Inhibition of Glycosylation Induces Formation of Open Connexin-43 Cell-to-Cell Channels and Phosphorylation and Triton X-100 Insolubility of Connexin-43*

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We transfected the cDNA for the cell-to-cell channel protein connexin-43 (Cx43) into Morris hepatoma H5123 cells, which express little Cx43 and lack gap junctional communication (open cell-to-cell channels). We found that cells overexpressing Cx43 nonetheless lacked open cell-to-cell channels, but that inhibition of glycosylation by tunicamycin induced open channels in these cells.

Tunicamycin also induced biochemical changes in Cx43 protein; the level increased, and a considerable fraction became phosphorylated and Triton X-100 insoluble, in contrast to untreated cells where Cx43 was non-phosphorylated and Triton X-100 soluble. Although tunicamycin caused the formation of open channels, channels were not found aggregated into gap junctional plaques, as they are when they have been induced by elevation of intracellular cAMP. The results suggest that although Cx43 itself is not glycosylated, other glycosylated proteins influence Cx43 posttranslational modification and the formation of Cx43 cell-to-cell channels.

Cell-to-cell channels mediate intercellular communication by providing a direct pathway for the exchange of molecules up to 1–2 kDa (Schwarzmann et al., 1981). The molecules transferred include signaling molecules, which may play important roles in tissue homeostasis (Loewenstein, 1981), cell growth control (Loewenstein and Rose, 1992), and embryonic development (Warner, 1992). The channels are known to cluster into often quite large aggregates, forming the so-called gap junctions. In electron microscopic images of freeze-fractured gap junctions, the channels show up as particles of uniform size that span the two adjoining membranes (Krautziger, 1968; Goodenough and Revel, 1970). The channels are formed from membrane proteins called connexins (Beyer et al., 1990; Kumar and Gilula, 1992). More than a dozen different connexins have been identified in vertebrates, all of which share similar topology and amino acid sequence (Kumar and Gilula, 1992). One well studied and widely expressed connexin is connexin-43 (Cx43), found in the heart (Beyer et al., 1987) and many other tissues (Beyer et al., 1987; Micevych and Abelson, 1991) as well as in a number of established cell lines (Musil et al., 1990; Mehta et al., 1992).

Progress has been made in understanding how cell-to-cell channels are formed. For example, it is now known that after synthesis, Cx43 (and probably the other connexins, too, see Rahman et al. (1993)) is first assembled into multimers called connexons or hemichannels in the Golgi (Musil and Goodenough, 1993); the hemichannels are then transported to the plasma membrane where they find counterparts on the adjoining cell’s membrane to form the cell-to-cell channels proper. Yet, little is known about how the cell-to-cell channels are concentrated into the gap junction plaques. In some cells, a phosphorylated and Triton X-100 insoluble form of Cx43, but not its non-phosphorylated and Triton X-100 soluble form, is localized in the gap junctional plaques (Musil and Goodenough, 1991). It is not clear whether Cx43 becomes Triton X-100 insoluble as a consequence of hemichannel interlocking, i.e. channel formation, or as a consequence of the clustering into gap junction plaques. Neither is it clear what role phosphorylation has in Cx43 channel or gap junction formation.

We have found previously that inhibition of glycosylation by tunicamycin (Tm) greatly increases the formation of open channels in a variety of Cx43-expressing cells (Wang and Mehta, 1995). In the present study, we investigate whether any biochemical changes in Cx43 are associated with Tm-induced channel formation. For this, we constitutively expressed Cx43 in a cell line that lacks open cell-to-cell channels and studied the effects of tunicamycin on cell communication via Cx43 channels and on phosphorylation, Triton X-100 solubility, and cellular localization of Cx43.

EXPERIMENTAL PROCEDURES

Materials—All culture media were from Life Technologies, Inc.; fetal bovine serum was from HyClone Laboratories (Logan, UT). Lucifer Yellow CH was from Molecular Probes, and forskolin was from Calbiochem. Phosphodiesterase inhibitor Ro-20-1724 was a gift from Dr. P. Sorter (Hoffman-LaRoche). Rhodamine- or fluorescein isothiocyanate-labeled goat antirabbit IgG and alkaline phosphatase were from Boehringer Mannheim; rhodamine-labeled lectins, wheat germ agglutinin, and Dolichos biflorus agglutinin were from EY laboratory (San Mateo, CA); all other reagents (molecular biology grade or highest purity) were from Sigma.

