Restoration of Cell-to-Cell Communication in Thyroid Cell Lines by Transfection with and Stable Expression of the Connexin-32 Gene

IMPACT ON CELL PROLIFERATION AND TISSUE-SPECIFIC GENE EXPRESSION*

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Gap junctions (GJ) are ubiquitous intercellular junctions allowing the cell-to-cell exchange of small cytoplasmic molecules. These exchanges have long been thought to play a role in the regulation of cell growth and cell differentiation in a number of tissues (1, 2). GJ proteins, connexins (Cx),1 belong to a multigenic family, each member presenting its own tissue-specific distribution. Despite strong overall homologies between Cx, functional properties (including permeability properties) differ from one Cx to the other. In most tissues, the determination of the role of a given Cx is complicated by the coexpression of several different Cx (for reviews, see Refs. 3–7). This applies to endocrine glands (8, 9) including the thyroid gland functioning are not known. Cx32 GJ could play a prominent role since Cx32 expression correlates with the expression of thyroid histotypic differentiation, i.e. follicle formation. To try to document the functional impact of cell-to-cell communication via Cx32 GJ, we have chosen to transfact two rat thyroid-derived cell lines (FRT and FRTL-5) that are communication-deficient with the rat Cx32 cDNA. Several clones that stably expressed high levels of Cx32 have been isolated from each cell line. All of them presented a high level of GJ-mediated cell-to-cell communication and exhibited a decreased growth rate as compared with the corresponding parental cells. Interestingly, stable expression of Cx32 by differentiated FRTL-5 cells led to up-regulation of the thyroglobulin gene.

EXPERIMENTAL PROCEDURES

Expression Vector—The 1.5-kb pCx32 cDNA (containing the entire coding region) was isolated from the pGEM-3 plasmid (kindly provided by Dr. D. L. Paul) (13) by EcoRI site digestion. Cx32 cDNA was inserted into the pSVK3 plasmid (Pharmacia Biotech Inc.) after linearization and dephosphorylation at its unique EcoRI site, yielding pSVK3-Cx32. The proper orientation of the cDNA insert was controlled by electrophoretic analysis of KpnI and PstI restriction enzyme digestion fragments and nucleotide sequencing of the appropriate region.

Cell Culture and Establishment of Stable Transfectants—FRT and FRTL-5 cells were kindly provided by Prof. L. Nitsch (Dipartimento di Biologia e Patologia Cellulare e Molecolare “L. Califano,” Universita Degli Studi di Napoli Federico II, (Naples, Italy). These two cell lines, derived from normal thyroids of Fischer rats, have been previously characterized (14, 15). FRTL-5 cells were grown in complete culture medium composed of Coon’s modified Ham’s F-12 medium (Seromed, Berlin, Germany) containing 100 units/ml penicillin and 100 μg/ml streptomycin and supplemented with 5% calf serum and a five-hormone mixture (10 μg/ml insulin, 10 ng/ml growth hormone, 5 μg/ml transferrin, 10 ng/ml glycyly-L-histidyl-L-lysine acetate, and 1 milliunits/ml TSH) as described (15). All hormones were from Sigma. FRT cells were cultured in the same medium as FRTL-5 cells, except that TSH was omitted and calf serum was replaced by fetal calf serum (Sigma). Cell cultures were maintained at 37 °C under a 95% air and 5% CO2 humidified atmosphere and were routinely subcultured by trypsinization with a change of medium twice weekly.

FRT and FRTL-5 cells (∼3–5 × 105 cells/100-mm Petri dish) were cotransfected with two plasmids (pSVK3-Cx32 and pCMV-neo) using the calcium phosphate precipitation procedure followed by a glycerol

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1 The abbreviations used are: GJ, gap junction(s); Cx, connexin(s); TSH, thyrotropin; kbp, kilobase pair(s); TTF-1, thyroid transcription factor-1.
shock. The optimal conditions of transfection were slightly different for the two cell lines. FRT cells were incubated for 3–4 h with calcium phosphate in the presence of 20–30 μg of pSVK3-Cx32 and 2–3 μg of pCMV-neo and then subjected to a 1-min glycerol shock. For FRT-L5 cells, the incubation in the presence of calcium phosphate was reduced to 1 min and then cells were subjected to a 3-min glycerol shock. For both cell types, 48 h after the onset of transfection, the neomycin analogue, G418 (Life Technologies, Inc.), was added to the medium at a concentration of 0.4 mg/ml. The medium containing G418 was changed every 3 days. After 3 weeks of selective pressure in the presence of G418, neomycin-resistant FRT colonies were picked by trypsinization in cloning rings and grown separately under nonselective conditions. In the case of FRT-L5 cells, only neomycin-resistant colonies judged to be well coupled by microinjection of lucifer yellow (see “Measurement of Intercellular Communication”) were picked up and grown for subsequent analyses. For controls, cells were cotransfected under the conditions described above, but with pCMV-neo and the pSVK3 vector lacking Cx32 cDNA. FRT and FRT-L5 transfectants were maintained in the appropriate media containing G418 at a concentration of 0.05 mg/ml.

