Tetracycline-regulated expression enables purification and functional analysis of recombinant connexin channels from mammalian cells

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INTRODUCTION

Gap junctions are arrays of connexin channels, each with an aqueous pore that spans two closely apposed cell membranes. They allow direct communication between the cytoplasmic contents of coupled cells and permit the intercellular diffusion of ions, metabolites and cytoplasmic messenger molecules smaller than ~1000 Da [1–4]. Intercellular communication mediated by gap junctions is necessary for many biological processes, including growth and differentiation, metabolic homeostasis, tissue synchronization in secretion and muscle contraction, and synchronization of neural activity [5–9].

Each gap junction channel is formed by end-to-end docking of two hemichannels, hexamers of connexin protein, expressed on neighbouring cells [10,11]. To date, about 20 connexin isoforms have been identified [12]. Connexin expression is ubiquitous, with most cells in multicellular organisms expressing more than one isoform. Isoform composition confers distinct molecular selectivity and modulatory sensitivity to the channels [13–17], leading to unique disease phenotypes associated with genetic defects in each isoform. These include neuronal demyelination, deafness, cardiac functional and developmental defects, cataracts, and skin disorders (reviewed in [17]).

Each connexin subunit has four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic N- and C-termini. Each hemichannel can be formed from identical connexin subunits (homopentameric) or from subunits of different isoforms (heteropentameric). Channels formed from Cx32 (connexin32) or Cx26 only will be referred to as homomeric Cx32 or Cx26 hemichannels respectively, while those formed from a mixture of Cx26 and Cx32 will be referred to as heteromeric Cx26/Cx32 hemichannels.

There are established techniques for the functional analysis of purified connexin hemichannel permeability and modulation [13,14,18–20], but, to date, the purification of recombinant connexin channels from mammalian cells in amounts sufficient for biochemical and functional analyses has remained a challenge. In addition to the usual difficulties encountered with heterologous channel expression, significant complicating factors in the design of connexin expression systems are the requirement for cell–cell contact in order to establish fully processed junctional channels and the apparent cytotoxic effects of high levels of connexin expression, presumably due to formation of active plasma membrane hemichannels. The insertion of Cx43 into plasma membrane gap junction plaques, for example, is accompanied by phosphorylation, a process that is deficient in non-communication-competent cell lines [21,22].

A recent report described the use of High-Five insect cells for the purification of solubilized His6-tagged Cx43 [23]. While the protein was shown to be functional when reconstituted into liposomes, it was likely derived from endoplasmic reticulum, since there are no cell–cell contacts in the suspension-grown cells. Therefore it is not clear whether the channels are in their mature processed state, and what the functional consequences are of the absence of formation of plasma membrane junctional plaques. Another approach has been the purification of Cx26 gap junction plaques from HeLa cells using successive detergent extraction steps [24]. However, the preparations were not pure connexin, as

Abbreviations used: AM, acetoxymethyl ester; Cx32, connexin32 (etc.); DFP, di-isopropyl fluorophosphate; HA, haemagglutinin; (HN)x, (His-Asn)x; IP3, inositol 1,4,5-trisphosphate; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; OG, n-octyl-β-D-glucopyranoside; rtTA, reverse tetracycline-controlled transactivator; TRE, tetracycline response element; TRITC, tetramethylrhodamine β-isothiocyanate, TSF, transport-specific fractionation.

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conditions were optimized for junctional plaque image analysis. There are no reports of functional connexin expression in bacteria or yeast.

Here we present a technique to express and purify homomeric or heteromeric connexin channels from cultured mammalian cells that form functional gap junctions. This method typically produces 10–20 µg of purified connexin per 2.5 ml that is suitable for both biochemical studies and functional analysis of channel permeability. We used the Tet-On inducible expression system in HeLa cells to obtain cell lines with reproducible stable connexin expression levels and a one-step immunoaffinity purification procedure, based on an influenza HA (haemagglutinin) tag, to obtain pure functional solubilized connexin hemichannels.

