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Expression of gap junction channels in communication-incompetent cells after stable transfection with cDNA encoding connexin 32

(ion channels/SKHep1 hepatoma cells/electrical coupling/single channel recording/immunocytochemistry)

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ABSTRACT **The gene family encoding gap junction proteins (connexins) consists of several known members, and multiple connexins are frequently coexpressed by coupled cells. To characterize the channel properties of the major rat liver gap junction protein (connexin 32) in isolation from other gap junction proteins, we have introduced the cDNA encoding it into a human hepatoma cell line (SKHep1) in which we have identified a gap junction deficiency. In this cell line, dye coupling was absent and junctional conductance was near zero. Connexins and connexin 32 mRNA were not detectable by immunocytochemistry and Northern blot analysis. After transfection and selection, cells were strongly coupled with regard to dye and electrical current, and connexin 32 mRNA and punctate connexin 32-immunoreactive membrane contacts were abundant. Functional gap junction channels were still expressed after 19 passages of the cells, indicating stable transfection. When junctional conductance was rendered reversibly low by exposing the cells to agents that uncouple other cell types, currents through single gap junction channels could be observed. The unitary conductance of these expressed channels was about 120–150 pS, a value that is distinctly larger than in heart cells, which express a different gap junction protein.**

Gap junction channels provide a pathway for exchange of metabolites and second messenger molecules between cells of most tissues and transformed cell lines (see refs. 1 and 2). The cDNAs encoding gap junction proteins [connexins (3)] in a variety of tissues have recently been sequenced, revealing strong overall sequence homologies and a similar predicted structural motif (3–10). Despite these similarities, the functional properties of gap junctions in various tissues differ (11) and determination of properties specific for each connexin is complicated by coexpression of two or more proteins (12–14). Furthermore, studies of regulation of expression and of the roles played by gap junctions in cellular processes would be aided by controlled manipulation of connexin molecules in a common cell type.

One approach to this problem has been the use of the *Xenopus* oocyte expression system (8, 15–17). This approach is potentially compromised by the expression of endogenous connexins; furthermore, the large size and low input resistance of the *Xenopus* oocyte do not permit identification of the unitary conductances of gap junction channels, which can be obtained only with whole cell recording from cell pairs. Another approach, used previously for study of another membrane channel protein [the acetylcholine receptor (18)], is to introduce cDNA encoding the protein into a cell line in which expression is normally lacking.

We here report the identification of a cell line (SKHep1 cells) in which coupling is quite low or altogether absent, and in which connexins and connexin 32 mRNA were not detectable. We have transfected these cells with a vector containing connexin 32 cDNA and have established cell lines that stably express connexin 32 and its mRNA and are strongly coupled with respect to dye and electrical current. We have used these cells to resolve unitary junctional currents corresponding to connexin 32 gap junction channels.

MATERIALS AND METHODS

Cell Culture. The experiments described here were undertaken with SKHep1 cells, which are derived from a highly metastatic human hepatoma (19, 20). Cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (GIBCO) and were routinely fed every 5 days and subcultured at confluence.

Immunocytochemistry. Cells were plated on glass coverslips and cultured until they approached confluence. Cells were fixed in 70% (vol/vol) ethanol at −20°C for 20 min, rinsed in Dulbecco's phosphate-buffered saline (PBS), and incubated for 1 hr at room temperature in monoclonal antibody R5.21 (ref. 21; 1:50 dilution of culture supernatant). The second incubation was carried out for 1 hr at room temperature in the presence of fluorescein-labeled goat antibody to rat IgG (1:100 dilution). After rinsing in PBS, a drop of *p*-phenylenediamine/glycerol [0.1% *p*-phenylenediamine in a 10:1 (vol/vol) mixture of glycerol and PBS] was added to the coverslip, which was inverted onto a microscope slide. Epifluorescence was examined on a Nikon diaphot microscope using the B filter set.