**Northern Blot Analysis—** cDNAs encoding rat Cx32 (clone Cx32-1; 1.1 kbp), rat Cx32 (1.5 kbp), and rat Cx43 (clone G1; 2.5 kbp) originate from Drs. B. J. Nicholson (16), D. L. Paul (13), and E. C. Beyer (17), respectively. cDNA clone G1 contains 92% of the coding region for Cx43; the other two cDNAs contain the complete coding regions of Cx32 and Cx43. G1 cDNA was extracted from the Bluescript plasmid using EcoRI and then digested with StuI (Promega); this EcoRI-StuI fragment (1.03 kbp; positions 293–1323) was used for hybridization experiments. Cx32 cDNA as well as Cx62 cDNA were extracted from the pGEM-3 plasmid by EcoRI site digestion. The thyroglobulin cDNA probe corresponds to the human thyroglobulin 0.7-kb M1 fragment (18). The rat Pax-8 cDNA fragment (0.3 kbp), corresponding to the paired domain fragment (19), was extracted from the C2H B2/c22 plasmid by EcoRI and HindIII restriction site digestion. The rat TTF-1 cDNA fragment (0.6 kbp), corresponding to the 3′-untranslated region, was extracted from the Bluescript THA plasmid (20). cDNA probes, prepared from DNA fragments isolated by electrophoresis and purified using the QIAEX gel extraction kit (QIAGEN Inc.), were labeled with [α-32P]dCTP by random hexanucleotide primed synthesis. Total RNA was isolated from wild-type FRT and FRT-L5 cells, stable transfected clones, and control rat tissues (liver, cervix, and heart) using the guanidinium isothiocyanate/acid phenol extraction method of Chomczynski and Sacchi (21). Hybridization with a [α-32P]dCTP-labeled rat cDNA fragment conjugated to fluorescein (Sigma) was used as secondary antibody. Immunofluorescent images were taken using a SIT camera (LHESA Electronique, Cercy Pontoise, France) installed on an Axiophot microscope (Zeiss, Oberkochen, Germany) coupled to an image-processing system (Sapphire, Quantel, Montigny-le-Bretonneux, France). Photomicrographs were prepared using a video printer (UP 5000 P, Sony, Tokyo).

**Western Blot Analysis—** Cells at confluence in 100-mm culture dishes were collected in cold Earle’s medium (pH 7.0) by scraping. After centrifugation, the cells were resuspended in 1 ml NaHCO3 supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mM NaF, 2 μg/ml leupeptin, and 1 μg/ml pepstatin (buffer A) and lysed by sonication. Cell lysates were centrifuged at 100,000 × g for 30 min at 4 °C, and the resulting pellets were resuspended in buffer A containing 0.25% N-lauroylsarcosine sodium salt (Sigma) and incubated for 20 min at 20 °C as described previously (10). After centrifugation at 100,000 × g for 30 min at 4 °C, the final pellets were resuspended in 0.1% (w/v) sodium dodecyl sulfate, 2% (w/v) SDS, and 10 mg/ml bromphenol blue (pH 6.7). Proteins were separated by electrophoresis on SDS-12% acrylamide gel and transferred to Immobilon-P membrane (Millipore Corp., Bedford, MA). The transfer membrane was saturated in Blotto solution (40 mM Tris, 5% (w/v) skim milk, and 0.1% (v/v) Tween 20) for 1 h at room temperature and then incubated with rabbit anti-Cx32 antibodies (1:500 final dilution in Blotto) for 2 h at room temperature. After extensive washings in 10 mM Tris, 150 mM NaCl, and 0.2% Tween 20 (pH 8.0), immunocomplexes were visualized using alkaline phosphatase-conjugated anti-rabbit IgG and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium substrates (Sigma) according to the manufacturer’s instructions. Western blot analyses of thyroglobulin were performed on total cell extracts.

