility is that the antibody used to immunoprecipitate the Cx43 protein (CT-360) may not be as effective in immunoprecipitating this highly phosphorylated (44 kDa) form of the protein. Although concern for this latter possibility stems from the knowledge that the CT-360 antibody was raised against a serine-rich segment of Cx43 (Laird & Revel, 1990), the fact that similar results were obtained with an antibody (AT-2) raised against the *N*-terminus of Cx43 would strongly suggest that phosphorylation does not inhibit CT-360 binding.

The identification of three forms of Cx43 in cardiomyocytes is similar to observations made in vole fibroblasts (Crow *et al.*, 1990). These authors demonstrated that the 45 and 47 kDa forms of the protein are phosphorylated and sensitive to alkaline phosphatase digestion. In our culture system, as far as can be ascertained by immunofluorescent labelling, Cx43 was found only in cardiac myocytes and not in the contaminating fibroblasts or endothelial cells (Laird & Revel, 1990). In any case, we found that the vole Cx43-like protein and our cardiomyocyte-derived gap junction protein behaved differently (see below).

Sites and kinases involved in Cx43 phosphorylation

Early sequence analysis of gap junction proteins resulted in speculation that these proteins (in particular Cx32 and Cx43) are suitable substrates for various protein kinases (Paul, 1986; Beyer *et al.*, 1987). To date, most of the evidence concerning the extent and role of phosphorylation has been confined to Cx32. Protein kinase C and cyclic AMP-dependent protein kinase have both been shown to phosphorylate Cx32 (Saez *et al.*, 1986; Traub *et al.*, 1987; Takeda *et al.*, 1987). In adult cardiac tissue it is known that the Cx43 gap junction protein is also phosphorylated (Laird & Revel, 1990). In this report we have unequivocally shown that Cx43 in cardiac myocytes is a phosphoprotein. In addition, Crow *et al.* (1990) have shown that the Cx43-like protein in vole fibroblasts is phosphorylated almost exclusively on serine residues. Based on labelling studies with P_i, the dynamics of phosphate turnover on both the 42 and 44 kDa forms of Cx43 yields a half-life of approx. 2 h, which is quite similar to the turnover time of the polypeptide backbone of the protein. This would suggest that once a Cx43 protein becomes phosphorylated it remains phosphorylated for the duration of its life. This is consistent with the observation that the apparent molecular mass of the [^{35}S]Met-labelled 42 kDa band is not decreased during the chase period, which would be expected if the protein was being rapidly dephosphorylated. It is possible that a pool of slowly turning over [^{32}P]P_i-labelled ATP or phosphocreatine was generated during the pulse period, thus resulting in the continual rephosphorylation of Cx43 with a radiolabelled phosphate group. This possibility cannot be completely ruled out; however, our results suggest that the turnover of the phosphate groups on Cx43 in these cardiac muscle cells is significantly longer than the half-life of the intracellular radiolabelled pool.

These results were markedly different from the observations for the Cx43-like protein in vole fibroblasts, where the process of phosphorylation–dephosphorylation was shown to occur within 30 min (Crow *et al.*, 1990). The consequence of a once-only phosphorylation and rapid dephosphorylation of the Cx43-like protein suggests that, in vole fibroblast gap junction plaques, the protein would be essentially in a dephosphorylated form. We have shown here that in adult cardiac gap junction plaques the protein is essentially all in a phosphorylated form (92.5%), and our turnover studies suggest strongly that it would be primarily phosphorylated in cultured cardiomyocyte gap junction plaques. It would thus appear that Cx43 in cardiac cells is under the control of different phosphorylation mechanisms from that observed for the Cx43-like protein in vole fibroblasts, which may in turn be related to differences between immortal cell lines which express Cx43 and primary cardiomyocyte cultures or cardiac tissue.

It remains unclear as to whether Cx43 in cardiac myocytes is phosphorylated by different kinases or by one kinase at several sites. Analysis of the primary sequence of Cx43 reveals that the polypeptide chain has consensus sites for protein kinase C and possibly for Ca^{2+}/calmodulin-dependent protein kinase (Edelman *et al.*, 1987), but no clear consensus sites for cyclic AMP-dependent protein kinase. Virtually all of these possible phosphorylation sites are located on the *C*-terminus of the Cx43 molecule.

Role of Cx43 phosphorylation

It has been shown, principally by indirect methods using

tumour promoters, that increased protein kinase C activity plays an important role in down-regulating gap junctions (Enomoto *et al.*, 1981; Yancey *et al.*, 1982; Fitzgerald *et al.*, 1983). Cyclic AMP has been shown by many investigators to up-regulate gap junction communication (Flagg-Newton *et al.*, 1981; Loewenstein, 1981; Azarnia *et al.*, 1981; Radu *et al.*, 1982; Wiener & Loewenstein, 1983; Kanno *et al.*, 1984; Traub *et al.*, 1987; Saez *et al.*, 1989). In most cases the evidence for the role of protein kinases was confined either to cells which expressed the Cx32 protein or to cells in which the connexin responsible for communication was unknown. Nevertheless, sequence and structural similarities among the various connexins allow one to speculate that Cx43 may also be regulated by phosphorylation.

An important issue that has remained virtually unexplored is whether phosphorylation affects gap junction gating directly or whether it serves to prevent or excite down-regulation (turnover) of the connexin protein. In cholesterol synthesis, for example, the phosphorylation of 3-hydroxy-3-methylglutaryl CoA reductase has been shown to act as a signal for enzyme degradation (Miller *et al.*, 1989). In a recent report, Saez *et al.* (1989) showed that cyclic AMP delayed the uncoupling of gap junctions in rat hepatocytes, possibly by decreasing the removal of junctional protein from the membrane. We are now in a position to determine not only the kinase(s) that are involved in phosphorylating Cx43, but also the effect that each may have on cardiac myocyte cell–cell communication. It is likely that different phosphorylation sites will control various aspects of gap junction metabolism and function.

Note added in proof (received 12 November 1990)

Phosphoamino analysis of Cx43 in rat cardiomyocytes has revealed that the protein is phosphorylated on serine residues, with no detectable phosphate on threonine or tyrosine (D. W. Laird, K. L. Puranam & J.-P. Revel, unpublished work).

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