Cell Culture—A subclone (MHD1) of Morris hepatoma H5123 cells (Borek et al., 1969) was isolated based on its communication-enhance ment response to elevation of cAMP. Cells were grown as described previously (Wang and Mehta, 1995).

Transfection of Cells—The expression construct (pSGRcx43A) was made by inserting c043 cDNA from rat heart (Beyer et al., 1987) into the BamHI site of plasmid pSG5 (Stratagene) as described in Mehta et al. (1991). In pSGRcx43A, c043 expression is driven by the SV40 promoter. Subconfluent cells were harvested and cotransfected by electroporation with pSGRcx43A and the expression vector for the genidin resistance.
gene, pMC1neo-polyA (Stratagene). Cells were then cultured in medium containing 400 μg/ml genetin (G418), and G418-resistant colonies were isolated, propagated in 200 μg/ml G418, and screened by Western blots for the expression of Cx43 protein.

Treatments—Cells were seeded at 2 × 10^5/35-mm dish. 2 days later, when near confluent, they were treated for experiments by replacing their medium with fresh medium containing the relevant drug at the desired concentration. Forskolin, Ro-20–1724, and tunicamycin were added from stock solutions in dimethyl sulfoxide (Me₂SO), with final Me₂SO not exceeding 0.4%, a concentration which did not affect any of the parameters we measured. For controls, cultures received fresh medium containing 0.4% Me₂SO.

Cell-Cell Transfer of Lucifer Yellow—Micro-injection of fluorescent dye Lucifer Yellow was performed as described (Wang and Mehta, 1995). The number of fluorescent cells (excluding the injected one) was noted 5 min after injection.

Immunostaining and Lectin Binding to Cell Surface—Immunostaining with affinity-purified anti-Cx43 antibody and surface binding of rhodamine-labeled lectins were performed as described (Wang and Mehta, 1995). Stained cells were viewed on a Nikon Diaphot fluorescence microscope with a 100× oil immersion objective (for Cx43 immunostaining) or 40× objective (for lectin binding). Images were photographed or captured on an optical disk (Panasonic model TQ-2026F) with an ST66 (DAGE MTI) video camera and reproduced on a video printer (Hitachi).

Western Blot—Lysis of cells, protein separation by SDS-PAGE, and Western blot analysis for Cx43 were performed as described (Wang and Mehta, 1995). The protein concentration was determined with the Pierce BCA protein assay.

De-phosphorylation of Cx43 by Alkaline Phosphatase—After appropriate treatment, cells were lysed in alkaline phosphatase buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 5 mM MgCl₂ plus 2 mM PMSF and 0.6% SDS). The lysates were boiled, sheared with 27-gauge needles, and diluted with 4 volumes of alkaline phosphatase buffer. Half of the sample was treated with alkaline phosphatase (200 units/ml sample) at 37°C for 4 h, and the other half was incubated untreated. The reaction was terminated by adding Laemmli sample buffer and boiling for 5 min. Phosphatase-treated and untreated samples were separated by SDS-PAGE and analyzed by Western blot.

Separation of Triton X-100 Soluble and Insoluble Fractions—The separation of Triton X-100 soluble and insoluble material was done essentially according to the method of Musil and Goodenough (1991). After appropriate treatment, cells from 6-cm dishes were scraped into 4 ml of phosphate-buffered saline containing 2 mM PMSF, 10 mM NaF, 10 mM NEM. Cells were then spun down, resuspended in 1 ml lysis buffer (5 mM Tris base, 5 mM EGTA, 5 mM EDTA plus 2 mM PMSF, 10 mM NaF, 10 mM NEM), incubated at 4°C for 10 min, and disrupted by passing through a 25-gauge needle 25–30 times. The resulting cell lysates were brought to isotonicity by addition of 100 mM of phosphate-buffered saline containing 2 mM PMSF, 10 mM NaF, 10 mM NEM, incubated at 4°C for 10 min, and disrupted by centrifugation, the supernatant (Triton X-100 soluble fraction) was carefully removed. 4× Laemmli buffer was added to it and the total cell lysate to a final 1× concentration. The pellet (Triton X-100 insoluble fraction) was resuspended with 1067 μl of lysis buffer containing 2 mM PMSF, 1× phosphate-buffered saline, 1% Triton X-100, and 1× Laemmli buffer. Equal volumes of total, Triton X-100 soluble, and insoluble proteins were separated by SDS-PAGE, and Cx43 was analyzed by Western blot.