Northern Blots. Double-stranded connexin 32 cDNA which had been inserted into the *Eco*RI site of plasmid pGEM-3 was isolated by *Eco*RI site digestion. This plasmid was used to transform *Escherichia coli* strain HB101 and was isolated by guanidine thiocyanate/CsCl gradient centrifugation (22). Connexin 32 cDNA was ligated in the plasmid pcEXV-3 (purified as described above) after being linearized and dephosphorylated at its unique *Eco*RI site. RNA samples were prepared by the guanidine thiocyanate/CsCl method (22); 7 μg of total RNA was loaded into each gel lane. Agarose gels were blotted onto nitrocellulose and hybridized at 65°C, using a 0.5-kilobase (kb) gap junction cDNA probe for riboprobe hybridization.

Transfection and Selection. Plasmid DNA [10–20 μg of pcEXV-3 containing connexin 32 cDNA insert (Fig. 2*A*) and 1–2 μg of pSV2-neo] were introduced into SKHep1 cells (approximately 5×10^5 cells per 100-mm dish) by using the calcium phosphate precipitation technique (23, 24). Control cells were untreated or treated with pSV2-neo alone. The cells were incubated for 4–6 hr in the presence of calcium phosphate and then subjected to a 2.5-min glycerol shock (24). Between 24 and 48 hr after exposure to DNA, the antibiotic G418 (0.4–0.5 mg per dish) was added to the medium. Medium containing the drug was changed every 5

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days. After 2–3 weeks colonies judged well coupled with regard to intercellular Lucifer yellow diffusion were picked by trypsinization in cloning cylinders and maintained separately for analysis.

Lucifer Yellow Injection. Lucifer yellow CH (5% in 150 mM LiCl) was injected iontophoretically through microelectrodes (resistance 20–50 MΩ) under sterile conditions and cells were examined with a Nikon diaphot microscope equipped with xenon epiillumination and the Nikon B fluorescence filter set.

Electrophysiology. Dual whole cell voltage clamp used polished patch electrodes [3–6 MΩ, containing 135 mM potassium glutamate, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 10 mM ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 0.5 mM $CaCl_2$, 10 mM KCl, 5 mM glucose, and 5 mM NaATP; adjusted to pH 7.2 with KOH]. High-resistance (1–10 GΩ) seals of pipettes to surfaces of each cell were obtained with the aid of gentle suction; cell membranes under the electrode were ruptured with brief strong suction. Cells were voltage clamped to a common holding potential (usually −40 mV) and junctional conductance was calculated from current through junctional membrane evoked by command steps as described in the legend of Fig. 4.

RESULTS AND DISCUSSION

SKHep1 Cells Are Normally Deficient in Gap Junction-Mediated Intercellular Communication. Human hepatocytes normally express a gap junction protein (5) that is highly homologous to the major gap junction protein of rat liver, connexin 32 (4). Immunofluorescence with a monoclonal antibody prepared against connexin 32 (21) did not reveal discrete intercellular gap junction contacts in cultured SKHep1 cells (Fig. 1*A*), in contrast to strongly positive staining of dissociated rat hepatocytes and in sections of rat liver (25). Similarly, no discrete staining was observed in experiments using polyclonal antibodies to rat connexins 32, 26, or 43 (negative data not shown).

