

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

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Intercellular coupling mediated by gap junction channels composed of connexin protein underlies numerous physiological processes, such as cellular differentiation, tissue synchronization and metabolic homeostasis. The distinct molecular permeability of junctional channels composed of different connexin isoforms allows cellular control of coupling via regulation of isoform expression. However, the permeability properties of most connexin isoforms have not been well characterized due to the difficulty of manipulating and measuring the diffusible concentrations of cytoplasmic messenger molecules and metabolites, and to a lack of control over channel isoform composition, *in vivo*. Here we present a method to express and purify active connexin hemichannels of a single isoform or a consistent ratio of two isoforms from cultured cells using the Tet-On inducible expression system and one-step anti-haemagglutinin immunoaffinity purification.

The procedure yields 10–20 μg of pure connexin protein from 2.5×10^8 HeLa cells. The purified channels are shown to be useful for *in vitro* permeability analysis using well established techniques. This method has substantial advantages over existing methods for heterologous connexin expression, such as the ease of co-expression of two isoforms at a constant ratio, consistently high expression levels over many passages, and the ability to study channel properties *in situ* as well as in purified form. Furthermore, the generic cloning site of the new pBI-GT vector and the commercial availability of anti-haemagglutinin (clone HA-7)–agarose make this affinity tagging and purification procedure easily applicable to other proteins.

Key words: connexin, gap junction, hemichannel, inducible expression, permeability, purification.

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 cytoplasm 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 (homomeric) or from subunits of different isoforms (heteromeric). 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)₆, (His-Asn)₆; IP₃, inositol 1,4,5-trisphosphate; MALDI-TOF, matrix-assisted laser desorption ionization–time of flight; OG, n-octyl β -D-glucopyranoside; rTA, reverse tetracycline-controlled transactivator; TRE, tetracycline response element; TRITC, tetramethylrhodamine β -isothiocyanate; TSF, transport-specific fractionation.

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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.

pBI-G26T and pBI-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.

pBI-26T-32 and pBI-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-Cx26 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 μ l of a dried 10 mg/ml phosphatidylcholine/phosphatidylserine/phosphatidylethanolamine lipid mixture (2:1:0.03 molar ratio) was solubilized using 180 μ l of the purified protein solution. Rhodamine-labelled phosphatidylethanolamine was used to allow visualization of liposomes. A 20 μ l aliquot of 10 \times 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 \times 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 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 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 μ M 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 \pm S.E.M.

RESULTS

DNA constructs

To enable inducible expression of recombinant connexin 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 \times HN tag. To obtain a C-terminally tagged protein as similar as possible to the native sequence, thrombin was used because it leaves only four amino acids (Leu-Val-Pro-Arg) attached the protein after cleavage [34]. There is no protease currently available that allows complete removal of a C-terminal tag. An N-terminal tag could not be used due to our findings that N-terminally His-tagged connexins failed to form gap junction plaques (not shown), and other reports indicating that placement of tags on the N-terminus affected either channel gating and conductance or the ability to form functional channels, depending

on the connexin isoform [35–37]. On the other hand, C-terminal tags have at most minimal effects on channel assembly, and functional differences are limited to effects on voltage dependence [35,37,38]. Although a C-terminal tag may affect gap junction plaque assembly by blocking interactions with protein binding partners, as in the case of the interaction of the Cx43 C-terminus with ZO-1 [39], the connexins examined in the present study are not known to interact with ZO-1 or other proteins.

The *Spe*I restriction site incorporated into the first 5 nucleotides of the thrombin cleavage site sequence allows in-frame ligation to any protein-coding sequence, as described in the Materials and methods section. Thus any protein can be cloned in-frame adjacent to the tag without the addition of extra amino acids. This cloning strategy is illustrated in Figure 1 using the Cx26 sequence to create the vector pBI-G26T.

Establishment of stable gap junction-expressing HeLa cells

Stable connexin-expressing HeLa cells were obtained following transfection of HeLa-TetOn cells with the pBI-G26T (homomeric Cx26tag), pBI-G32T (homomeric Cx32tag), pBI-26T-32 (heteromeric Cx26tag/Cx32) and pBI-32T-26 (heteromeric Cx26/Cx32tag) vectors. After selection, 100–150 stable clones from each transfection were screened for either β -galactosidase or connexin expression, followed by immunofluorescence-based detection of gap junction formation. The criteria for determining stable connexin channel expression were the presence of: (1) gap junctions detectable by indirect immunofluorescence and (2) intercellular dye spread in response to doxycycline induction through 25 passages in culture.

