Analysis of Connexin43 Expression under the Control of a Metallothionein Promoter

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Abstract

Transfection of C6 glioma cells with connexin43 (Cx43) cDNA under a constitutive promoter resulted in increased expression of Cx43 protein, an increase in functional gap junctions, and decreased growth under in vitro and in vivo conditions (D. Zhu et al., Proc. Natl. Acad. Sci. USA, 88: 1883–1887, 1991). To allow for precise temporal and quantitative control of Cx43 gene expression, the Cx43 cDNA was inserted into an expression vector [pSV2-M(26)] containing a modified metallothionein promoter. Upon transfection of this vector into C6 cells, clones were isolated that expressed increased levels of inducible Cx43 protein and dye coupling. The level of induction of Cx43 expression increased with increasing concentration of Zn2+1, thus enabling the use of the same clone with different levels of gap junctions present. Although we observed no change in cell growth under in vitro conditions following exposure to Zn2+ or Cd2+, clones with inducible expression of Cx43 were characterized by reduced growth in vivo. Within tumors, the level of expression of Cx43 mRNA and protein corresponded to that seen in vitro following the addition of Zn2+. The suppression of tumor growth in vivo correlated with the level of induced Cx43 expression.

Introduction

One of the major characteristics of neoplastic cells is their uncontrolled, rapid growth. Many aspects of tumor growth have been studied, including the implication that loss of gap junctional communication is associated with cancer development. Since the initial suggestion by Loewenstein and Kanno (1) that gap junctional defects may lead to aberrant cell growth in cancers, a number of observations have provided support for this hypothesis (2). Gap junctions are under tight regulatory control by multiple mechanisms, which can either increase or decrease communication over a wide spectrum of time (milliseconds to hours). Thus, any agent(s) that affect the connexin gene (mutation), the transcriptional rate of connexin genes, the stability and translation of connexin transcripts, posttranslational modification of connexins, their subsequent oligomerization into connexons, insertion into the membrane, the formation into gap junctional plaques, and the removal from the membrane could lower the coupling, resulting in an increased risk or promotion of neoplastic growth within a tissue. Examination of these agents in transformation have been reviewed recently (3–6).

The consensus from reviews of published data indicates that a decrease in gap junctional communication occurs early in tumor promotion, but not in all tumors, and is always lacking throughout progression (2–5). This decrease appears between daughter cells following initiation. Continual exposure to a promoting agent can result in a permanent decrease in functional gap junctions, presumably due to secondary mechanisms. These daughter cells are weakly coupled but do not communicate to surrounding normal cells. This creates an isolated environment freed of growth controls, resulting in rapid change to a neoplastic phenotype (7–10). There is evidence suggesting a correlation between the level of gap junctional communication and the metastatic ability of tumor cells. Highly metastatic tumors demonstrate the lowest level of coupling (11, 12). It is not known if this reduction in communication is the cause or the effect of the metastatic phenotype.

Recently, the introduction and overexpression of connexin cDNA in tumor cells by transfection has shown that the presence of functional gap junctions can suppress growth and/or tumorigenicity of some types of transformed cells. Transfection and expression of Cx43 (13–16), Cx32 (17, 18), Cx26, and Cx40 (14) in various cells resulted in increased communication. However, only some connexins caused a slower growth in vitro and in vivo that correlated with the level of expression.

The aim of this project was to transfect the Cx43 cDNA coding sequence under the control of an inducible promoter into the C6 cell line and determine what effect differential expression of connexin protein would have on cell growth under both in vitro and in vivo conditions. The C6 cell line is a chemically transformed rat astrocytoma (19) that exhibits extremely low levels of intercellular communication (20). We have previously transected C6 cells with Cx43 cDNA under the control of a constitutive promoter (13). Under in vitro and in vivo conditions, transformed clones demonstrated a reduced growth rate compared with the parental line (13, 21). These clones continuously express the Cx43 protein; thus, the expression cannot be controlled or altered. Therefore, we transfected C6 cells with a construct composed of the heavy
metal-inducible human metallothionein I promoter (22) ligated to the Cx43 cDNA. The resultant transfected clones display inducible expression of the Cx43 protein and gap junctional intercellular communication. The effect of this induction on cell growth was characterized in vitro and in vivo.