MATERIALS AND METHODS

Materials

All components of the Tet-On expression system (HeLa-TetOn cells, doxycycline, Tet System approved fetal bovine serum, and the DNA vectors pBl, pBl-G and pTK-Hyg) were purchased from BD Biosciences (Palo Alto, CA, U.S.A.). Dulbecco’s modified Eagle’s medium, G418 sulphate, hygromycin B and lipofectamine 2000 were from Life Technologies (Rockville, MD, U.S.A.). All other reagents were from Sigma (St. Louis, MO, U.S.A.). Secondary antibodies for Western blotting were from Amersham Biosciences Corp. (Piscataway, NJ, U.S.A.). Secondary antibodies for immunofluorescence were from Dako Corp. (Carperteria, CA, U.S.A.). Agarose-conjugated and free anti-HA clone HA-7 mouse IgG were from Sigma (St. Louis, MO, U.S.A.). Cell labelling dyes CM-Dil and calcine-AM (acetoxy-methyl ester) and the SlowFade Light Antifade Kit were from Molecular Probes (Eugene, OR, U.S.A.). Lipids for reconstitution were from Avanti Polar Lipids (Alabaster, AL, U.S.A.). All other reagents were from Sigma, unless stated otherwise.

Tet-On inducible mammalian expression system

The Tet-On expression system allows tetracycline- or doxycycline-regulated gene expression under the control of the rtTA (reverse tetracycline-controlled transactivator) protein and the Tet operator DNA sequence (tetO), both of which are regulatory elements derived from the Escherichia coli tetracycline resistance operon [25]. When a vector containing the gene of interest preceded by a TRE (tetracycline response element, which consists of seven repeats of the tetO sequence) is introduced into a cell line stably expressing the rtTA, expression of the gene can be controlled by the level of doxycycline in the culture medium. The rtTA can only bind to the TRE and initiate transcription of the gene in the presence of doxycycline. Furthermore, co-regulated co-expression of two genes can be obtained by using Bidirectional Tet Expression Vectors which contain a central bidirectional TRE element [26]. For the work presented in this paper, the bidirectional vectors pBl, containing two available multiple cloning sites, and pBl-G, containing an available multiple cloning site in one direction and a LacZ gene in the other, were used.

DNA constructs

We created a modified version of the bidirectional Tet vector pBl-G, called pBl-GT, in which a sequence coding for a thrombin-cleavable C-terminal purification tag is added to a gene cloned into the upstream multiple cloning site. A double-stranded oligonucleotide encoding a thrombin cleavage site (Leu-Val-Pro-Arg-Gly-Ser), an influenza HA tag (Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Leu), a (HN)₆ [(His-Asn)₆] tag and a stop codon, with NotI- and Sall-compatible overhangs, was synthesized by in vitro annealing of two 96-mer oligonucleotides: 5′-GGCGGTCACTAGTGCCAGGGGCAGCTACCCCTAGACGTGGCGACTGCTGTCAACACCACAACCAACACCAACAACCAACACTGAG-3′ and 5′-TGACTCAATGGTTGTGTTGTGTTGTGTTGTGTTGTGTTGTGTTGTGTTGTGTTGT-GTGCAAGGCGTATCGGCAGTGGTGGGGAAGGATTGTGCCATGTTCGATGCGACGTCGATGCCCTTGGGCACTAGTAC-3′ (italics indicate overhangs, the SpeI restriction site is underlined, and the stop codon is in bold). This oligonucleotide was ligated into the pBl-G vector precursor with NotI and SafI. The resulting vector, pBl-GT, contains the LacZ gene in the downstream cloning site and a thrombin-cleavable HA–(HN)₆ tag sequence adjacent 3′ to the upstream multiple cloning site.

An SpeI restriction site (underlined in the above sequence) was incorporated into the first 5 nucleotides of the thrombin cleavage site sequence to produce a 5′-CTAG overhang upon digestion of the pBl-GT vector with SpeI, allowing in-frame ligation to a protein-coding sequence that is digested at its 3′ end with AvrII, NheI, XbaI or SpeI. The restriction site used at the 3′ end is selected based on the final nucleotide of the sequence, which forms the first residue of the restriction site: AvrII if it is a C, NheI if G, SpeI if A and XbaI if T. To ensure that the protein-coding sequence is followed-in-frame by the tag sequence without the addition of extra amino acids, the restriction site is added by PCR-based amplification of the sequence using a reverse primer that includes the first 5 nucleotides of the restriction site. The 5′ end can be ligated using an EagI, NotI or PstI restriction site.