Because overexpression of connexin is cytotoxic, inducibility of connexin expression was confirmed by Western blotting or immunofluorescence, and only inducible cell lines were propagated to ensure expression stability in culture. Furthermore, connexin expression levels sufficient for functional analysis of purified protein and proper trafficking of the C-terminally tagged connexin were confirmed by Western blotting and immunofluorescence, respectively, with anti-HA antibody. Following induction, connexin 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 connexin expression was achieved following induction with 1.0 μ g/ml doxycycline for 48 h. Immunofluorescence analysis of pBI-G26T- and pBI-G32T-transfected cell lines confirmed the localization of tagged connexin 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 connexin 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 μ g/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

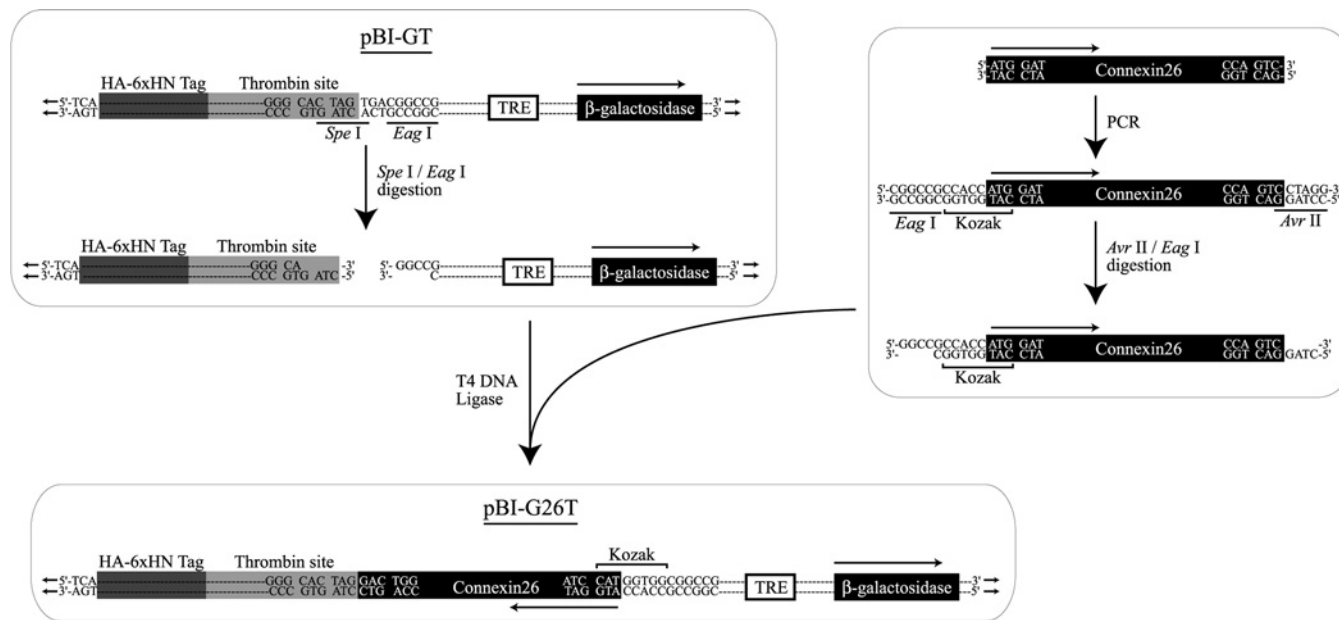


Figure 1 Subcloning Cx26 cDNA into the pBI-GT vector

The vector contains the sequence for a C-terminal thrombin cleavage site followed by HA and (HN)₆ tags and a stop codon. The vector is prepared for cloning by digestion with *Eag*I and *Spe*I. The connexin sequence, which ends in a cytosine residue (without a stop codon), is inserted adjacent to the thrombin cleavage site using *Eag*I (5') and *Avr*II (3') overhangs generated by digestion of the PCR-amplified sequence. A Kozak consensus translation start site (CCACCATG) was incorporated into the sequence as part of the forward PCR primer to produce higher expression levels [27]. Note that ligation of the insert cDNA into the pBI-GT vector requires flipping of the sequence, resulting in transcription from the minus strand. The individual domains represented by shaded blocks and the spaces between them are not drawn to scale. Only the relevant portions of the circular plasmids are shown in linear form to conserve space.