Results

Characterization of Plasmid Vectors

To obtain an inducible Cx43 gene product, Cx43 cDNA was subcloned into the Sal1 site of the plasmid pSV2M(2)6, and the orientation of the insert was determined by HindIII restriction enzyme mapping (Fig. 1). No eukaryotic selection gene marker is present in this plasmid; therefore, a neomycin selection plasmid, pSV2neo (23), was cotransfected with pSV2M(2)6-S43 to allow selection of neomycin-resistant transformants. Stable transformants carrying both plasmids were analyzed in experimental studies.

Expression of Cx43 cDNA following Induction with Zinc

Induction of Cx43 mRNA. Cytoplasmic RNA was extracted from several transformed clones and parental C6 cells following induction for 18 h with and without zinc (100 μM). Northern blot analysis with a radiolabeled Cx43 cDNA probe was performed to determine the level of Cx43 mRNA expression. Exposure of C6 cells to zinc did not appear to change the endogenous 3.0-kb Cx43 expression level (Fig. 2, top). Also, the amount of endogenous Cx43 mRNA within each transfected clone did not vary with the presence of zinc. A number of clones demonstrated induction of a Cx43 mRNA species of 2.25 kb that corresponds to the size of mRNA transcribed from the plasmid. The level of Cx43 mRNA induction varied between clones. The clone C6-13, which was previously transfected with the pLTR containing Cx43 cDNA
Fig. 3. A. Western blot analysis of the synthesis of Cx43 protein in several clones. Proteins were extracted and separated by SDS-PAGE from an equal number of cells (5 x 10^5) grown with (+) or without (-) 100 μM zinc for 18 h. Immunoreactivity to Cx43 protein on the blot was seen as three bands, M, 42,000, M, 44,000, and M, 46,000 (black arrows). Standard molecular weight markers were used to determine the positions of M, 68,000 and M, 41,000. B. Location of Cx43 protein in MS122 cells following induction by zinc. An equal number (5 x 10^5) of control (-) cells or cells treated with 100 μM (+) zinc were split into crude membrane and cytoplasmic fractions and separated by SDS-PAGE. Immunoreactivity to Cx43 following Western blotting of these fractions was observed to be similar in molecular weight (black arrows) as seen in A. Total protein lysates (100 μg) from MS122 cells treated with varying concentrations of zinc (0, 50, or 100 μM) were also analyzed by Western blotting.

(13), demonstrates a higher level of expression of the transfected cDNA than any of the inducible clones.

The addition of increasing amounts of zinc to the transfected clone MS122 for 18 h showed a corresponding increase in Cx43 mRNA (Fig. 2, bottom). Increasing the zinc from 50 to 100 μM resulted in a large increase in the 2.25-kb Cx43 mRNA, suggesting a more efficient induction at 100 μM. Increasing concentrations of zinc did not have an effect on the expression of the 3.0-kb Cx43 mRNA in the parental C6 cells (Fig. 2, bottom, A).

Synthesis of Cx43 Protein. The synthesis of Cx43 protein was investigated by Western blot analysis of crude protein lysates from various transfected clones, cultured in the presence or absence of 100 μM zinc for 18 h (Fig. 3). Each lane in Fig. 3A contains the protein from an equal number of cells (5.0 x 10^5). The presence of zinc did not change the Cx43 protein profile of C6-13 clone, which shows a large percentage of protein as the M, 42,000, form, and a small amount as the M, 44,000 and M, 46,000 forms. Cx43 protein content in the inducible clones exhibits similar profiles, but induced levels per cell were lower that that observed in clone C6-13. Comparison of the amount of Cx43 protein synthesized in the inducible clones, following exposure to zinc, demonstrates a direct relationship between the level of expressed Cx43 mRNA and protein. Protein was not readily detected in clone MS89 and MS75 under both conditions. Clones MS49 and MS1 under normal conditions exhibited a low level of Cx43 protein and a corresponding increase following induction by zinc. Clone MS122 exhibited the highest level of induction.

