Immunochemical and electrophysiological characterization of murine connexin40 and -43 in mouse tissues and transfected human cells

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Human HeLa or SkHep1 cells, defective in intercellular communication through gap junctions, were transfected with coding sequences of murine connexin40 (Cx40) and -43. The transfected cells were restored in gap junctional coupling as shown by 100-fold increased electrical conductance. When studied by the double whole-cell patch-clamp technique, Cx40 HeLa transfecants exhibited single channel conductances of $\gamma = 121 \pm 7 \text{ pS}$ and $\gamma = 153 \pm 5 \text{ pS}$. They were voltage gated with an equivalent gating charge of $z = 4.0 \pm 0.5$ for a voltage of half-maximal inactivation $U_a = 44 \pm 7 \text{ mV}$. The corresponding values of connexin43 (Cx43) HeLa transfecants are: $\gamma = 60 \pm 4 \text{ pS}$ and $\gamma = 40 \pm 2 \text{ pS}$ as well as $z = 3.7 \pm 0.8$ and $U_a = 73 \pm 7 \text{ mV}$. Transfer of the dye Lucifer Yellow was always considerably lower in Cx40- than in Cx43-transfectedants though their total junctional conductance was similar or even higher than for Cx43-transfectedants. In order to characterize cell and tissue distribution as well as phosphorylation of connexin40 and -43 proteins, antibodies to C-terminal oligopeptides of these proteins were prepared and used for immunoblotting, immunoprecipitation, and immunofluorescence analysis of transfected cells where they exhibited the punctate pattern characteristic of gap junctions on contacting membranes. Phosphorylation of connexin40 was shown by immunoprecipitation from $^{32}$P-labeled, transfected SkHep1 cells. Analyses of protein distribution in tissues revealed that the amount of connexin40 detected in heart was higher than in lung which is the inverse of the level of connexin40 mRNA in these tissues, suggesting posttranscriptional control of expression. Connexin40 protein in adult mouse heart and skin is about 20-fold more abundant than in the corresponding embryonic tissue. Connexin43 in adult mouse heart appears to be more highly phosphorylated than in embryonic heart or in transfected human cells.

Introduction

Intercellular communication via gap junctions is mediated by connexin proteins, which are coded for by a multigene family consisting of at least twelve members in mammals [5, 14, 18, 36, 37]. Connexin protein subunits, probably six, form a hemichannel that can dock to its counterpart in the plasma membrane of contacting cells. After injection of different connexin cRNAs into oocytes of Xenopus laevis, the expressed connexin proteins can form functional homogeneous gap junction channels between pairs of oocytes (homotypic channels). This has been accomplished for rat or mouse connexins (Cx): Cx26 [2], Cx32 [30, 34], Cx37 [38], Cx40 [15], Cx43 [30], Cx46 [25], and Cx50 [36]. Furthermore, by pairing two oocytes of which each one had previously been injected with a different connexin cRNA, coupling through heterotypic gap-junction channels could be demonstrated for Cx32 and Cx26 [2], as well as Cx40 and Cx37 [15, 38], but surprisingly not for Cx40 and Cx43 hemichannels [8]. Expression of endogenous Xenopus Cx38 in oocytes was specifically inhibited by previous injection of anti-sense oligonucleotides [2, 8, 15].

In addition, functional expression of mammalian connexins was studied after transfection of the coding sequences under control of expression vectors into human SkHep1 cells, (Cx32, Cx43; [12, 13]). C6 rat glioma cells (Cx43; [39]) N2A mouse neuroblastoma cells (Cx37; [26]), and human HeLa cells (Cx40; [15]). The recipient cells are largely deficient in expression of endogenous connexin transcripts and can be restored to high levels of dye transfer and electrical conductance by connexin transfection. In this paper we have used expression of murine Cx40 [15] and Cx43 in human HeLa and SkHep1 cells for characterization of antibodies prepared to C-terminal connexin peptides. Functional properties of the corresponding gap-junction channels in HeLa transfecants were investigated by double whole-cell patch-clamp measurements and Lucifer Yellow spreading. We show by immunoprecipitation...
that mouse Cx40 is phosphorylated in transfected SkHep1 cells. Furthermore, the antibodies were used to compare the relative amounts of Cx40 and Cx43 proteins in heart and lung of adult and embryonic mouse.