cells per donor; homomeric 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

The recombinant channels solubilized in OG were immunoaffinity purified using a monoclonal anti-HA antibody (clone HA-7). We found that the tagged protein could be bound completely to immobilized HA-7 antibody matrix and eluted efficiently using a high-salt pH 4.0 pulse. Gold-stained blots and Western blots of the purified material from pBI-G26T and pBI-G32T cells confirmed the presence of Cx26tag and Cx32tag respectively (Figures 4A and 4B). Elution with HA peptide was not efficient, even following a 1 h incubation with 1 mg/ml peptide (results not shown). We found that inclusion of 0.5 M NaCl in the elution buffer allowed the use of the relatively mild pH 4 elution conditions with approx. 50% elution efficiency (assuming 100% at pH 2.5), compared with <10% elution with 0.15 M NaCl (results not shown). The protein was purified to near homogeneity and the identity of the bands on zinc-stained gels was ascertained by in-gel tryptic digestion and MALDI-TOF (matrix-assisted laser desorption ionization–time of flight) MS.

Essentially complete cleavage of the tag by thrombin could be achieved without non-specific degradation of connexin (Figure 4C). Tag cleavage did not affect channel activity measured by TSF. Purification of connexin using immobilized metal affinity chromatography (Ni²⁺ or Co²⁺) with imidazole elution failed to achieve purity comparable with that achieved using the anti-HA resin. Pure connexin could be obtained by thrombin-mediated cleavage/elution from Co²⁺ beads, but with unacceptably low yield.

Purification of heteromeric 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 4(E), one pBI-32T-26 cell line yielded Cx26/Cx32tag preparations that had a Cx32tag/Cx26 ratio of 0.85 (labelled #1), while another yielded channels with a Cx32tag/Cx26 ratio of 11 (#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 through 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,