Cytoplasmic versus Membrane Location of Cx43 Protein. Crude membrane and cytoplasmic extracts were made from MS122 cells following 18 h with or without zinc (100 μM; Fig. 3B). No detectable Cx43 protein in MS122 cells could be seen in the membrane fraction (Fig. 3B, MEM), and there were barely observable amounts in the cytoplasmic fraction (Fig. 3B, CYT) before induction. After induction, the three species of Cx43 could be seen in the membrane fraction (Fig. 3B, MEM, +), whereas the vast majority in the cytoplasmic fraction was the M, 42,000 form (Fig. 3B, CYT, +). Cx43 protein present in total protein lysates of MS122 cells increased following exposure to varying amounts of zinc (0, 50, and 100 μM) for 18 h. The elevated levels of protein corresponded with previously observed increases in mRNA expression induced by increasing concentrations of zinc.

Immunolocalization of Cx43 Protein in Transfected Clones. Immunocytochemical visualization of Cx43 protein was performed on newly confluent monolayer of clones MS75, MS122, MS1, MS49, and MS89 (Fig. 4). In the absence of zinc, no immunofluorescence could be seen in these clones (Fig. 4, A and C), whereas in the presence of
zinc, Cx43 immunoreactivity was greatest in MS122 (Fig. 4D), with decreased amounts in MS1 (Fig. 4F) and MS49 (Fig. 4E). The immunoreactivity to Cx43 protein appeared mostly as punctate staining against a diffuse fluorescent background. No punctate immunoreactivity to Cx43 could be seen in either MS89 (Fig. 4B) or MS75 (data not shown) clones in the presence of zinc.

**Functional Gap Junctional Analysis**

_Microinjection into Single Cells._ The procedure of microinjection of CF into individual cells was used to determine the number of cells coupled to each other over a time frame of 3 min. The highly inducible MS122 clone was compared with the C6 parental cells. Twenty separate injections were carried out for each cell type treated with or without zinc. Typical representations are shown in Fig. 5. Each parental C6 cell appeared to be coupled on average to less than one cell in the absence (Fig. 4, G-J) or the presence (Fig. 4, J-L) of zinc. MS122 cells exhibited similar coupling as the parental cell when untreated (Fig. 4, A-C), but after 18 h in the presence of 100 μM zinc, coupling increased to an average of 10 cells (Fig. 4, D-F).

_Scrape Loading._ The six inducible clones and parental C6 cells were subjected to scrape loading as a comparative analysis of inducible gap junctional coupling (Table 1). The degree of inducible coupling was determined by the ratio of the number of cells labeled with CF divided by the number of cells labeled with Dylithium rhodamine along 600 μM at the scrape edge (i.e., CF/Dx). C6 and MS89 cells showed virtually no CF transfer to neighboring cells whether in the presence or absence of zinc (i.e., CF/Dx = 1). The other clones exhibited dye coupling, which correlated with the level of inducible Cx43 expression: CF/Dx for MS75 = 1.24; for MS49, 1.81; for MS1, 1.38; and for MS122, 3.42.

**In Vitro Growth Analysis**

The growth curves and rates were determined for C6 parental, MS122, MS89, MS1, and MS49 under normal conditions and in the presence of 100 μM zinc or 1.0 μM cadmium over a 4-day interval. The presence of zinc or cadmium had no
significant effect on the growth of the parental cells or on any of the inducible clones (data not shown). The growth of C6, MS89, and MS122 cells was examined up to 2 weeks in culture. No difference in saturation density was observed (data not shown).