Materials and methods

Cells and culture conditions

Human cervix carcinoma HeLa (ATCC CCC2, cf. [11]) cells and human adenocarcinoma SkHep1 cells (ATCC HTB 52) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 µg/ml streptomycin (standard medium) at 37°C in a moist atmosphere of 10% CO2.

For patch-clamp experiments, HeLa cells were grown to about 70% confluency in plastic dishes without addition of selective drugs. For preparation of cell pairs, the cells were treated with trypsin for 5 to 12 min at 37°C and dissociated in extracellular saline (ESw, 95 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 10 mM HEPES/NaOH, pH 7.4) or culture medium and reseeded on glass coverslips (14 mm diameter). The cells were allowed to attach to the glass surface for 0.5 to 1.5 h at 37°C before performing the experiments.

Preparation of connexin constructs and transfection of mammalian cells

The following connexin gene fragments were used for ligation to DNA of the vector pBESH pac18 [16] that contains the SV40 early promoter as well as polyadenylation signals and a gene conferring resistance to puromycin:

1) The 1.2 kb BamHI/KpnI fragment of the mouse Cx40 gene derived from the pBluescript SK/Cx40 construct containing the 11 kb coding region in the HincIII site [15].

2) The 1.4 kb EcoRI fragment of the rat Cx43 cDNA [6]. The sense orientation of the insert was verified by sequence analysis.

HeLa and SkHep1 cells (2 × 106 each) were transfected according to Chen and Okayama [9] with 20 µg of each of the recombinant plasmid vector pBEHpac18, harboring the Cx40 and Cx43 coding sequences. Sixty-four hours after transfection, the medium was replaced by fresh standard medium containing 1 µg puromycin per ml (for HeLa cells) and 2 µg puromycin per ml (for SkHep1 cells). Individual clones were picked after 3 weeks and grown up in selective medium for subsequent analyses.

Dye transfer

Glass micropipettes were pulled from capillary glass (Hilgenrein Glas, Malsfeld/Germany) with a vertical pipette puller (700 C, David Kopf Instruments, Tujunga, CA/USA), and backfilled with 4% (w/v) solution of Lucifer Yellow CH (Sigma, St. Louis, MO/USA) in 1 M LiCl. The dye was injected iontophotographically into a monolayer cell for 20 s with a negative current of about 20 nA supplied by the iontophoresis unit of a microelectrode amplifier (LM-1, modification 500 M2, List-Electronic, Darmstadt/Germany). The electrode was withdrawn from the cells and 2 min after finishing the iontophotographic injection photographs were taken with an Olympus OM2 camera on Kodak T-MAX 400 films under phase contrast and epifluorescence conditions (Zeiss Standard RA, Filter sets 05 or 09; Oberkochen/Germany). Coupling was quantified by counting the number of fluorescent neighbors for every cell injected with Lucifer Yellow.

Current recording and data processing

We have used the double whole-cell recording technique [23] to study total gap junctional conductance in monolayers and current fluctuations through single gap-junction channels in isolated cell pairs. For patch-clamp experiments the coverslips were removed from the dish, washed with ESw, and transferred to an experimental chamber containing extracellular saline in which sodium was exchanged for barium chloride (ESw, 70 mM BaCl2, 5 mM KCl, 1.4 mM CaCl2, 10 mM HEPES/NaOH, pH 7.4) as bath medium. Pipettes were pulled from soft glass capillaries and heat polished to a final resistance of 1 to 5 MO. Pipette solution consisted of 119 mM KCl, 2.9 mM MgCl2, 5 mM EGTA (ethyleneglycol-bis-(β-aminobenzylic ether) N,N,N',N'-tetraacetic acid) and 10 mM HEPES adjusted to pH 7.4 with KOH. Current recordings were made using two List EPC-7 patch-clamp amplifiers. For current to voltage analysis, voltage ramps (0.2-0.02 Hz, 50 mV or 100 mV) were applied to one cell while the neighboring cell was kept at a constant voltage near its resting potential. Current recordings from both cells were low pass filtered at 250 to 500 Hz and stored on video tape via a modified digital audio processor [4]. Since this system provides only two dc-inputs, it is not possible to concurrently record the currents and voltages for two cells. For voltage ramp experiments, therefore, we used the audio input of the video recorder to store trigger pulses, which mark the start and end of each ramp. Thus, the respective voltages at each time point can easily be numerically reconstructed.