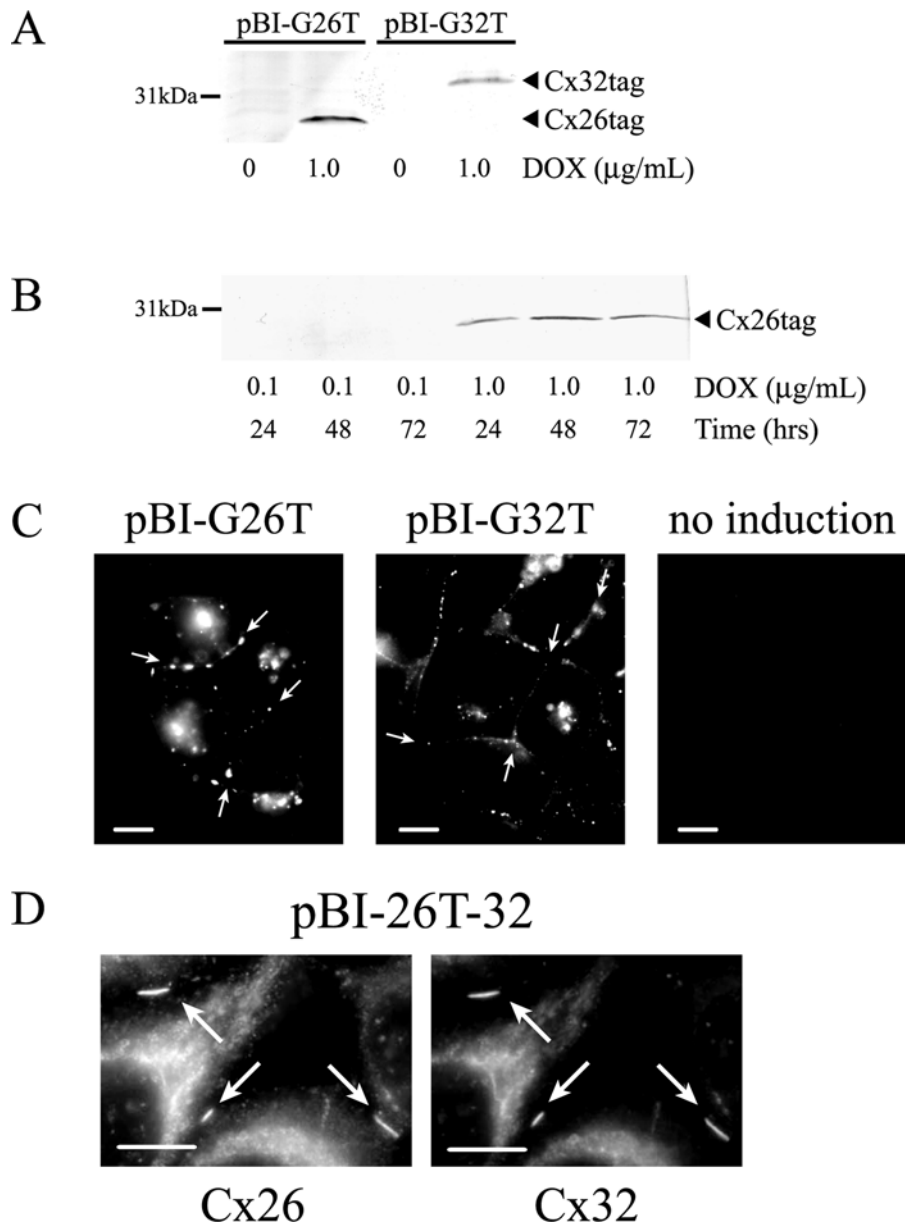


Figure 2 Induction and localization of tagged Cx26 and Cx32 in stable cell lines

(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

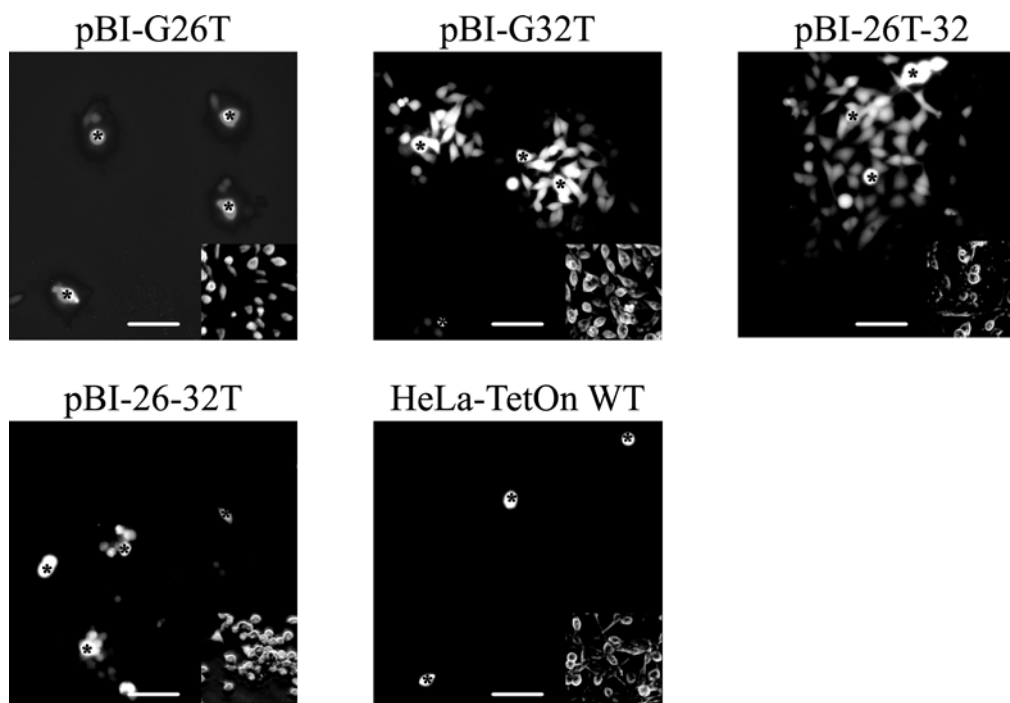


Figure 3 Dye transfer assay of stable connexin-transfected cell lines

Calcein/CM-Dil 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 IP_3 , 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,