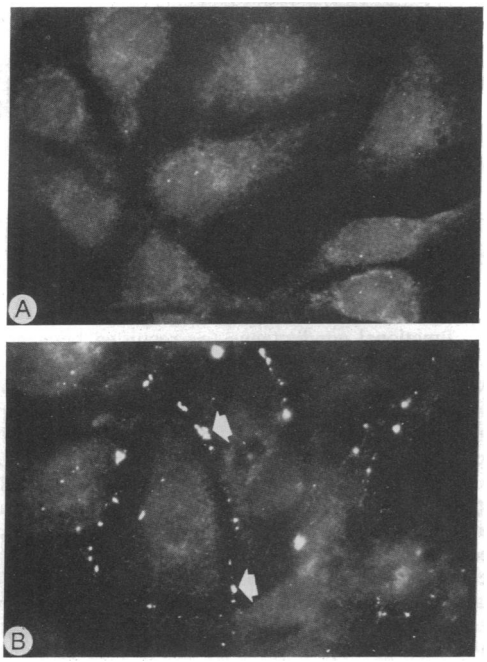

FIG. 1. Immunofluorescence with anti-connexin 32. (×600.) The major rat liver gap junction protein, connexin 32, is absent between normal SKHep1 cells (*A*) but is abundant after transfection with connexin 32 cDNA (*B*). Punctate immunofluorescence at the interfaces of transfected cells, presumably corresponding to intercellular gap junction plaques, is indicated by arrows in *B*.

Consistent with the lack of antibody recognition of gap junction proteins, connexin mRNAs were not detected by using hybridization to connexin 32 antisense RNA (riboprobe; Fig. 2*B*) or to a random-primed connexin 32 cDNA probe under conditions of either high or low stringency (not illustrated). Although we have performed hybridization studies using probes for only connexin 32 cDNA, it should be realized that one strategy used to clone the other connexins involved hybridization to connexin 32 at low stringency (3, 6–9); our findings suggest that mRNAs corresponding to members of the connexin gene family are not abundant, and are possibly even absent, in normal SKHep1 cells.

Our electrophysiological and immunocytochemical findings more conclusively demonstrate the gap junction deficiency in these cells. SKHep1 cells are not coupled with respect to the dye Lucifer yellow CH (Fig. 3*A*), whose intercellular diffusion is diagnostic of the presence of patent gap junction channels between cells. High-resolution electrical recordings using the dual whole cell recording technique with patch-type pipettes revealed in most cell pairs that coupling was totally absent. [In these experiments we occasionally were able to record a low degree of electrical coupling: In 4 of 52 cell pairs where resolution of low junctional conductance (g_j) was possible (nonjunctional resistances of both cells 0.5 GΩ or higher), g_j was between 30