Data processing was performed off-line using an IBM-AT386 compatible microcomputer equipped with an appropriate A/D-converter board. Programs for acquisition and analysis of patch-clamp data written in ASYST (Keithley, Germering/Germany) were used for data processing [11].

For current to voltage analysis, the data were sampled in sweeps of 1024 points per trace with the pulse from the audio trace serving as a trigger signal. Each data file consisted of an ensemble of 10 to 100 current records each of which corresponded to one voltage ramp. Channel conductances were determined as the slope of the ridges in current-voltage surfaces [27] of these records. We have also estimated the voltage dependent open probability $p_0(U)$ of the channel within the range of the voltage ramp using a modified $H_n^\alpha$ technique [11]. The channel open probability distribution was fitted with a Boltzmann distribution of the form

$$p_0(U) = \frac{1}{1 + e^{\frac{q(U-U_s)}{kT}}}$$

where $q$ is the elementary charge, $k$ is the Boltzmann constant, $T$ is the absolute temperature in K. The equivalent gating charge $z$ and the voltage for half maximum blockage $U_s$ were estimated and used together with the single-channel conductance to characterize the respective gap-junction channel species.

Preparation of peptide-specific antibodies to Cx40 and Cx43

Antisera to Cx40 and Cx43 proteins were prepared by immunizing rabbits with synthetic peptides conjugated to keyhole limpet hemocyanin, according to Beyer et al. [7]. Oligopeptides were synthesized semiautomatically using an apparatus of the Bachem Company (Bubendorf/Switzerland). The Cx40 peptide corresponded to amino acid residues 337 to 358 of the mouse Cx40 protein. The purity of this peptide was assessed by high performance liquid chromatography. The Cx43 peptide represented amino acid residues 360 to 382 of rat Cx43. Crude antisera were initially characterized by dot blot analysis using the corresponding peptide and screened subsequently for reactivity with cross section tissues or cultured cells by immunohistochemical and immunoblot analysis.

The peptides were coupled to KLH (Keyhole limpet hemocyanin) as described above. Antisera were screened for reactivity to the peptides by modified enzyme-linked immunosorbent assay (ELISA). Unconjugated peptides were dot-blotted onto nitrocellulose membranes. The titer of the antisera was determined, using different dilutions, reaction with [125I]-protein A, and autoradiography.

Affinity purification of peptide-specific antibodies

Peptides were coupled to AH- and CH-Sepharose 4B (Pharmacia, Freiburg/Germany) according to the Pharmacia coupling protocol. The crude rabbit anti-peptide Cx40 serum was applied onto this column and processed as previously described [7, 33]. For some experiments, these affinity-purified peptide Cx40 antibodies were further affinity purified using electrophoretically separated cell lysates of Cx40 transfected SkHepl cells, and transferred onto nitrocellulose paper, according to Olmsted [24].

Immunochemoanalyses

For immunoblot analysis, rat heart gap junctions were isolated according to Manjunath and Page [20]. Purification of plasma membranes from cultured cell lines and conditions for immunoblot analyses have been described [32]. Lysates of different cell lines were obtained in Laemmli sample buffer [19].

Rabbit antibodies were detected according to Altherr et al. [1] using an anti-rabbit Ig antibody conjugated to alkaline phosphatase (Dianova, Hamburg/Germany) and bromochloroindolyl phosphate (BCIP)/nitroblue tetrazolium (NTB) (Boehringer Mannheim, Mannheim/Germany).

Immunofluorescence

Labeling of Cx40 and Cx43 proteins by immunofluorescence was carried out in cultured cell lines and on cryostat sections (6–8 µm) of different tissues. The labeling procedure for both connexins followed the scheme as described [10].

Immunoprecipitation

Cells were metabolically labeled by addition of [32P]-sodium orthophosphate (100 µCi per ml of culture medium) to phosphate-free standard medium for 1 to 4 h. In order to terminate the incorporation of [32P], the labeling medium was removed and the cell layer washed three times with phosphate buffered saline without calcium or magnesium ions. The cells were treated then for 20 min with RIPA buffer (10 mM sodium phosphate buffer, pH 7.2, 40 mM NaF, 2 mM EDTA, 1% Triton, X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1% Trasylol (Bayer AG, Leverkusen/Germany), in order to facilitate solubilization of membrane proteins. The supernatants were agitated four times on a vortex mixer. After centrifugation at 13,000g (30 min, 4°C), the supernatants were aspirated, stored as aliquots at −70°C, or used directly for immunoprecipitation as described [32].