**In Vivo Growth Analysis**

Following s.c. injection of several clones into nude mice, the volume of the tumors was measured every 3.5 days for 3 weeks (Fig. 6). The growth and size of the tumors did not vary whether the nude mice were maintained on regular lab chow or chow supplemented with 25 mM ZnSO₄ in the drinking water. All subsequent experiments involving tumors were done without supplementing zinc to the diet. The volume of the tumors derived from parental C6, MS89, and MS49 cells were not significantly different ($P > 0.05$). Growth of clones MS75, MS1, and MS122 was significantly different ($P < 0.05$) when compared to the former three at each time interval. Also, each of these tumors (MS75, MS1, and MS122) were significantly different ($P < 0.05$) from each other at every time point. At 3 weeks following inoculation of cells, these tumors were removed, and their wet weights were determined (Fig. 7). The weights of C6, MS89, and MS49 tumors were found not to be significantly different ($P > 0.05$) from each other. Clones MS75, MS1, and MS122 derived tumors were significantly different ($P < 0.05$) in weight from each other as well as C6 and MS89. The greatest reduction in tumor volume (25-fold) and tumor wet weight (22-fold) was observed with clone MS122 cells.

**Expression of Cx43 mRNA in Tumors**

Cytoplasmic RNA was extracted from these tumors after 3 weeks growth in vivo. RNA from two separate tumors of each clone was analyzed by Northern blotting for expression of Cx43 mRNA (Fig. 8). The parental C6, MS1, and MS49 clones exhibited expression of endogenous Cx43 mRNA (Fig. 8, open arrow). The inducible clones all show expression of transfected Cx43 mRNA (Fig. 8, solid arrow), varying from the highest in MS122 to barely detectable amounts in MS89 tumors.
Table 1 Comparison of dye-coupling by scrape loading

<table>
<thead>
<tr>
<th>Clone</th>
<th>Zn±</th>
<th>Dx±</th>
<th>CF±</th>
<th>CF/Dx</th>
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<tr>
<td>MS122</td>
<td>-</td>
<td>38.0 ± 1.8</td>
<td>39.8 ± 2.2</td>
<td>1.05</td>
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<tr>
<td></td>
<td>+</td>
<td>38.3 ± 3.0</td>
<td>131.0 ± 4.6</td>
<td>3.42</td>
</tr>
<tr>
<td>MS1</td>
<td>-</td>
<td>37.0 ± 2.0</td>
<td>51.3 ± 2.3</td>
<td>1.38</td>
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<tr>
<td></td>
<td>+</td>
<td>34.0 ± 2.3</td>
<td>100.3 ± 5.3</td>
<td>2.95</td>
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<tr>
<td>MS49</td>
<td>-</td>
<td>41.0 ± 3.3</td>
<td>44.0 ± 3.2</td>
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<tr>
<td></td>
<td>+</td>
<td>38.3 ± 2.6</td>
<td>61.8 ± 2.6</td>
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<tr>
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<td>-</td>
<td>45.3 ± 2.8</td>
<td>45.3 ± 2.8</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>41.5 ± 2.1</td>
<td>51.3 ± 3.1</td>
<td>1.24</td>
</tr>
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<td>43.7 ± 1.9</td>
<td>44.0 ± 2.0</td>
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<td>+</td>
<td>46.0 ± 1.4</td>
<td>46.3 ± 1.4</td>
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<tr>
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<td>39.0 ± 2.7</td>
<td>42.0 ± 2.6</td>
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<td>35.5 ± 3.9</td>
<td>38.4 ± 4.2</td>
<td>1.08</td>
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* Number of cells labeled with Dx-rhodamine.
* Number of cells labeled with CF.