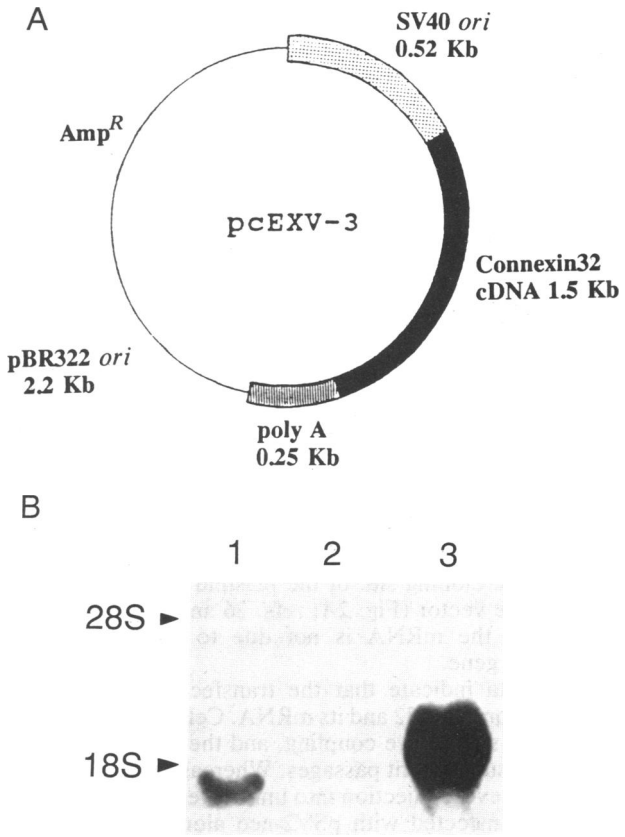

FIG. 2. (*A*) Vector used in this study, with its connexin 32 insert. The plasmid used for transfection consisted of pcEXV-3, a hybrid of plasmids pcDV1 and pL1 (26, 27) containing the simian virus 40 origin of replication (SV40 *ori*), a segment of the ampicillin resistance gene (Amp^R), and SV40 late region polyadenylylation signal (poly A) of sizes indicated, and connexin 32 insert. (*B*) mRNAs detected in transfected and untransfected cells by using hybridization to the connexin 32 probe. mRNA encoding connexin 32 is undetectable in Northern blots of SKHep1 cell extracts (lane 2) and is expressed in transfected cells (lane 3) as a hybridization product of slightly larger molecular weight than the corresponding liver mRNA (lane 1). Blot was exposed for 20 hr at −70 to −80°C.

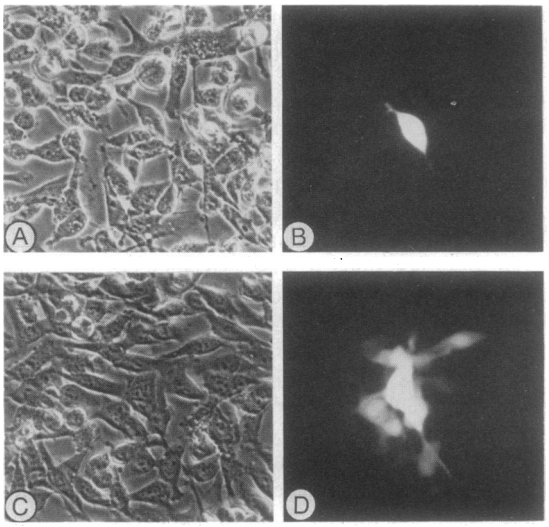

FIG. 3. (*A* and *C*) Phase-contrast micrographs. (*B* and *D*) Fluorescent micrographs of same cells. Micrographs were taken 1–2 min after injection of Lucifer yellow. (×300.) Dye injected into single SKHep1 cells (brightest cell in each field) is confined to the injected cell in controls (*A*, *B*) but spreads rapidly to adjacent cells in transfected colonies (*C*, *D*).

and 500 pS; in the remaining 48 pairs g_j was below the limit of resolution (20 pS or less).]

Connexin 32-Transfected Cells Express Gap Junction-Mediated Intercellular Communication. Punctate immunofluorescent staining with antibodies to connexin 32 was seen to various extents in both of the transfected colonies that were tested; a typical staining pattern is shown in Fig. 1*B*. In addition to brilliant staining of appositional regions (arrows, Fig. 1*B*), staining of cytoplasmic locations was sometimes observed (not illustrated). Northern blot analysis detected mRNA hybridizing to the connexin 32 probe in all four of the transfected and selected colonies that were tested (a Northern blot comparing mRNAs in one of the transfected colonies to those of normal liver cells is shown in Fig. 2*B*). Relative abundance of hybridizing RNA in the transfected cells was apparently even higher than in rat liver. The hybridizing mRNA of transfected cells exhibited a slightly larger size (about 2 kb) than the 1.5- and 1.6-kb native mRNAs from rat and human liver (Fig. 2*B*; cf. refs. 4 and 5). This larger size presumably reflects the expected transcription of a small piece of plasmid DNA [the size difference corresponds to the cDNA in the cloning site of the plasmid plus the promoter region of the vector (Fig. 2*A*; refs. 26 and 27)] and demonstrates that the mRNA is not due to expression of an endogenous gene.

These data indicate that the transfected SKHep1 cells expressed connexin 32 and its mRNA. Cells were selected on the basis of strong dye coupling, and the cells retained this property in subsequent passages. Whereas dye coupling was absent after every injection into untransfected SKHep1 cells or those transfected with pSV2-neo alone (more than 100 intracellular Lucifer yellow injections), injection of dye into cells transfected with connexin 32 revealed rapid spread to adjacent cells and to higher-order neighbors (Fig. 3*D*).