Phosphatase treatment of immune complexes

The immune complexes were washed twice with buffer J [28], once with the phosphatase buffer, and then treated with alkaline or acid phosphatase (Sigma-Chemie, Deisenhofen, and Boehringer Mannheim) at 30°C for 30 min, in the presence of apronin, in the presence of 1% Triton (1 µg/ml) and 1 mM phenylmethylsulfonyl fluoride as protease inhibitors. After phosphatase treatment, the immunoprecipitates were washed again, and analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Results

Functional analysis of Cx40 and Cx43 transfectants

Functional expression of connexins was assayed using double whole-cell patch-clamp recording which revealed total gap junctional conductances of 50 nS and 40 nS for HeLa-Cx40 and HeLa-Cx43 transfectants, respectively, corresponding to a 100-fold increase of coupling compared to wild-type HeLa cells (Tab. I). Coupling in transfectants was not constant, however, since the conductances increased to 78 nS for HeLa-Cx40 and 66 nS for HeLa-Cx43 during a two-year cultivation period.

Single-channel conductance and voltage-dependent gating of gap-junction channels were determined using voltage ramps. An example of single-channel analysis is given in Figure 1 concerning Cx40 HeLa transfectants. The current measurement in a cell clamped to a constant voltage resolves the current fluctuations caused by opening and closing of gap-junction connecting this cell to an adjacent one which was subject to a voltage ramp (Fig. 1a).

[Graph and diagram]

Fig. 1. Schematic representation of voltage ramp experiments (a) Upper two traces: voltage and current records of the "ramped" cell. Lower two traces: voltage and current records of the cell kept at constant voltage. Recordings from this cell were used to construct a two-dimensional histogram or current-voltage surface (b), which was then used for further analysis.
these measurements, a current - voltage surface (Fig. 1b) was computed which allowed estimation of two channel conductances $\gamma = 121 \pm 7 \text{ pS}$ and $\gamma = 153 \pm 5 \text{ pS}$ for Cx40 and $\gamma = 40 \pm 2 \text{ pS}$ and $\gamma = 60 \pm 4 \text{ pS}$ for Cx43 channels, respectively (Figs. 2a, c and Tab. I). The channel-open probability can be derived for different voltages from the data obtained with voltage ramp experiments. The open-probability distribution follows a bell-shaped curve where the maximum open probability is at $0 \text{ mV}$ transjunctional voltage, which corresponds to the physiological situation in coupled cells by gap junctions. The voltage-dependent component for both HeLa transfectants is about 50% of the open probability and can be fitted to a Boltzmann distribution. For the presented experiments an equivalent gating charge $\zeta = 3.9$ and a voltage for half maximal inactivation $U_o = 38 \text{ mV}$ was estimated for Cx40 (Fig. 2b) and $\zeta = 3.8$ and $U_o = 76 \text{ mV}$ for Cx43 (Fig. 2d).

The combined data for both Cx40 and Cx43 HeLa transfectants are summarized in Table I. As can be seen, HeLa Cx40 transfectants exhibit two different single-channel conductances of 121 pS and 153 pS which are significantly different from the 40 pS and 60 pS conductances of HeLa

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Total conductance $G_t (nS)$</th>
<th>Single-channel conductance $\gamma (pS)$</th>
<th>Boltzmann parameters $U_o (mV)$, $\zeta$</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa</td>
<td>$0.4 \pm 0.8$ (n = 179)</td>
<td>$26 \pm 6$ (n = 16)</td>
<td>$49 \pm 10$ (n = 8)</td>
</tr>
<tr>
<td>HeLa Cx40</td>
<td>$50 \pm 6$ (n = 5)</td>
<td>$121 \pm 7$ (n = 9)</td>
<td>$44 \pm 7$ (n = 5)</td>
</tr>
<tr>
<td>HeLa Cx43</td>
<td>$40 \pm 2$ (n = 6)</td>
<td>$40 \pm 2$ (n = 13)</td>
<td>$73 \pm 7$ (n = 17)</td>
</tr>
</tbody>
</table>

* Data were taken from [11]. - All values are given as mean ± SD - n: Number of cell pairs studied.