Synthesis of Cx43 Protein in Tumors

Aliquots of protein (100 μg) from extracted cells of MS1, MS122, and MS89 tumors were analyzed by Western immunoblot using antisera against Cx43 and GFAP (Fig. 9). Immunoreactivity to GFAP on the blot revealed an intense band of M, 46,000 (Fig. 9B), indicating that transfected C6 cells were present in the sample. Cx43 immunoreactivity on a similar blot identified three bands, M, 42,000, M, 44,000, and M, 46,000 for each sample (Fig. 9C). MS122 tumors contained predominately the M, 46,000 protein species. The majority of the Cx43 in the MS1 tumors appeared to be evenly distributed between the M, 42,000 and M, 46,000 forms. The MS89 tumors expressed a small amount of the M, 42,000 Cx43 protein.

Immunolocalization of Cx43 within the Tumors

Following excision, tumors were sectioned and reacted with antisera to Cx43 and GFAP together to colocalize these two proteins in the same cell. Tumor sections of MS122 (Fig. 10, A and B) displayed both punctate and diffuse staining for Cx43 protein in GFAP-immunoreactive cells. In contrast, no punctate Cx43 immunofluorescent staining could be seen in GFAP reactive cells in MS89-derived tumors (Fig. 10, C and D).

Discussion

The lack of gap junctional coupling in many neoplastic cells has sparked an intensive search for the role(s) of connexin proteins in the control of cell growth (reviewed in Ref. 6). We have used the C6 glioma cell line as a model to investigate the role of gap junctions in the control of cell growth (13, 17, 20, 21). This cell line was derived from a rat glial tumor induced by ethynitrosoourea (19). The expression of Cx43 in C6 glioma cells has been shown to be relatively low in comparison to primary cortical astrocyte cells in culture (24). In addition, intercellular coupling of C6 cells is very low, and their growth rate is relatively fast. C6 cells are capable of forming large tumors in nude mice (17) and aggressive neoplastic gliomas in the brains of rats (19, 25). Transfection of C6 cells with Cx43 cDNA resulted in a decrease in cell growth in vitro and in vivo (13, 20, 21, 26). To further characterize the role of gap junctional communication in this model, Cx43 cDNA was inserted into a plasmid containing a heavy metal-inducible metallothionein promoter and transfected into these cells. Following induction of the Cx43 gene product, their growth properties were analyzed.

In the present study, a 2.25-kb Cx43 transcript was induced in a number of selected clones following a minimum exposure of 12 h to zinc. Induction of Cx43 mRNA was not investigated following shorter heavy metal treatment intervals. The level of induction of Cx43 mRNA varied between clones. Furthermore, the expression of Cx43 transcripts and protein increased with increasing zinc concentration, confirming that the activity of the promoter in the current context could be regulated in a dose-dependent manner (22). Immunocytochemical localization of Cx43 protein revealed punctate staining in plasma membrane areas of contact between cells, as well as a diffuse staining in the cytoplasm. The separation and identification of Cx43 protein by Western immunoblots showed three different forms, approximately M, 42,000, M, 44,000, and M, 46,000 kDa, similar to that reported for different phosphorylated forms in previous studies (27–29). The location of the three forms of Cx43 within the cell following induction were identified by separation of cellular protein into membrane and cytoplasmic components and analysis by immunoblotting. Within the cytoplasm, only the M, 42,000 form was detectable, whereas all three forms were observed in the membrane. These results generally agree with the findings of Musil et al. (27–29). The lowest molecular weight form of Cx43 has been shown not to be phosphorylated and to be present only in the cytoplasm, whereas all three forms were found in the plasma membrane (29).