The vectors for the expression of homomeric Cx26 or Cx32 channels were made by insertion of rat Cx26 or Cx32 cDNA respectively into the pBl-GT vector. The cDNAs were amplified by PCR with the addition of a 5′ EagI restriction site (bold italics in forward primers), a 5′ Kozak consensus translation start sequence (underlined) [27], and a 3′ AvrII restriction site (bold italics in reverse primers) using the following primers: for Cx26: Cx26fwd, 5′-AAACGGCGCCGACCATGGATTGCGGCACTACAGAG-3′; Cx26rev, 5′-CACCTAGGACTGGTTCTTTGAGCTCCCTG-3′; for Cx32: Cx32fwd, 5′-AAACGGCGCCGACCATGGATTGCGGCACTACAGAG-3′; Cx32rev, 5′-CACCTAGGACGCGTGAGCTCCCTG-3′. The PCR amplicons digested with AvrII and EagI were ligated into the pBl-GT vector using the EagI and SpeI sites to yield the vectors pBl-G26T and pBl-G32T for regulated co-expression of β-galactosidase and Cx26 or Cx32 respectively.

The expression vectors for heteromeric Cx26tag/Cx32 and Cx32tag/Cx26 channels were based on the pBl vector, which has two available multiple cloning sites. Cx26 and Cx32 cDNAs were subcloned into the downstream cloning site of pBl using the EcoRV and NheI sites to make pBl-26 and pBl-32 respectively. Cx26tag and Cx32tag constructs were excised from pBl-G26T and pBl-G32T and subcloned into the NotI/Sall restriction sites of the upstream cloning site of pBl-32 and pBl-26, respectively, to create vectors pBl-26T-32 and pBl-26T-32.

All PCR amplicons were fully sequenced in both directions at the Molecular Resource Facility (New Jersey Medical School, Newark, NJ, U.S.A.) following ligation into the pBl vectors, to verify sequence integrity.

Plasmid transfection and stable cell line screening

HeLa cells were used for transfection because they have been widely utilized for heterologous connexin expression due to their near-complete lack of endogenous connexins [28]. HeLa-TetOn cells were maintained in 100 µg/ml G418 sulphate until transfection. The cells were co-transfected with one of the four plasmids (pBl-G26T, pBl-G32T, pBl-26T-32 or pBl-32T-26) and
the selection plasmid pTK-Hyg in 10:1 and 20:1 ratios using lipofectamine 2000™. Stable double-transfected clones were selected in medium containing 400 µg/ml hygromycin B in addition to 100 µg/ml G418 sulphate. Following screening, stable cell lines were maintained in 200 µg/ml hygromycin B and 100 µg/ml G418 sulphate. Connexin expression prior to purification, staining or dye transfer experiments was induced with 1 µg/ml doxycycline for 48 h, unless stated otherwise.

pB1-G26T and pB1-G32T transfectants were first screened for β-galactosidase expression using the X-Gal Staining Assay Kit (Gene Therapy Systems Inc., San Diego, CA, U.S.A.) pre- and post-doxycycline induction. Lines that were β-galactosidase-positive in an inducible manner were then screened for gap junction staining by indirect immunofluorescence using anti-HA mouse IgG.

pB1-26T-32 and pB1-32T-26 transfectants were first screened for connexin expression by immunoblotting with anti-HA mouse IgG pre- and post-induction. Positive inducible clones were then screened for gap junction staining by indirect immunofluorescence with antibodies against each connexin isoform.

**Western blotting, immunocytochemistry and gold staining**

Whole-cell lysates for Western blotting were prepared by washing the cells three times with cell wash buffer (0.01 M PBS, 0.138 M NaCl, 0.02 % NaN3, pH 7.4) followed by a 2 h incubation in lysis buffer [50 mM NaH2PO4, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 80 mM OG (n-octyl β-D-glucopyranoside), 1 mM β-mercaptoethanol, 0.5 mM DFP (di-isopropyl fluorophosphate), pH 7.5] at 4 °C using 0.05 ml/cm². For Western blotting and gold staining, either cell lysates (10 µg) or purified connexin preparations (10 µl) were separated by SDS/PAGE [29] in 13 % Tris-glycine mini-gels and transferred to a PVDF membrane.

For gold staining, the membranes were blocked [0.01 M PBS, pH 7.4, 0.3 % (v/v) Tween-20] for 30 min at 37 °C. This was followed by two 5 min washes with blocking solution and three 5 min washes with double-distilled water at room temperature. The blots were stained with Colloidal Gold Total Protein Stain (Bio-Rad Laboratories, Hercules, CA, U.S.A.) until the desired band intensities developed, washed with double-distilled water, and air-dried.