**Measurement of Intercellular Communication—** Intercellular communication was analyzed using the low M, fluorescent probe lucifer yellow CH (Sigma), originally described by Stewart (23). The probe was microinjected into one cell, and after diffusion to adjacent cells, the number of lucifer yellow-labeled cells was counted as described previously (12). Lucifer yellow was never observed in the very low level of junctional coupling analyzed by lucifer yellow microinjection. In FRT-L5 cells, lucifer yellow was never transmitted from the injected cells to surrounding cells as illustrated Fig. 3 panel b). In FRT cells, the dye was sometimes detected in some cells (two to five cells) adjacent to the injected cell several minutes after microinjection (Fig. 3A, panel b). The low level of coupling observed in FRT cells could be either related to the presence of a low number of GJ channels composed of Cx32, Cx43, or Cx26 expressed in minute amounts or dependent on channels composed of another Cx than those normally expressed in thyroid cells.

**RESULTS**

FRT and FRTL-5 cells do not express the Cx expressed in normal thyrocytes. No Cx32 transcript was detected in total RNA from FRT and FRTL-5 cells by Northern blotting (Fig. 1A, lanes 2 and 7, respectively); likewise, Cx43 and Cx26 transcripts could not be found (data not shown). The same negative results were also obtained at the protein level. Neither Cx32 (Fig. 1B, lanes 2 and 7) nor Cx43 (data not shown) was detected by Western blot analyses of FRT or FRTL-5 detergent-resistant membrane extracts. Immunofluorescence labeling with anti-Cx32 (Fig. 2, A and E) or anti-Cx43 (data not shown) antibodies was also negative. The inability to detect any of the three Cx (Cx32, Cx43, and Cx26) was in keeping with the absence of or the very low level of junctional coupling analyzed by lucifer yellow microinjection. In FRTL-5 cells, lucifer yellow was never transmitted from the injected cells to surrounding cells as illustrated Fig. 3B (panel b). In FRT cells, the dye was sometimes detected in some cells (two to five cells) adjacent to the injected cell several minutes after microinjection (Fig. 3A, panel b). The low level of coupling observed in FRT cells could be either related to the presence of a low number of GJ channels composed of Cx32, Cx43, or Cx26 expressed in minute amounts or dependent on channels composed of another Cx than those normally expressed in thyroid cells.

**Cx32 Expression in FRT and FRTL-5 Cells Transfected with the Rat Cx32 cDNA—** Cx32-transfected FRT cells were identi-
Transfection of Thyroid-derived Cells Lines with Connexin Gene

Evidence that intercellular communication is restored in Cx32-transfected FRT and FRTL-5 cells. Lucifer yellow was injected into the cytoplasm of one cell (identified by the arrows), and the distribution of the fluorescent probe was examined 5 min after microinjection. Panels a and c correspond to phase-contrast images, and panels b and d give the fluorescence images of the corresponding fields. Panel e, FRTL-5 cells; panels a and b, wild-type cells; panels c and d, Cx32-transfected cells (clone A). B, FRTL-5 cells. Panels a and b, wild-type cells; panels c and d, Cx32-transfected cells (clone Q).

FIG. 2. Immunofluorescence detection of Cx32 in transfected FRT and FRTL-5 cells. Indirect immunofluorescence labeling was performed on fixed and permeabilized cells attached to Petri dishes using anti-peptide antibodies as described under “Experimental Procedures.” A, wild-type FRT cells; B, Cx32-FRT cells (clone A); C, Cx32-FRT cells (clone B); D, Cx32-FRT cells (clone C); E, wild-type FRTL-5 cells; F, Cx32-FRTL-5 cells (clone 1); G, Cx32-FRTL-5 cells (clone P); H, Cx32-FRTL-5 cells (clone Q). Bars = 20 μm.

FIG. 3. Evidence that intercellular communication is restored in Cx32-transfected FRT and FRTL-5 cells. Lucifer yellow was injected into the cytoplasm of one cell (identified by the arrows), and the distribution of the fluorescent probe was examined 5 min after microinjection. Panels a and c correspond to phase-contrast images, and panels b and d give the fluorescence images of the corresponding fields. A, FRT cells. Panels a and b, wild-type cells; panels c and d, Cx32-transfected cells (clone A). B, FRTL-5 cells. Panels a and b, wild-type cells; panels c and d, Cx32-transfected cells (clone Q).