RESULTS

Overexpression of Cx43—We used Morris hepatoma H5123 cells (Borek et al., 1969), which express a low level of Cx43 mRNA but neither Cx26 nor Cx32 mRNA (Mehta et al., 1992). We transfected a subclone of Morris hepatoma cells, MHD1 (see “Experimental Procedures”), with cx43 cDNA from rat heart (Beyer et al., 1987). Several overexpressing clones were obtained, and three of them (MHD1–43A, -B, and -C) were used in this study. They all gave the same results.

The expression of Cx43 protein in MHD1 cells and in one Cx43 overexpressing clone, MHD1–43A, is shown in Fig. 1A. The MHD1–43A cells express Cx43 abundantly, manyfold higher than the parental MHD1 cells. And like in the parental cells, Cx43 in the MHD1–43A cells is mainly in the non-phosphorylated form, appearing as one major band of 42 kDa on SDS-PAGE (Fig. 1A, lane 1; see also Fig. 1C, lane 1).

Connexin-43 Overexpression Induces Few Open Channels—Morris hepatoma cells lack gap junctional communication (open cell-to-cell channels) (Borek et al., 1969) and so do the cells of the subclone MHD1 (Wang and Mehta, 1995). We tested junctional communication in three clones of Cx43 overexpressing cells by micro-injecting the channel-permeable fluorescent tracer, Lucifer Yellow. Despite their high level of Cx43 protein, the overexpressors have few open channels; in most cases, the tracer remained confined to the injected cells. A typical example for MHD1–43A cells is shown in Fig. 2A (see also Fig. 2B).

Inhibition of Glycosylation by Tunicamycin Reduces Surface Carbohydrates and Induces the Formation of Open Channels—Surface carbohydrates may have an inhibitory effect on channel formation (Lin and Levitan, 1987; Levine et al., 1991), and a reduction of surface carbohydrates by inhibition of glycosylation has been found to correlate with increased channel formation in several cell types (Wang and Mehta, 1995). We examined the effect of a glycosylation inhibitor, tunicamycin (Tm) on the abundance of surface carbohydrates and on communication in the Cx43 overexpressers. As in parental MHD1 cells (Wang and Mehta, 1995), Tm greatly reduced surface carbohydrates as detected by lectin binding to the cell surface (Fig. 3). Concomitant with this decrease, there was a dramatic increase in communication; after an 8-h tunicamycin treatment, Lucifer Yellow consistently transferred to more than 20 neighboring cells (Fig. 2A, panel d, and 2B). The time course of the communication increase in clones MHD1–43A, -B, and -C is shown in Fig. 2B. After a 2-h delay, communication rose steadily to a maximum at 8–10 h. This result is different from that obtained with the parental MHD1 cells where tunicamycin per se failed to induce communication (Wang and Mehta, 1995).

Tunicamycin has been reported to inhibit protein synthesis (Elbein, 1987), and inhibition of general protein synthesis may somehow increase communication (Azarnia et al., 1981). To test whether tunicamycin’s effect on communication could be due to general inhibition of protein synthesis, we treated MHD1–43A cells with the protein synthesis inhibitor, cycloheximide. As seen in Fig. 2B, communication changed little, showing that the effect of tunicamycin cannot be explained by general inhibition of protein synthesis.