Dye coupling, immunofluorescent staining, and connexin 32 mRNA were stably expressed for at least seven passages after the initial selection (the longest times tested), suggesting stable incorporation of the cDNA into the host genome. [As an additional control, cells were transfected with pSV2-neo alone. Neither positive immunostaining nor connexin 32 mRNA was detected in any of the colonies stably transfected with pSV2-neo alone for as long as five passages (the longest time examined).]

Connexin 32-Transfected Cells Express Gated Gap Junction Channels. The cDNA used in this study encodes connexin 32, the main gap junction protein of rat liver. Properties of rat liver gap junctions [in which connexin 32 is coexpressed with the putative gap junction protein connexin 26 (10, 12, 13)] have been studied by using voltage clamp techniques in dissociated cell pairs (28, 29). Pairs of transfected SKHep1 cells were voltage clamped to determine the extent to which the properties of the gap junction channels expressed by transfectants matched those in normal hepatocytes. Mean junctional conductance of 23 such pairs in 12 separate cultures from three different colonies (the latest examined being the 19th passage) was 5.9 ± 1.2 (±SEM) nS, and conductance was reversibly reduced by 1 mM octanol (not shown) and 2 mM halothane [an agent which selectively blocks junctional channels (ref. 30; Fig. 4*A*)]. Under conditions of low junctional conductance, unitary current steps were detected corresponding to elemental conductances of about 120–150 pS (arrows Fig. 4*B*). In addition, much smaller events (about 20–30 pS) were occasionally seen in the transfected cells (resolved well in only one of 40 single-channel experiments) that fulfilled the criterion of being gap junction channels (equal size and opposite polarity in the two cells); these may correspond to a very low level of expression of an endogenous channel type or may represent infrequent occupancy of a low-conductance substate of the junctional channel.

The gap junction channel formed by connexin 32 thus has a predominant unitary conductance of about 120–150 pS,

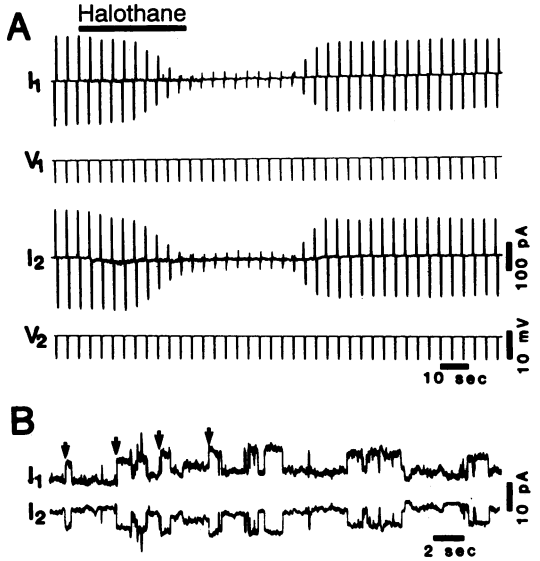

FIG. 4. Junctional conductance (g_j) recorded between voltage clamped pairs of transfected SKHep1 cells. (*A*) Macroscopic g_j was high between dissociated cell pairs (initially about 15 nS in this figure) and was reversibly reduced after exposure to 2 mM halothane. Cells were voltage clamped to a common holding potential (−40 mV) and command steps (V_1, V_2) were alternately delivered to each cell. Currents passed in the pulsed cell (I_1, I_2, downward deflections) represent the sum of currents flowing through junctional and nonjunctional membranes; currents passed through the electrode in the nonpulsed cell (I_1, I_2; upward deflections corresponding to V_2 and V_1, respectively) represent current flow through junctional channels. Junctional conductance is calculated as junctional current divided by the corresponding transjunctional driving force. (*B*) After uncoupling, unitary channel openings and closures were detectable when the transjunctional potential was maintained at 50 mV (a few are indicated by arrows, with conductances ranging from about 140 to 160 pS). For this recording, cell 1 was held at −50 mV, cell 2 at 0 mV after brief exposure of the pair to 2 mM halothane. At the beginning of the record, g_j is less than 10 pS. Upward transitions in I_1 and corresponding downward deflections in I_2 represent gap junction channel openings. Records were filtered at 30 Hz.