FRTL-5 cells expressing the Cx32 gene were identified by their high junctional coupling. Three clones (clones I, P, and Q) were selected; they expressed similar levels of Cx32 transcripts (Fig. 1A, lanes 9–11). Cx32 mRNA was absent in FRT and FRTL-5 cells cotransfected with the neomycin gene and the empty pSVK3 vector (Fig. 1A, lanes 3 and 8). The expression of the Cx32 gene in FRT and FRTL-5 cells was further analyzed by Western blotting and immunofluorescence labeling. The Cx32 protein of the expected size (27–28 kDa) was immunodetected in all the Cx32 mRNA-positive clones (Fig. 1B, lanes 4–6 for FRT cell-derived clones and lanes 9–11 for FRTL-5 cell-derived clones). As expected from mRNA data, the amount of Cx32 protein in clone B of FRT cells was lower than that detected in both clones A and C (Fig. 1B, compare lane 5 with lanes 4 and 6). In transfected FRTL-5 cells, the amount of Cx32 protein was similar in the three clones (Fig. 1B, lanes 9–11).

Fig. 2 illustrates the cellular distribution of Cx32 in transfected FRT and FRTL-5 cells. The immunofluorescence labeling of Cx32-transfected FRT cells was qualitatively similar for the three clones (Fig. 2, B–D); it appeared as discontinuous lines and/or dots delineating the region of cell-cell contacts. Bright dots were also visible over the cells. In Cx32-transfected FRTL-5 cells, the labeling profile was different. In the three clones (Fig. 2, F–H), the labeling appeared as rather large and round spots more randomly distributed over the cells. As FRTL-5 cells did not spread very well on the Petri dish, but rather remained tightly grouped, the location of the labeled spots was more difficult to establish; fluorescent spots were found in the regions of cell-cell contacts, but also in other regions of the plasma membrane or possibly inside the cells. In both cell types, Cx32-transfected cells were intensely labeled.

The level of Cx32 expression (assessed by mRNA and protein measurements) remained stable beyond 30 and 20 passages in transfected FRT and FRTL-5 cells, respectively. These results show that the Cx32-transfected cells we selected stably overexpressed the exogenous rat Cx32 gene, the major part of the protein being addressed to the plasma membrane.

Cell-to-Cell Communication in FRT and FRTL-5 Cells That Stably Express Cx32—Cx32-transfected cells exhibited a high level of GJ-mediated intercellular communication. When microinjected into one cell, lucifer yellow was detected within seconds in numerous cells in the vicinity of the microinjected one; this is illustrated for FRT cell-derived and FRTL-5 cell-derived clones in Fig. 3 (A and B, respectively). The level of cell-to-cell communication or junctional coupling of each clone was quantified; the results are presented in the form of histograms of frequency (Fig. 4) that allow the collection and comparison of data from multiple microinjection tests on the different clones. Among the Cx32-transfected FRT cells, clones A and C, which presented the highest level of Cx32 expression, showed the highest dye-coupling capacity. Indeed, >50% of the cells from clones A and B and only 15% from clone C communicated with >20 neighbor cells. The three clones of Cx32-transfected FRTL-5 cells exhibited similar junctional coupling levels. These data unequivocally establish that Cx32 protein synthesized by FRT and FRTL-5 cells from the exogenous Cx32 cDNA is competent to form functional gap junction channels. In this study, FRT and FRTL-5 cells that stably express Cx32 are designated Cx32-FRT and Cx32-FRTL-5, respectively.

Growth Rate of Cx32-FRT and Cx32-FRTL-5 Cells—The proliferation rate of Cx32-transfected cells was compared with that of control cells (wild-type cells and cells expressing the neomycin resistance gene: FRT-neo or FRTL-5-neo). The results of Fig. 5A show that the proliferation rate of the three clones of Cx32-FRT cells was significantly lower than that of control cells. Interestingly, cells from clone B (the clone among
Transfection of Thyroid-derived Cells Lines with Connexin Gene

This study clearly demonstrates that, as a consequence of transfection with and expression of the exogenous Cx32 gene that led to the restoration of cell-to-cell communication, the rate of proliferation of FRT and FRTL-5 cells was significantly reduced. In all the selected clones, the levels of Cx32 mRNA prohormone, and two transcription factors (TTF-1 and Pax-8), as shown in Fig. 7 (lane 1 in A–C, respectively). TTF-1 and Pax-8 mRNA levels were similar in Cx32-FRTL-5, wild-type FRTL-5, and control FRTL-5-neo cells; but a huge increase in the amount of thyroglobulin mRNA was reproducibly observed in the three clones of Cx32-FRTL-5 cells (Fig. 7A, compare lanes 3–5 with lanes 1 and 2). The increase of thyroglobulin gene expression in Cx32-transfected cells was confirmed at the protein level. The Western blot analysis results reported in Fig. 7E show that thyroglobulin was present in higher amounts in Cx32-FRTL-5 cells (lanes 3–5) than in control cells (lanes 1 and 2).