Under standard culture conditions, the transfected C6 clones were as poorly dye-coupled as the parental C6 cells, but the addition of zinc resulted in a significant increase in coupling. The inducible clones did not demonstrate any significant difference in growth in culture after treatment with zinc or cadmium for 2 weeks. There are several possible explanations for this. In comparison to C6 clones transfected
with Cx43 under the control of the constitutive pLTR promoter (13), the present inducible clones express less Cx43 mRNA and protein and display a much lower level of dye-coupling. It may be that much higher levels of Cx43 expression are required to elicit effects on cell growth. The presence of the phosphorylated forms of Cx43 are necessary for functional coupling (27, 30), although it is not known what level of coupling is required for various physiological functions of gap junctions. We have noticed differences in Cx43 transfected clones that correlate with the level of gap junctional coupling, including cell proliferation (13) and expression of IGF binding proteins (31). In addition, the life history of these clones differs dramatically. The pLTR transfected clones have continually expressed Cx43 since their initial transfection, selection, and subcloning. In contrast, these inducible transfected cells only express Cx43 subsequent to induction with zinc. Although we have not observed any apparent effect of zinc on the expression of Cx43 in pLTR clones, zinc has been shown to affect expression of a number of genes (32, 33). In addition, zinc may disrupt intracellular calcium homeostasis by acting at calcium binding sites, including calmodulin (34), influencing the effects of calcium on gating of gap junctions (35, 36).

In contrast, when these inducible clones were implanted s.c. in nude mice, we observed significant reduction of growth that correlated with the level of induction of Cx43 expression in tissue culture. The clones containing inducible expression of Cx43 protein exhibited reduced growth, as measured by tumor volume and wet tumor weight over a 3-week period in the absence of zinc supplement. Also, the reduced growth was seen during the entire 3-week interval, suggesting that the growth rate of each inducible clone was constant and not a problem with establishment of the tumor. An examination of the tumors for the expression of Cx43 mRNA and protein demonstrated that the inducible clones expressed the transfected Cx43 cDNA to similar levels as seen in tissue culture. Although there were likely other cell types that may express Cx43 in these tumors, such as endothelial cells, histological analysis and GFAP immunocytochemistry confirmed that the tumors consisted predominantly of C6 glioma cells. This metallothionein promoter has the basal enhancer sequences removed and additional heavy metal response elements inserted, producing a hyper-inducible promoter (22). Spontaneous induction of the metallothionein promoter in vivo likely results from this increased sensitivity due to additional metal response elements and suggests a significant level of circulating heavy metals.

Fig. 6. In vivo tumor growth of C6 and several inducible clones. Cells (1.0 × 10⁶) were injected s.c. in the dorsum of nude mice, and the volumes of the tumors were measured every 3.5 days. The volumes of tumors derived from C6, MS89, and MS49 cells did not demonstrate any significant difference at any of the intervals, whereas MS122, MS1, and MS75 tumors were significantly different (P < 0.05) from the above three throughout the 3 weeks. Starting at 1.5 weeks, the growth of tumors generated by MS122, MS1, and MS75 were significantly different (*, P < 0.05) from each other. The sample size for each group was four. Bars, SE.

Fig. 7. Tumor growth measured as wet weight after 3 weeks in vivo. Three weeks after s.c. injection into nude mice, the tumors were removed, and their wet weights were determined. The weight of C6-, MS89-, and MS49-derived tumors were not significantly different from each other, but those derived from MS122, MS1, and MS75 were significantly different (*, P < 0.05) from C6 and MS89 and among themselves. Sample size was four mice per group. Bars, SE.
Fig. 8. Expression of the transfected Cx43 mRNA in cells within tumors after 3 weeks in vivo. Tumors were excised from the mice 3 weeks after inoculation, and a portion was processed to isolate cytoplasmic RNA. Ten μg of RNA from two tumors derived from each cell type inoculated were subjected to Northern analysis. Hybridization to a Cx43 radiolabeled cDNA probe is shown in A, demonstrating expression of the endogenous (open arrow) and transfected (black arrow) Cx43 mRNA. The X-ray film in A was exposed for 24 h before developing. The same Northern blot was rehybridized to a radiolabeled 18S cDNA probe, and the X-ray film was exposed for 1/2 h, which is shown in B.