Fig. 3. Lucifer Yellow spreading and total junctional conductance in Cx40 (left side) and Cx43 transfectants (right side). Phase contrast (a, e) and epifluorescence illumination (b, f) of monolayer cells 2 min after Lucifer Yellow injection. (Arrowheads indicate injected cells.) Distribution of dye-coupled neighbors (c: n = 46; g: n = 51). Distribution of total gap junctional conductance ($G_t$) in monolayer cells (d: $G_t = 78 \pm 42 \text{ nS}$, n = 24; h: $G_t = 66 \pm 46 \text{ nS}$, n = 26; values: mean ± SD). - Bar 25 μm.

Cx43 transfectants and from the 26 pS channels found in wild-type HeLa cells.

Transfer of the fluorescent dye Lucifer Yellow between HeLa Cx43 transfectants is higher than between HeLa Cx40 transfectants, even with a higher total junctional conductance for HeLa Cx40. In addition, the fluorescence intensity of dye-coupled neighbors was always considerably smaller in Cx40 than in Cx43 transfectants. These data are summarized in Figure 3. In Cx43 transfectants, some of the cells were always excluded from dye transfer, even when they were in direct contact with the injected cell (see Fig. 3f). These cells probably correspond to a population with low total junctional conductance found in the electrical measurements (Fig. 3h, first two bins).

Expression of Cx40 protein in transfected human cells and in mouse tissues

Peptide-specific antibodies to the C-terminal 22 amino acids of mouse Cx40 were raised in rabbits and affinity purified using the corresponding synthetic peptide. The specificity of the affinity-purified antibodies was assessed by immunoblot analysis using lysates of Cx40-transfected SkHep1 or HeLa cells (Fig. 4).

The antiserum as well as affinity-purified antibodies specifically recognized a 40 kDa protein component which is only expressed in the Cx40 transfectants. In addition, the antibodies recognized a protein band of an apparent molecular mass of 46 kDa in Cx40-transfected SkHep1 cells (Fig. 4, A, lane 1), HeLa cells (Fig. 4, A, lane 3) as well as in non-transfected SkHep1 cells (Fig. 4, A, lane 2), non-transfected HeLa cells (Fig. 4, A, lane 4), and human tissue (data not shown) but not in murine cells and tissue. In order to improve the specificity of the antiserum, it was further affinity purified using electrophoretically separated lysates of Cx40-transfected SkHep1 cells [24].

Antibodies bound to the 40 kDa protein band were eluted from nitrocellulose. Panel B in Figure 4 shows in lanes 1 and 2 that these purified antibodies recognize this antigen but still showed very weak cross-reactivity with the 46 kDa protein. When isolated plasma membranes of transfected cells were subjected to immunoblot analysis, only the 40 kDa protein, but not the 46 kDa protein, was detected (Fig. 4, C, lanes 1, 2). This suggests that the 46 kDa protein is not located in the plasma membrane. This conclusion was confirmed by immunofluorescence analysis of transfected cells using antibodies eluted, according to Olmsted [24], from the 40 kDa and the 46

Fig. 5. Tissue-specific and development-dependent expression of Cx40. 100 µg of proteins from adult mouse tissues: lung (lane 1), heart (lane 2), kidney (lane 3), skin (lane 4), and embryonic tissues: lung (lane 5), heart (lane 6), kidney (lane 7), and skin (lane 8) were analyzed after electrophoresis by immunoblot using affinity-purified Cx40 antibodies, labeling with [125] protein A, and autoradiography. In lane 9 lysates from Cx40-transfected HeLa cells were analyzed under the same conditions.

Fig. 6. Immunofluorescence analysis of cell lines using rabbit affinity-purified Cx40 antibodies and goat anti-rabbit IgG-fluorescein isothiocyanate. Nontransfected SkHep1 cells (a) and nontransfected HeLa cells (c) do not show immunofluorescence on cell contacts, but Cx40-transfected HeLa cells (e) as well as Cx40-transfected SkHep1 cells (g) exhibit the punctate pattern characteristic of gap junctions. - b, d, f, h. Phase-contrast micrographs of the same viewfields analyzed by immunofluorescence. - Bar 40 µm.
kDa protein bands. The electrophoretic position of the 40 kDa protein band was not changed after treatment with alkaline phosphatase (cf. Fig. 4, C, lanes 1 and 2).