For Western blotting, membranes were blocked with 5 % (w/v) skimmed dry milk in wash buffer [0.01 M PBS, pH 7.4, and 0.05 % (v/v) Tween-20], stained with primary antibody in 1 % (w/v) BSA in wash buffer, washed, stained with alkaline phosphatase-conjugated secondary antibody in wash buffer, and developed in Nitro Blue Tetrazolium/5-bromo-4-chloroindol-3-yl phosphate solution (Pierce, Rockford, IL, U.S.A.).

For Western blotting of Cx26, we used mouse anti-Cx26 IgG (Zymed Laboratories Inc., San Francisco, CA, U.S.A.) as primary antibody at 1:500 dilution, with alkaline phosphatase-conjugated goat anti-mouse IgG as secondary antibody. Blotting for Cx32 was carried out with M12.13 mouse anti-Cx32 IgG (30) (primary) at 1:20 000 dilution and alkaline phosphatase-conjugated goat anti-mouse IgG (secondary). Blotting of HA-tagged connexin was carried out with mouse anti-HA clone HA-7 IgG (primary) at 1:40 000 dilution and alkaline phosphatase-conjugated goat anti-mouse (secondary). Both secondary antibodies were used at 1:10 000 dilution.

For indirect immunofluorescence, cells were cultured to confluence on glass coverslips or chamber slides with and without 1 µg/ml doxycycline for 48 h and fixed with 100 % methanol at −20 °C for 10 min, followed by three washes in 0.01 M PBS, pH 7.4. They were then incubated with primary antibody in 1 % (w/v) BSA in wash buffer, washed, and incubated with secondary antibody in 1 % (w/v) BSA wash buffer. Coverslips were mounted using the SlowFade Light Antifade Kit. Cells were viewed and photographed on a Zeiss Axiovert 100 epifluorescence microscope equipped with a Zeiss AxioCam MRm CCD camera.

The antibodies used for immunofluorescence were M12.13 mouse anti-Cx32 at 1:1200 or mouse anti-HA at 1:200 (primary) with TRITC (tetramethylrhodamine β-isothiocyanate)-conjugated rabbit anti-mouse secondary at 1:50, and rabbit anti-Cx32 IgG primary (gift from Dr Bruce Nicholson, Department of Biochemistry, University of Texas Health Sciences Center, San Antonio, TX, U.S.A.) at 1:400 with FITC-conjugated swine anti-rabbit secondary at 1:50 dilution.

**Dye-coupling ‘parachute’ assay**

The assay was performed essentially as described by Goldberg et al. [31]. Donor and receiver cells were grown to confluence and induced simultaneously with 1 µg/ml doxycycline for 48 h in 35 mm cell culture dishes. The donor cells were double-labelled with 5 µM CM-DiI, a gap junction-impermeable membrane dye, and 5 µM calcein-AM, which is converted into the gap junction-permeable dye calcein intracellularly, for 30 min. The donor cells were then trypsinized and seeded on to the receiver cells at a 1:150 donor/receiver ratio. The cells were allowed to attach for 3–4 h at 37 °C and then examined on a Zeiss Axiovert 100 fluorescence microscope and photographed.