Several other labs have transfected different connexins into different cell types, resulting in a common observation that the presence of gap junctions results in a reduced growth rate of tumors in vivo (14, 16, 18). The unique presence of certain connexins in different cell types suggests functional cell specificity for connexins. This concept is supported by the finding that chemically transformed mouse fibroblasts (16) and rat glioma cells (21), which are deficient in Cx43 expression, exhibit reduced tumorigenicity following transfection of Cx43 cDNA. A similar observation was seen following transfection of human hepatoma cells with Cx32, which is normally present in hepatocytes (18) and following Cx26 introduction into HeLa cells, which were derived from cervical cells (14).

An interesting aspect of the current study concerns the observed increase in the amount of the phosphorylated forms of Cx43 in the slower growing tumors. Highly inducible cells exhibited a low proportion of the P1 and P2 protein forms in vitro, but under in vivo conditions, these phosphorylated forms predominate. This increase in phosphorylated Cx43 in vivo suggests there may be more functional gap junctions and, thus, would be consistent with a role of gap junctional communication in the observed decrease in cell growth. The reason for the difference in phosphorylation between the in vitro and in vivo environment is presently unknown. Clearly, humoral factors, adhesion molecules, and growth factors could play a role in vivo, as well as interaction of the transfected cells with normal surrounding cells at the site of implantation. Heterologous interactions between tumor cells and normal cells have been shown to lead to reduced growth in vivo (37). Previous reports have also shown a similar discrepancy between growth of Cx32-transfected cells in vitro and in vivo (17, 18, 38). The possible differences in phosphorylation have not been examined in...
these cases, and the role of phosphorylation in the function of gap junctional communication and growth control in vivo remains to be clarified.

**Materials and Methods**

**Cell Lines and Culture Conditions.** The C6 astrocytoma cell line (American Type Culture Collection) and the C6-13 cell line transfected with Cx43 cDNA (13) were maintained as monolayer culture on 100-mm plates in DMEM (Life Technologies, Inc.) supplemented with 10% FCS (HyClone), 10 units/ml penicillin, and 10 mg/ml streptomycin at 37°C in a humidified atmosphere containing 95% air and 5% CO2. When plating a specific cell number, the cells were counted in a Coulter counter (Coulter Electronics) following exposure to trypsin and diluted appropriately.

**Expression Vector Construction.** The protein coding sequence of Cx43 was obtained as a 1.4-kb cDNA fragment ligated into the EcoRI restriction site of Bluescript M13+ plasmid (Ref. 39; gift from Dr. E. Beyer, Washington University School of Medicine, St. Louis, MO). The expression vector pSV,M2(6) (22) used in this study was kindly donated by Dr. J. McNeall (University of North South Wales, Kensington, New South Wales, Australia). Cx43 cDNA insert was removed from the Bluescript plasmid by double endonuclease digestion with BamHI and SalI, blunt ended, and ligated into the SalI site of pSV,M2(6) (40). The recombinant plasmids were cut with HindIII to determine orientation. Clones containing the insert in the sense orientation were designated pSV,M2(6)-S43.

**Transfection of C6 Cells with Cx43 cDNA.** C6 cells were transfected with Cx43 cDNA in the sense orientation within the plasmid pSV,M2(6)-S43 using the reagent Lipofectin (Life Technologies, Inc.), as reported previously (13, 41). PSVs,neo was cotransfected with pSV,M2(6)-S43 because the latter plasmid does not contain a selectable marker for eukaryotic cells, whereas the former conveys neomycin resistance. At 48 h posttransfection, the cells were trypsinized, replated onto 100-mm plates at a dilution of 1 in 50 in DMEM with 10% FCS plus 600 mg/ml of G418 (Life Technologies, Inc.) to initiate selection. After 14 days of selection, stable transfectants were expanded in selection medium for another 2 weeks. A portion of each was frozen as stocks, and the remainder was investigated by Northern blot analysis to determine which clones expressed the transfected Cx43 cDNA.