Figure 5 illustrates the immunoblot analysis of the Cx40 protein in different adult and embryonic mouse tissues. A strong, single band of about 38 kDa apparent molecular mass was found in adult heart (lane 2), whereas the same band in embryonic heart (lane 6) was about 20-fold weaker according to densitometric evaluation of the autoradio-

graph. In lung homogenate, the Cx40 antibodies recognized a protein of 38 kDa which was present in monomeric and multimeric forms (lane 1). In adult skin (lane 4), very weak expression of the 38 kDa monomeric form of the Cx40 protein was found. This protein could not be detected by immunoblot in adult and embryonic kidney (lanes 3, 7). For comparison, lane 9 illustrates again the results of immunoblot using a lysate from Cx40-transfected HeLa cells. The Cx40 protein in transfected cells migrates...
slightly slower than the 38 kDa protein detected by the same antibodies in heart. Possibly, posttranslational modifications of the Cx40 protein may differ in transfected HeLa cells and mouse heart.

**Location of Cx40 in cultured cells and tissues**

The affinity-purified Cx40 antibodies labeled their antigen in contacting plasma membranes of transfected HeLa and SkHep1 cells (Figs. 6c and g, respectively) in a punctate pattern, typical for the location of gap junctions. Some fluorescent spots were seen in the cytoplasm of transfected cells that may correspond to Cx40 protein during processing. In addition, the antibodies labeled nuclei to a variable extent. This is likely due to non-specific fluorescence since it is also seen with preimmune serum. Non-transfected SkHep1 cells (Fig. 6a) and non-transfected HeLa cells (Fig. 6c) do not exhibit immunofluorescence on contacting plasma membranes. Figure 7 illustrates that the affinity-purified Cx40 antibodies recognize their antigen in vascular endothelial cells in brain (Fig. 7a), liver (Fig. 7c), kidney (Fig. 7e), and lung (Fig. 7g).

**Phosphorylation of Cx40 protein in transfected cells**

In order to investigate whether mouse Cx40 is phosphorylated in transfected cells we used the affinity-purified Cx40 antibodies for immunoprecipitation of Cx40 from transfected SkHep1 cells metabolically labeled with [32P]orthophosphate. The results illustrated in Figure 8 show phosphate incorporation into the 40 kDa protein (Fig. 8, A, lane 4) and increase of phosphorylation when the cells were grown for 1 h in the presence of dibutyryl cyclic adenosine monophosphate (db-cAMP) (Fig. 8, B, lane 4) or phorbol 12-O-myristate-13-acetate (TPA) (Fig. 8, C, lane 4). In addition to the 40 kDa protein, we also detected immunoprecipitates of a 125 to 130 kDa protein showing the same labeling intensity as the 40 kDa protein. Possibly, the 125 to 130 kDa protein represents an aggregated form of the 40 kDa band. Neither the 40 kDa band nor the 125 to 130 kDa band were detected when control immunoprecipitations were carried out without Cx40 antibodies (data not shown). Dephosphorylation of Cx40 catalyzed by acid phosphatase is more effective than by alkaline phosphatase (cf. the corresponding lanes 1 or 2 with lanes 3 in A, B and C of Fig. 8).

**Expression of Cx43 protein in transfected human cells and in mouse tissues**

Peptide-specific antibodies to the C-terminal 22 amino acids of rat Cx43 were raised in rabbits and affinity purified using the synthetic peptides. The specificity of the affinity-purified rabbit antibodies was assessed by immunoblot analysis using rat and mouse heart homogenates (Fig. 9). In adult mouse heart, Cx43 shows different states of phosphorylation (Fig. 9, lane 2), previously described by Musil et al. [22]. In embryonic mouse heart (19 dpc), Cx43 is only about half as abundant as in adult mouse heart and shows predominantly the faster migrating bands corresponding to lesser phosphorylation [22] (Fig. 9, lane 1). In contrast to embryonic heart, part of the Cx43 protein is detected as slower migrating form, i.e., presumably more highly phosphorylated in adult heart and transfected human cells (Fig. 9, HeLa in lane 3 and SkHep1 in lane 4). Possibly, the activity of Cx43 phosphorylating protein kinase(s) is different in mouse myocardium and human transfected cells.

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**Fig. 8.** Cx40 transfected cells were labeled with 32P inorganic phosphate for 4 h (panels A, B, C), lysed and immunoprecipitated with affinity-purified Cx40 antibodies. The immunoprecipitates were subjected to electrophoresis and autoradiography. In addition, before the end of the 32P-labeling period, the cells of panel B were incubated for 1 h with cAMP and cells in panel C with TPA. The cell extracts were treated with acid phosphatase: 0.5 units (lane 1), 2.5 units (lane 2), or alkaline phosphatase: 25 units (lane 3), or incubated in the absence of phosphatase (lane 4).