**Connexin purification and tag cleavage**

Cells were seeded at 35 % confluence and induced for 48 h with 1 µg/ml doxycycline in four 500 cm² dishes (Corning Inc., Corning, NY, U.S.A.). Each dish was washed three times with 25 ml of cell wash buffer (0.01 M PBS, 0.138 M NaCl, 0.02 % NaN3, pH 7.4) and solubilized with 20 ml of solubilization buffer (50 mM NaH2PO4, 50 mM NaCl, 5 mM EDTA, 5 mM EGTA, 60 mM OG, 1 mM β-mercaptoethanol, 0.5 mM DFP, 0.75 mg/ml azolectin, pH 7.5) for 2 h at 4 °C with gentle rocking. Solubilization of gap junctions with OG has been shown previously to yield connexin hemichannels [19]. The lysate was transferred to a beaker and incubated on ice for 10 min, followed by centrifugation at 100 000 g for 30 min at 4 °C in a Beckman Ti45 rotor to remove unsolubilized material. The supernatant was incubated with 0.25 ml of agarose-immobilized anti-HA mouse IgG clone HA-7 in a 150 ml glass bottle overnight at 4 °C with shaking. The antibody matrix was collected by gentle centrifugation (700 g for 1 min at 4 °C) and transferred to a column. It was then washed by gravity flow with 20 ml of high-salt wash solution (0.01 M PBS, 1 M NaCl, 80 mM OG, 1 mg/ml azolectin, pH 7.4) followed by 20 ml of wash solution (0.01 M PBS, 0.138 M NaCl, 80 mM OG, 1 mg/ml azolectin, pH 7.4). The protein was eluted with 4 ml of elution buffer (50 mM sodium acetate, 500 mM NaCl, 10 mM KCl, 1 mM EDTA, 80 mM OG, pH 4.0) and 0.6 ml fractions were collected into tubes containing 0.05 ml of neutralization buffer (1 M NaHCO3, 0.01 M KCl, 80 mM OG, pH 9). The final pH of all samples was verified to be in the 7.3–7.5 pH range.

Tag cleavage was carried out using restriction-grade thrombin (Novagen Inc., Madison, WI, U.S.A.). A 200 µl aliquot of purified protein was incubated with 2 units of thrombin for 0–36 h at 4 °C; an equivalent volume of thrombin storage/dilution buffer was used as a control. To stop the digestion, DFP was added to a final concentration of 0.75 mM. A 180 µl aliquot of the reaction was used for reconstitution, and the remainder for Western blotting.
Connexin reconstitution and TSF (transport-specific fractionation)

Hemichannel reconstitution and TSF were carried out as described previously [20]. Briefly, 20 µl of a dried 10 mg/ml phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine lipid mixture (2:1:0.03 molar ratio) was solubilized using 180 µl of the purified protein solution. Rhodamine-labelled phosphatidylethanolamine was used to allow visualization of liposomes. A 20 µl aliquot of 10× urea buffer (4.59 M urea, 0.01 M KCl, 0.01 M NaHCO3, 0.1 mM EDTA, pH 7.4) was then added and the solution incubated at 4 °C overnight. The reconstitution was carried out on a gel filtration column equilibrated with 1× urea buffer (0.459 M urea, 0.01 M KCl, 0.01 M NaHCO3, 0.1 mM EDTA, pH 7.4). Elution fractions containing rhodamine-labelled liposomes were pooled prior to TSF.

TSF was used to assess the permeability of the reconstituted connexin hemichannels to two solutes – urea and sucrose. The proteoliposomes were loaded on to an iso-osmolar linear urea/sucrose density gradient and centrifuged in a Beckman SW 60 Ti rotor at 300,000 g for 3 h at 37 °C. Liposomes that do not contain any active channels are buoyed by the entrapped less dense urea solution and form a diffuse band near the top of the gradient. Liposomes that contain active channels permeable to urea and sucrose are able to equilibrate the internal urea/sucrose solution with the denser external urea/sucrose solution, and hence move down in the gradient to form a sharp band at the density level equivalent to the lipid density. The bands can be then be visualized by illumination of the gradient from the top through a green (no. 58) Wratten filter and observation or photography through a red (no. 23A) Wratten filter. The upper (inactive) and lower (active) bands can be recovered from the gradient and the overall channel activity calculated from the ratio of the fluorescence of the lower band to that of the upper band.

IP3 (inositol 1,4,5-trisphosphate) permeability assay

To assess the permeability of the reconstituted hemichannels to IP3, 100 µM IP3 was loaded into the liposomes during reconstitution by inclusion on the column and in the protein–lipid solution, as has been described previously for other tracers [20]. Following TSF of the liposomes, the upper and lower bands were recovered and analysed for the amount of entrapped IP3 using a fluorimetric enzyme-linked assay as previously described [32,33]. The IP3/rhodamine ratio of the lower band divided by that of the upper band indicates the percentage of active channels that are impermeable to IP3. Permeability data are represented as means ± S.E.M.