Tunicamycin Treatment Induces Cx43 Phosphorylation and an Increase in Total Cx43—To find out whether the Tm-induced formation of open channels is associated with any biochemical changes in Cx43, we compared the protein from

FIG. 3. Western blot analysis of Cx43 in MHD1 and MHD1–43A cells. A, overexpression of Cx43 in Morris hepatoma cells. Lane 1, MHD1; lane 2, MHD1–43A. B, effect of Tm on Cx43 in MHD1 cells. Lane 1, control (0.4% Me₂SO, 8 h); lane 2, Tm-treated cells (4 μg/ml, 8 h). C, effect of Tm on Cx43 in MHD1–43A cells. Lane 1, control; lane 2, Tm, dephosphorylation of Cx43 from Tm-treated MHD1–43A cells by alkaline phosphatase. Lysates treated with (lane 1) and without (lane 2) alkaline phosphatase. The positions of molecular mass markers (in kDa) are indicated on the left for Western blots A and B and on the right for Western blots C and D (30 μg total protein/lane).
treated and untreated MHD1–43A cells in Western blots. One dramatic change was the appearance of Cx43 of higher molecular mass in Tm-treated cells (Fig. 1C), representing phosphorylated Cx43; it disappeared when cell lysates were treated with alkaline phosphatase (Fig. 1D). A second prominent change was an increase in total Cx43 level (Fig. 1C). In contrast, no such changes were seen in Cx43 from MHD1 cells after tunicamycin treatment (Fig. 1B).

Tunicamycin Treatment Induces Triton X-100 Insoluble Cx43—Musil and Goodenough (1991) have shown that in some communication-incompetent cells, Cx43 is mainly non-phosphorylated and Triton X-100 soluble. When these cells were made communication-competent by expression of cell-cell adhesion molecules, a form of Cx43 appeared that was phosphorylated and Triton X-100 insoluble. Since tunicamycin treatment induced communication and phosphorylation of Cx43 in Cx43-overexpressing cells but not in the parental MHD1 cells, we examined Cx43 Triton X-100 solubility in both cells before and after tunicamycin treatment to see whether communication correlated with Cx43 phosphorylation and Triton X-100 insoluble Cx43.

Under control conditions, Cx43 in the non-communicating MHD1 cells was predominantly Triton X-100 soluble (Fig. 4, lanes 1–3); Triton X-100 insoluble Cx43 was barely detectable, and this was so also after Tm treatment (Fig. 4, lanes 4–6). In contrast, forskolin treatment, which increases Cx43 expression and induces communication as well as Cx43 phosphorylation in...
MHD1 cells (Wang and Mehta, 1995), induced Triton X-100 insoluble Cx43 (Fig. 4, lanes 7–9). This Triton-insoluble fraction included both phosphorylated and non-phosphorylated Cx43, while the soluble fraction consisted of non-phosphorylated Cx43.

The Cx43 in untreated, basically non-communicating MHD1–43A and MHD1–43B cells was also mainly Triton X-100 soluble (Fig. 5, lanes 1–3 and 7–9). But after tunicamycin treatment, with the appearance of open channels and of phosphorylated Cx43 (the two upper bands in lanes 4, 6, 10, and 12 of Fig. 5), part of the Cx43 became Triton X-100 insoluble. As in the forskolin-treated MHD1 cells, this insoluble Cx43 consisted of both non-phosphorylated and phosphorylated forms of Cx43 (Fig. 5, lanes 6 and 12), while the soluble Cx43 was mainly non-phosphorylated (Fig. 5, lanes 5 and 11).

**DISCUSSION**

In the present study, we transfected cx43 cDNA into cells that lack open cell-to-cell channels and express little Cx43 and found that cells which overexpressed Cx43 nonetheless had few open channels. The lack of open channels in the Cx43 overexpressors seems not due to any null mutation in the cx43 cDNA. The ability of cDNA-derived Cx43 to form open cell-to-cell channels is clearly evident from the difference in communication between the parental MHD1 and the overexpressor cells after tunicamycin treatment; extensive cell-to-cell transfer of tracer was induced in Cx43 overexpressing cells (Fig. 2) but not in parental MHD1 cells (Wang and Mehta, 1995). Instead, the failure of the exogenous Cx43 to make open channels points to some cellular condition non-permissive for Cx43 to make open channels, and inhibition of glycosylation remedies this condition, allowing channel formation.