**Fig. 9.** Expression of rat Cx43 protein in transfected human cells and embryonic and adult mouse heart. Proteins from embryonic and adult mouse heart and the following cell lysates were analyzed by immunoblot using affinity-purified Cx43 antibodies: embryonic mouse heart (lane 1, 50 µg) and adult mouse heart (lane 2, 50 µg), Cx43-transfected HeLa cells (lane 3, 6 µl), Cx43-transfected SkHep1 cells (lane 4, 10 µl), as well as the corresponding nontransfected HeLa cells (lane 6, 10 µl), nontransfected SkHep1 cells (lane 6, 10 µl), and Cx40-transfected HeLa cells (clone B35) (lane 7, 5 µl). The position of the immune complexes was detected by incubation with anti-rabbit Ig conjugated to alkaline phosphatase and BCIP/NTB color reaction [1].
Discussion

The connexin-transfected HeLa cells serve two experimental purposes: First, they demonstrate that expression of different exogenous connexins leads to a large increase in transcellular conductance, as shown in this paper, and can restore dye transfer. Thus, we conclude that the defect in gap junctional communication in HeLa cervix carcinoma cells is due to lack of functional connexins. We have been unable to detect transcripts of 10 different connexins in total RNA from HeLa cells by Northern blot hybridization (C. Elfang, unpublished results). It appears that HeLa cells may have a defect in transcription of connexin genes, leading to largely reduced gap junctional communication. However, residual cell-cell coupling was demonstrated in HeLa cells [11] suggesting that these cells (like other recipient cells for connexin DNA transfection) must contain a low level of connexin transcripts and protein. Currently we do not know the identity of the connexin(s) expressed in non-transfected HeLa cells. Similar bioelectric properties were reported for an unidentified endogenous connexin in SkHepl cells [29] and N2A cells [35]. As has been described [11], HeLa cells are advantageous for expression of connexins due to their low level of endogenous gap junctional conductance, their low single-channel conductance, and their stability in whole-cell patch-clamp experiments.

The total junctional conductance of Cx40 or Cx43 HeLa transfectants is about 100-fold higher than the total junctional conductance of HeLa parental cells (Tab. 1). This is unlikely that the single-channel currents which were measured in HeLa transfected are due to endogenous connexins. The single-channel conductances in transfected HeLa cells of 121 pS or 153 pS and 40 pS or 60 pS for mouse Cx40 and -43 respectively, correlate well with similar results recently reported for transfected N2A [35] and SkHepl cells [13]. Interestingly, Cx40 is preferentially located in conducting rat myocardium [3] and dog Purkinje fibers [17].

During two years the total gap junctional conductance and the Lucifer Yellow spreading was measured repeatedly in transfected HeLa cells. The mean value of electrical coupling was always higher in Cx40 (50-78 nS) than in Cx43 transfectants (40-66 nS). Assuming a single-channel conductance of 150 pS for Cx40 and 60 pS for Cx43 channels, the number of open channels in Cx43 transfectants is approximately twice that in Cx40 transfectants. If the same number of dye molecules pass an open pore, regardless of its bore size, one would expect twofold higher spreading of Lucifer Yellow in Cx43 transfectants. Thus, from the total junctional conductance of different cell types the gap junctional permeability for larger molecules (e.g., Lucifer Yellow or cAMP) cannot be predicted. In addition, different channel types may have different specificities for larger (charged) molecules but not for smaller ions due to electrostatic interaction of these molecules with the pore wall. The fluorescence intensity of dye coupled neighboring cells is always considerably higher with Cx43 than with Cx40 transfectants, indicating that Cx43 channels may have a higher permeability for Lucifer Yellow than Cx40 channels.

The second purpose of the connexin-transfected HeLa cells is to provide biological material for standardization of antibodies directed to the corresponding connexins. Primary cells or established cell lines usually express more than one type of connexin in contrast to HeLa transfectants that express mainly homotypic gap-junction channels consisting of the same connexin protein.