which is consistent with sizes of one class of gap junctional channels detected in acinar cells (31, 32) and in isolated liver junctional membranes incorporated into lipid bilayers (33, 34). In these preparations, connexin 32 is coexpressed with at least one other gap junction protein, perhaps explaining why other channel sizes were also recorded. The 120- to 150-pS elemental conductance seen in connexin 32-containing cells and exogenous expression systems is distinctly larger than the unitary gap junction conductance seen between heart cells (50 pS; see refs. 35 and 36), where connexin 43 is the major junctional protein (3). It thus appears that connexin type may be the primary determinant of channel size.

CONCLUSIONS

We conclude that we have achieved the stable transfection of a normally communication-deficient cell line with cDNA encoding connexin 32. The resulting gap junction channels are gated by treatments that reduce junctional conductance between hepatocyte pairs and have a unitary size corresponding to one class of channels seen in cells expressing connexin 32 and in liver junctional membranes incorporated into lipid bilayers. These studies indicate that connexin 32 can form gap junction channels, confirming reports that injection of connexin 32 into oocytes leads to enhancement of intercellular communication after they are paired (15, 17). The expression of gap junctions after transfection of cells which (unlike oocytes) have few or no endogenous gap junctions and which express only the exogenous mRNA (marked by its unique size resulting from an attached portion of its vector) unequivocally demonstrates that connexin 32 cDNA encodes a gap junction protein and that this protein can form intercellular channels in the absence of other connexins.

Identification of the communication-incompetent cell line described here provides another approach to the study of gap junction channels. We have used these cells, together with the technique of stable transfection, to establish the association of the 120- to 150-pS channel with connexin 32 in this study. These findings indicate that there is a diversity of single channel conductances for gap junction channels and that connexin type may dictate gap junction channel properties.

Stable transfection of this communication-incompetent cell line with gap junction cDNAs offers the further opportunity to analyze properties of other connexins and of chimeric pairs of otherwise identical cells expressing different channel types, to test hypotheses of channel gating domains (37) by using site-directed mutagenesis, to study routes of trafficking of the gap junction protein within these cells, and to evaluate whether addition of gap junctions modifies behavior of this highly metastatic cell line.

These studies depended on the availability of full-length cDNA encoding connexin 32 (provided in the vector pGEM by D. Paul, Harvard Medical School), the SKHep1 cell line originally isolated by J. Fogh (Sloan Kettering, Rye, NY) and obtained from L. Reid at Einstein, the pcEXV-3 (provided by J. Ravetch, Sloan Kettering) and pSV2-neo vectors (from K. T. Montogomery), and helpful advice from M. Zern of the Albert Einstein Liver Center. We thank K. Spiegel for advice in riboprobe hybridization procedures. C. Roy performed all the immunofluorescence studies illustrated in this paper, for which we are extremely grateful. We thank R. Dermietzel (University of Regensburg, F.R.G.) for screening untransfected SKHep1 cells by using anti-connexin antibodies; polyclonal antibodies used to substantiate the absence of connexins were provided by O. Traub and K. Willecke (University of Bonn) (connexins 26 and 32), and by E. Beyer (Washington University, Saint Louis, MO) (connexin 43). This work was supported in part by National Institutes of Health Grants NS 16524 (to D.C.S.), NS 20778 (to J.A.K.) and NS 07512 (to M. V. L. Bennett).

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