RESULTS

DNA constructs

To enable inducible expression of recombinant connxin with a cleavable C-terminal tag, the pBI-GT vector was created based on pBI-G by insertion into the multiple cloning site of a sequence encoding a 3.3 kDa tag that includes a thrombin cleavage site, an HA epitope and a 6 x HA epitope for Western blotting and immunofluorescence, respectively, with anti-HA antibody. Following induction, connxin expression was detectable as bands of 28 kDa and 33 kDa for representative pBI-G26T and pBI-G32T cell lines respectively (Figure 2A). This is slightly below the predicted molecular mass of the coding sequence plus tag, but connexins are known to migrate anomalously fast on SDS/PAGE gels [40]. The time- and concentration-dependence of doxycycline induction was tested by Western blotting of whole-cell lysates from a representative pBI-G26T cell line (Figure 2B). The results for the other cell lines were similar (not shown), and for all lines connxin expression was achieved following induction with 1.0 µg/ml doxycycline for 48 h. Immunofluorescence analysis of pBI-G26T- and pBI-G32T-transfected cell lines confirmed the localization of tagged connxin to structures corresponding to gap junction plaques (Figure 2C), indicating a lack of interference of the C-terminal tag in channel assembly and trafficking. Immunofluorescence analysis of pBI-26T-32-transfected cells showed co-expression of both connxin isoforms and superimposable staining of gap junctions with antibodies specific for each isoform (Figure 2D).

Functionality of the expressed gap junction channels was assessed by intercellular dye spread using the ‘parachute’ assay. All cell lines had coupling levels above that of control parental HeLa-TetOn cells treated with 1 µg/ml doxycycline (Figure 3). All cell lines except the Cx26tag line showed extensive dye coupling, as indicated by the clusters of calcein-positive receiver cells surrounding the calcein and DiI double-positive donor cells. As expected, Cx26tag-expressing cells showed low coupling efficiency, with calcein transfer detectable in only one or two receiver cells surrounding the calcein and DiI double-positive donor cells.
cells per donor; homomorphic Cx26 channels have been shown to have lower permeability than Cx32 channels to molecules similar in size and charge to calcein [15,41]. Therefore the C-terminal tag does not seem to affect gap junction assembly or significantly alter permeability to low-molecular-mass dyes.

**Purification of HA-tagged connexin hemichannels**

Immunopurification from pBI-26T-32 and pBI-32T-26 cells resulted in the detection of bands on Western blots stained with antibodies specific for Cx26 and Cx32 (Figures 4D and 4E). This is consistent with co-purification of the tagged and untagged isoforms in the form of heteromeric channels. The amount of pure connexin, calculated from band densitometry of gold-stained blots, ranged from 10 to 20 µg, depending on the cell line, in 2.5 ml. Furthermore, purifications from different clonal HeLa cell lines yielded heteromeric channels with different isoform composition ratios. For example, as shown in Figure 4E, one pBI-32T-26 cell line yielded Cx26/Cx32 preparations that had a Cx32/Cx26 ratio of 0.85 (labelled #1), while another yielded channels with a Cx32/Cx26 ratio of 1.1 (#2), indicating a predominance of homomeric Cx32tag channels in the preparation. The isoform ratios of channels purified from the same cell line did not vary between preparations.

**Channel reconstitution and TSF**

Purified connexin hemichannels were reconstituted into rhodamine-labelled unilamellar phospholipid vesicles by gel filtration. The protein/lipid ratio used during reconstitution was such that 20–60% of liposomes had active channels. Channel activity was ascertained by TSF. The proteoliposomes were centrifuged though linear iso-osmolar urea/sucrose density gradients to separate liposomes containing active channels (lower band) from those containing inactive channels or no channels (upper band). Figure 5 shows photographs of TSF tubes containing homomeric Cx26tag, homomeric Cx32tag, heteromeric Cx26tag/Cx32 and heteromeric Cx32tag/Cx26 proteoliposomes. All connexin preparations produced active channels. Material obtained by purification from wild-type HeLa-TetOn cells did not produce a bottom band in TSF.
Figure 2  Induction and localization of tagged Cx26 and Cx32 in stable cell lines