The Cx40 antibodies that we raised in rabbits to the C-terminal 21 amino acids of the mouse Cx40 protein recognized in extracts of mouse tissues only the expected protein of about 38 kDa (and aggregates thereof) which corresponds to a protein of about 40 kDa in transfected human HeLa or SkHepl cells. The Cx40 antibodies, affinity purified with the immunogenic Cx40 peptide, recognized in human transfectants a second protein of about 46 kDa, presumably because of its partial sequence identity to the C-terminal Cx40 peptide used for immunization. Additional affinity purification of the Cx40 antibodies of the human Cx40 protein, however, led to antibodies which were specific to this protein and could be reliably used for analysis of localization and modification of the Cx40 protein in transfected human cells.

We showed by immunoprecipitation that the Cx40 protein can be phosphorylated in HeLa transfectants. Phosphorylation does not change the electrophoretic mobility of Cx40 in SDS-polyacrylamide gels, since treatment of plasma membranes from Cx40-transfected HeLa cells with alkaline phosphatase did not affect the electrophoretic position of the Cx40 protein. Increased incorporation of [32P]phosphate into the Cx40 protein during 1 h incubation with cAMP or TPA could be due to increased phosphorylation of the Cx40 protein or increased biosynthesis of the Cx40 protein.

We do not know whether phosphorylation of connexins is relevant to the assembly or gating of gap-junction channels. For rat Cx43 three different isofoms can be electrophoretically separated, a non-phosphorylated P0 form, a phosphorylated P1 form and a highly phosphorylated P2 form [22]. Recently it was reported, that cAMP- or cGMP-dependent phosphorylation alters the single-channel conductance of Cx43 gap-junction channels in cardiac myocytes or transfected SkHepl cells [21, 31]. It is assumed that successive phosphorylation decreases the conductance from about 90 pS for P0 to 60 pS for P1 and 40 pS for P2. Our data from HeLa Cx43 transfectants are consistent with these values: the predominant single-channel conductance is 60 pS; 40 pS occurs less frequently, whereas the 90 pS channel was not observed. Cx40 channels also exhibit two single-channel conductances (with 150 pS as the predominant conductance). Since Cx40 is also a phosphoprotein (as shown in this paper), one can speculate that phosphorylation may regulate gap junctional conductance in channels made of each connexin.

On immunoblots of protein extracts from mouse heart and skin the Cx40 antibodies recognized a protein of 38 kDa molecular mass which migrates differently from the 40 kDa protein in HeLa transfectants. Posttranslational modification of Cx40 may differ between these tissues and transfected cells. Surprisingly, the 38 kDa protein could only be detected in lung after treatment with 10%
mercaptopethanol. In this tissue, a 70 to 80 kDa protein was recognized by the Cx40 antibodies which could be the dimeric form of Cx40. Previously it had been described [14, 15] that Cx40 transcripts were much more abundant in lung than in heart. It seems that the level of Cx40 protein in lung does not correlate with the level of Cx40 mRNA in this tissue, suggesting posttranscriptional control of expression. The results of our immunoblot (cf. Figs. 5 and 9) show no cross-reactivity between the Cx40 and Cx43 antibodies. These antibodies can therefore be used reliably for quantitative estimation of Cx40 or Cx43 protein in tissues like myocardium, where both proteins are coexpressed.

Consistent with the finding that Cx40 transfectants of HeLa or SkHep1 cells show restored gap junctional coupling, the corresponding proteins can be seen after immunobiochemical analysis as a punctate pattern on the plasma membrane of contacting cells. This is the expected location of gap junctional plaques. Our immunofluorescence analysis demonstrated that Cx40 is localized in vascular endothelium of mouse brain, liver, as well as kidney (Fig. 8). Previously it was reported that Cx40 is localized in rat endothelial cells of lung [8] and heart [3, 8]. Furthermore, Cx40 is preferentially localized in conducting myocardium [3].

Transfected HeLa cells are useful for the characterization of connexins. For example, they may be used for checking the specific detection of the antigen by connexin antibodies, or, for functional studies like single-channel recording or dye transfer experiments. Furthermore, they can be used to study heterotypic coupling under conditions where two different connexin-transfected cells are contacting one another. It has recently been shown that Cx40 and Cx43 do not form functional gap-junctional channels when expressed by eRNA injected into Xenopus oocytes [8]. We found (C. Elfgang et al., in preparation) that the Cx40 and Cx43 HeLa transfectants characterized in this paper do not exchange microinjected Lucifer Yellow, and we are currently using this cell system to study intercellular coupling between HeLa cells transfected with several other connexins.

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