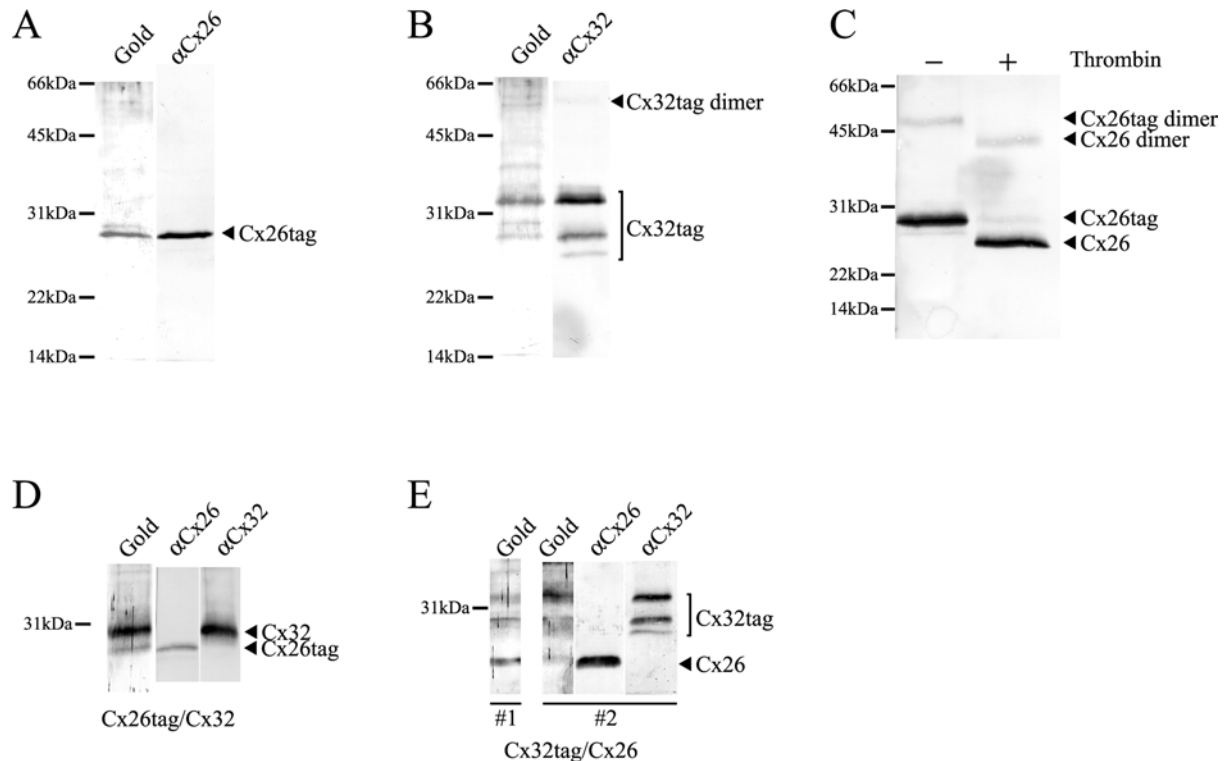


Figure 4 Gold-stained and Western blots of HA-purified connexin, and tag cleavage by thrombin

(A) Gold-stained and Western blots (mouse anti-Cx26; α Cx26) of protein purified from a pBI-G26T cell line show a Cx26tag band migrating at 28 kDa. (B) Western blot (mouse anti-Cx32) of a purification from pBI-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 μ l 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 pBI-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 pBI-32T-26 cell lines show the ability to purify heteromeric channels of different isoform stoichiometries (see the text for details).

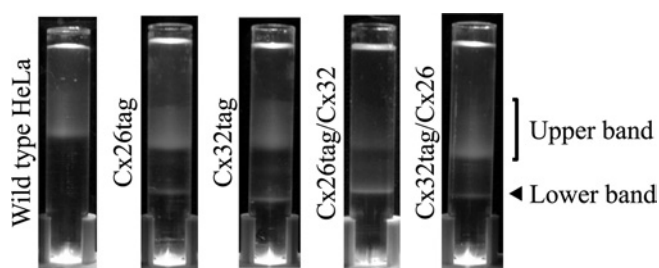


Figure 5 TSF of HA-purified reconstituted proteins

Cx26tag, Cx32tag, Cx26tag/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.

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.

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