(A) Western blot probed with mouse anti-HA IgG showing induction of Cx26tag and Cx32tag expression following 48 h of treatment of pBI-G26T and pBI-G32T stable cell lines, respectively, with 1 µg/ml doxycycline (DOX). (B) Western blot (mouse anti-HA IgG) showing a time course for the induction of Cx26tag expression using 0.1 and 1.0 µg/ml doxycycline for 24, 48 and 72 h. (C) Immunofluorescent localization of connexin in pBI-G26T and pBI-G32T stable cell lines with mouse anti-HA IgG and TRITC-labelled secondary antibody following 48 h induction with 1 µg/ml doxycycline, compared with the same pBI-G26T stable cell line without induction. Arrows indicate punctate staining at sites of cell–cell contact indicative of gap junction plaque formation. (D) Immunofluorescent co-localization of Cx26tag and Cx32 in a pBI-26T-32 stable cell line using rabbit anti-Cx26 IgG with FITC-labelled secondary antibody (left) and mouse anti-Cx32 IgG with TRITC-labelled secondary antibody (right). The arrows point to three large gap junction plaques. The staining of the two connexins is completely superimposable. Bar = 5 µm.

indicating that the urea/sucrose permeability of the liposomes was attributable to the expressed connexin channels.

Permeation properties of Cx26tag/Cx32 hemichannels

Since urea and sucrose are not the likely endogenous permeants through gap junction channels, the permeability of the recombinant channels to IP₃, an intracellular messenger molecule and physiological permeant [33,42,43], was measured and compared with that of Cx26/Cx32 hemichannels purified from mouse liver. The liposomes were loaded with IP₃ during channel reconstitution and analysed by TSF. The upper (inactive) and lower (active) liposome bands were recovered from the TSF gradients and analysed for IP₃ content using an enzymic fluorimetric assay [32,33]. There was no statistically significant difference between the fraction of functional hemichannels (lower band in TSF) permeable to IP₃ in the mouse Cx26/Cx32 and HeLa Cx26tag/Cx32 preparations (mouse, 84 ± 3.2%; HeLa, 74 ± 7.1%). The permeability is less than 100% due to the range of channel stoichiometries and arrangements present in each type of preparation, with some being permeable and some impermeable (see [13] for an explanation of analysis methods). These results indicate that
Figure 3  Dye transfer assay of stable connexin-transfected cell lines

Calcein/CM-DiI double-labelled donor cells were 'parachuted' on to unlabelled confluent receiver cells following induction. The presence of dye transfer is indicated by clusters of calcein-labelled receiver cells surrounding the double-labelled donor cells (marked with *). Insets are transmitted light images of a portion of the field to show confluence level of cell monolayer. WT, wild type. Bar = 50 µm.

The recombinant channels are capable of performing a function of native channels, i.e. permeability to IP₃, and have a comparable channel isoform distribution.

DISCUSSION

We describe here the development of a heterologous expression system and purification procedure that allows the non-denaturing purification of any connexin isoform, including heteromeric channels of various stoichiometries, from cells capable of forming gap junctions in amounts sufficient for biochemical and functional studies. This allows in vitro analysis of channel permeability using native and genetically modified connexins.

The Tet-On expression system has been widely used for the overexpression of toxic proteins, for studying effects of gene dosage, and for transgenic conditional knockout technology [44–46]. It has also been used for regulated connexin expression in studies examining the effects of connexin dosage on cell growth and other cellular functions [47–51]. Here we show that the technique can be applied to the expression of membrane proteins in mammalian cells at sufficient levels to consistently allow the purification of tens of micrograms of protein.

An alternative approach to the expression of heteromeric channels is the use of pIREs (internal ribosomal entry site) vectors to express two connexin isoforms from a single polycistronic mRNA. However, we found that translation efficiency from the second site was too low, yielding mostly homomeric channels consisting of the isoform cloned into the first site.

The pBI-GT vector that we developed facilitates the addition of a cleavable C-terminal double tag to the protein, leaving open the option of using either of the two purification methods, metal affinity or HA immunoaffinity, or both in tandem, to increase product purity. The ability to obtain material purified to near homogeneity from an expression system where the target protein constitutes a very small fraction of total protein using a single immunoaffinity purification step was quite surprising. We attribute it to a carefully selected set of binding, washing and elution conditions in conjunction with the high affinity and specificity of the HA-7 clone anti-HA antibody.

The level of stable connexin expression obtained using this inducible system far surpassed that attainable using constitutive cytomegalovirus promoter-driven expression (not shown), and the cell lines showed remarkable stability. All HeLa cell lines that had inducible gap junctions at initial screening retained their phenotype through at least 25 passages, and there was no connexin-related toxicity evident in these cells. In cell lines identified initially by high connexin expression but with high background (presence of gap junctions in the absence of induction), expression was nearly lost by passage 25.