FRT cells expressed Pax-8, but neither TTF-1 nor thyroglobulin. The level of Pax-8 mRNA was similar in Cx32-FRT and control cells (data not shown).

**DISCUSSION**

This study clearly demonstrates that, as a consequence of transfection with and expression of the exogenous Cx32 gene that led to the restoration of cell-to-cell communication, the rate of proliferation of FRT and FRTL-5 cells was significantly reduced. In all the selected clones, the levels of Cx32 mRNA...
TABLE I
Population doubling time of FRT and FRTL-5 cells transfected with the rat Cx32 gene

| Clones      | Population doubling time (h) |
|-------------|-----------------------------|
| FRT cells   |                             |
| Wild-type   | 23.2 ± 1.1                  |
| Neo         | 23.7 ± 1.6                  |
| Cx32-A      | 31.4 ± 1.6b                 |
| Cx32-B      | 25.4 ± 1.8                  |
| Cx32-C      | 31.5 ± 2.7b                 |
| FRTL-5 cells|                             |
| Wild-type   | 36.9 ± 3.0                  |
| Neo         | 36.4 ± 0.4                  |
| Cx32-I      | 50.8 ± 3.6b                 |
| Cx32-P      | 51.2 ± 3.3b                 |
| Cx32-Q      | 45.9 ± 0.5b                 |

*Statistically different from controls (p < 0.05).

FIG. 6. Effects of TSH, insulin, and serum on the growth of wild-type and Cx32-transfected FRTL-5 cells. Wild-type FRTL-5 and Cx32-FRTL-5 cells (clone P) were cultured in 24-well culture plates. A, proliferation of cells in the presence or absence of TSH. Cells were seeded at 10^5 cells/well in complete culture medium or in the same medium devoid of TSH. Cells were counted at the indicated times. B, cell proliferation in response to increasing concentrations of TSH. Cells were seeded at 15 × 10^4 cells/well in complete culture medium. Twenty-four h after plating, the medium was changed, and the cells were maintained for 8 days in the TSH-free medium. Then, increasing concentrations of TSH (0–10 milliunits/ml) were added, and the cells were counted 36 h later. C, cell proliferation in the presence or absence of insulin. Cells were seeded at 5 × 10^4 cells/well in complete culture medium. After 24 h, the medium was changed, and the cells were cultured for up to 10 days in complete culture medium or in the same medium devoid of insulin (Ins). Cells were counted at the indicated times. D, cell proliferation as a function of serum concentration. Cells were seeded at 5 × 10^4 cells/well in complete culture medium. Twenty-four h after plating, the cells were washed and cultured for 7 days in the presence of increasing concentrations of serum (0.5, 2, or 5%). In each panel, the symbols represent the mean of triplicate measurements in a representative experiment. Similar results were obtained with clones I and Q.
cretion. Recent data support this hypothesis; selective permeability toward chemical compounds have been found for channels composed of different Cx (34).

What are the possible targets of the regulatory molecules that could pass from cell to cell via GJ? There is some evidence that restoration of GJ-mediated cell-to-cell communication can affect the expression of proteins or factors involved in cell cycle control. Chen et al. (35) have reported changes in the expression of cell cycle regulatory proteins in TRMP cells transfected with Cx43 cDNA. The reduction of cell proliferation was accompanied by a decrease in cyclins A, D1, and D2 and cyclin-dependent kinases CDK5 and CDK6 and by a prolongation of both G1 and S phases. In another study, Cx43-transfected C6 glioma cells were found to produce a factor capable of reducing the growth rate of nontransfected parental cells (36); this factor could be an insulin-like growth factor-binding protein, IGFBP-4 (37), that inhibits the mitogenic action of insulin-like growth factors.

In our study, the reduction of Cx32-FRTL-5 cell growth does not seem to be secondary to the production of an inhibitory growth factor or an inhibitor of growth factor(s) since wild-type FRTL-5 cells cultured in conditioned medium of nontransfected or Cx32-transfected cells exhibited a similar growth rate (data not shown). The alteration of Cx32-FRTL-5 cell growth was probably not due to a change in the signaling pathway by which TSH activates cell multiplication because the TSH concentra-

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24716

Transfection of Thyroid-derived Cells Lines with Connexin Gene

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