Due to the lack of potential glycosylation sites in their extracellular loops, connexins are unlikely to be glycosylated. Connexin-32 is known not to be glycosylated (Hertzberg and Gilula, 1979; Rahman et al., 1993), and our result of a lack of a reduction of carbohydrates from cell surface proteins other than Cx43 is a more likely cause for the observed increase in communication.
From a priori considerations, glycoproteins on the cell surface can be expected to impose an inhibitory effect on the formation of cell-to-cell channels and gap junctions (Perachchia, 1985; Abney et al., 1987). It was shown previously that lectins induced or fostered intercellular communication in Aplysia neurons (Lin and Levitan, 1987) or in Xenopus oocytes (Levine et al., 1991), presumably by removing bulky glycoprotein from the plasma membrane, and we have shown that inhibition of glycosylation increased intercellular communication in a variety of mammalian cells (Wang and Mehta, 1995).

Carbohydrates may interfere with any one of the steps occurring on the membrane during the formation of open cell-to-cell channels and thereby result in decreased communication. The extracellular domain of connexins is no larger than 8–10 Å, smaller than that of many membrane glycoproteins. One possibility therefore is that large membrane glycoproteins interfere with hemichannel interlocking by hindering two adjoining plasma membranes to come close enough to allow hemichannel interaction. Little is known about how the hemichannels get to the cell-to-cell contact sites and how channels become concentrated in the junctional plaques. It is possible that hemichannels are transported onto the plasma membrane at random sites and then laterally diffuse to the cell membrane contacts; or, they could be directly inserted into these sites. Bulky membrane glycoproteins may impede the lateral movement of hemichannels on the plasma membrane, or the insertion of hemichannels into the plasma membrane at random or specific sites may involve some glycoprotein(s). Yet another possibility is that even when channels are formed, bulky surface carbohydrates in the immediate vicinity of channels produce some condition unfavorable for the channels to be in the open state.

Although tunicamycin treatment elevated the total Cx43 level in Cx43 overexpressors, it is unlikely that this caused the dramatic rise in communication. Indeed, the greater total Cx43 protein may reflect a higher stability of Cx43 protein in channels than in hemichannels. We therefore interpret the increase in Cx43 to be the consequence of hemichannel interlocking rather than the cause of it. In agreement with this interpretation is that in the parental MHD1 cells, where tunicamycin did not induce channel formation, the Cx43 protein level did not rise; in fact, it was slightly diminished. This is consistent with the reported inhibition of protein synthesis by tunicamycin in other cells (Elbein, 1987).

One unexpected result of this study is that tunicamycin treatment induced Cx43 phosphorylation in the Cx43 overexpressor cells, raising the possibility that tunicamycin activates a kinase. Activation of protein kinase A up-regulates junctional communication and induces Cx43 phosphorylation in a variety of cells, including MHD1 cells (Wang and Mehta, 1995). But because protein kinase A activation has other effects not seen with tunicamycin treatment, e.g. stimulation of Cx43 transcription (Wang and Mehta, 1995), it is unlikely that tunicamycin activates protein kinase A. It is even less likely that tunicamycin activates protein kinase C or a tyrosine kinase because, although these kinases cause Cx43 phosphorylation, they inhibit communication (Crow et al., 1990; Filson et al., 1990; Brissette et al., 1991; Berthoud et al., 1992; Kanemitsu and Lau, 1993), whereas tunicamycin increases communication.

It is therefore unclear how Cx43 gets phosphorylated after tunicamycin treatment. Cx43 hemichannels are probably not phosphorylated (Musil and Goodenough, 1993) and are in a closed conformation. When they interlock, they must undergo a conformation change, which enables them to switch to an open state. It is possible that after the conformation change, Cx43 becomes a substrate of an unidentified, constitutively active kinase and thus gets phosphorylated. The function of this Cx43 phosphorylation is not clear. There is no evidence that phosphorylation is a prerequisite for channels to open.

Musil and Goodenough (1991) showed in several cell lines that a certain form of phosphorylated and Triton X-100 insoluble Cx43 is correlated with its localization at the junctional plaques. We found this to be true also in MHD1 cells, where forskolin induced the appearance of junctional plaques concurrently with Cx43 phosphorylation and Triton X-100 insolubility. However, we noticed differences. In the cells used by Musil and Goodenough (1991), the Triton X-100 insoluble fraction contained primarily phosphorylated Cx43, whereas tunicamycin increased communica-

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