It is interesting to note that, following selection with hygromycin, >90% of clones were positive for either connexin expression by Western blot (pBI-26T-32 and pBI-32T-26) or β-galactosidase activity by X-Gal staining (pBI-G26T and pBI-G32T). However, only approx. 5% of all clones had gap junctions detectable by immunofluorescence, while the rest were characterized by barely detectable diffuse cytoplasmic staining. Therefore, in the case of connexin overexpression, detection of the protein on Western blots is not a sufficient screening method to identify cell lines that form gap junctions.

The combination of the bidirectional promoter and a C-terminal purification tag in the pBI-GT vector enables the use of this system to assay protein-protein interactions in mammalian cells using a single expression vector, and would be especially useful if one of the proteins is toxic. By insertion of a ‘bait’ protein into the tagging site and a potential binding partner into the second site,
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Figure 4 Gold-stained and Western blots of HA-purified connexin, and tag cleavage by thrombin

(A) Gold-stained and Western blots (mouse anti-Cx26, α-Cx26) of protein purified from a pBl-G26T cell line show a Cx26tag band migrating at 28 kDa. (B) Western blot (mouse anti-Cx32) of a purification from pBl-G32T cells shows three bands; the most abundant one at 33 kDa is full-length Cx32tag. The two lower-molecular-mass bands are C-terminal cleavage products of the Cx32tag, as shown by lack of reactivity with anti-HA mouse IgG (not shown). The two higher-molecular-mass Cx32 dimer bands are detectable on the gold-stained blot of the same preparation (left). A 40 kDa contaminant occasionally detected on gold-stained blots was identified as a fragment of cytokeratin 10 (K10) by tryptic digestion and MALDI-TOF MS (not shown). K10 is not appreciably expressed in HeLa cells [52] and is therefore probably a post-purification contaminant rather than an interacting co-purified protein. (C) Mouse anti-Cx26 Western blot of Cx26tag after incubation of 200 µl of protein with 2 units of thrombin (+) for 7 h shows essentially complete cleavage compared with incubation in thrombin dilution buffer alone (−). (D) Gold-stained and Western blots (mouse anti-Cx26 and mouse anti-Cx32) of protein purified from a pBl-26T-32 cell line indicate co-purification of a 30 kDa Cx32 band and a 28 kDa Cx26tag band. (E) Gold-stained blots of purifications from two different pBl-32T-26 cell lines show the ability to purify heteromeric channels of different isoform stoichiometries (see the text for details).

Figure 5 TSF of HA-purified reconstituted proteins

Cx26tag, Cx32tag, Cx28tag/Cx32 and Cx32tag/Cx26 preparations all produced two bands in TSF. TSF of reconstituted material purified from wild-type HeLa-TetOn cells resulted in an upper band only.

<table>
<thead>
<tr>
<th>Wild type HeLa</th>
<th>Cx26tag</th>
<th>Cx32tag</th>
<th>Cx28tag/Cx32</th>
<th>Cx32tag/Cx26</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Upper band</td>
<td>Lower band</td>
</tr>
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</table>

one can ensure the simultaneous expression of both proteins in all transfected cells, and can conveniently purify the complex using the C-terminal tag on the bait protein to determine the stoichiometry of association.

In summary, the technique presented here comprehensively addresses many desired features of a heterologous connexin expression and purification system. Specifically, the cytotoxicity of constitutive connexin overexpression is addressed by the use of Tet-On regulation. Purification, and monitoring of the expression level and cellular localization of connexin, are addressed by the tandem HA and (HN)₆ tags. Possible functional effects of the tag can be avoided by its cleavage with thrombin. A stable ratio of co-expression of connexin isoforms is addressed by the use of a single, bidirectional promoter. These are substantial improvements over existing methods for the purification of connexin channels from mammalian cells.

This work was supported by NIH grants GM36044 and GM61406 to A.L.H. We cordially thank Dr Thaddeus A. Bargiello (Albert Einstein College of Medicine, New York, NY, U.S.A.) for providing the connexin cDNA templates, Dr John P. Reeves (New Jersey Medical School) for allowing extensive use of his fluorescence microscope, and Dr Bruce J. Nicholson for the gift of the anti-Cx26 rabbit antibody. We also thank Dr Robert J. Donnelly (New Jersey Medical School) for fruitful discussions.
Purification of connexin channels