**Induction of Metallothionein Promoter.** The heavy metals, zinc (Zn2+) sulfate (BDH) and cadmium (Cd2+) sulfate (BDH) were prepared as filter-sterilized stock solutions, 100 and 1 µM, respectively. Medium containing these metals was prepared fresh for each use. C6 clones containing the metallothionein promoter with downstream Cx43 sequence were treated with either heavy metal for at least 12 h prior to analysis of Cx43 mRNA and protein expression.

**RNA Isolation and Northern Blot Analysis.** Cytoplasmic RNA was isolated from confluent cultures and tumors according to a modified procedure of Sambrook et al. (40). Ten µg of cytoplasmic RNA was ethanol precipitated overnight and subjected to gel electrophoreses and transferred to nitrocellulose as described previously (40). The resultant blots were hybridized and subsequently washed according to established protocols (13). Twenty-five ng of purified cDNA was labeled with [32P]dCTP by random primer labeling (Megaprime DNA Labeling Systems; Amersham Corp.) and purified away from the unincorporated nucleotides by passage through a DNA nick column (350; Pharmacia Biotech, Inc.). Ethanol precipitation of the probe and scintillation counting of the pellet in Biofluor (DuPont NEN) demonstrated 85–90% incorporation of radioactive label. The same blots were later reprobed with a 32P-radiolabeled cDNA for 18S RNA.

![Image](image_url)
Immunocytochemistry. For this study, the following primary antibodies were used: (a) affinity-purified rabbit polyclonal anti-Cx43 (generously supplied by Dr. Bruce Nicholson of SUNY, Buffalo, NY; diluted 1:200); (b) affinity-purified mouse monoclonal GAFAP (diluted 1:20; Boehringer Mannheim); and (c) rabbit polyclonal (serum) anti-Cx43 (diluted 1:500; generously supplied by Dr. Alan Lau of the University of Hawaii, Honolulu, HI). Secondary antibodies were obtained from Dimension Laboratories and included goat antirabbit IgG conjugated to FITC (diluted 1:100) and goat antimouse IgG conjugated to rhodamine (diluted 1:100). Some of the immunoreactions involved two antibodies mixed together; therefore, only rabbit and mouse primary antisera were mixed.

Cells were grown to confluency on glass coverslips, fixed in 95% ethanol/5% (v/v) acetic acid for 30 min at −20°C, washed with PBS (pH 7.4), blocked in PBS with 10% (v/v) normal goat serum and 1% (v/v) BSA for 30 min, and then incubated for 1 h with primary antibody in PBS with 1% (v/v) BSA. The coverslips were washed three times for 10 min each with PBS and then incubated for 1 h with the secondary antibody in PBS with 1% (v/v) BSA. Coverslips were again washed three times for 10 min each and were mounted on slides in PBS containing 50% (v/v) glycerol and 1% (w/v) p-phenylenediamine (Fisher Scientific). Fluorescent immunoreaction was visualized under UV light on a Zeiss Axioskop photomicroscope equipped with filter set 17 for FITC (exciter filter, BP 485; barrier filter, 515–565 nm) and filter set 15 for rhodamine (exciter filter, BP 546; barrier filter, BP 590). Tumors were flash frozen in isopentane (BDH) that had been chilled with liquid nitrogen, removed from the isopentane, and stored at −70°C. Frozen tissue was sliced into 15-μm sections using a cryostat, placed on sanded slides (coated with gelatin), and stored at −20°C. Slides were immersed in 90% (v/v) methanol for 15 min at −20°C to fix the sections and rehydrated with several changes of PBS over a 1-h interval at room temperature. Sections were first overlaid with blocking serum, then primary antibody, followed by secondary antibody, and finally mounted as described above.

Western Blot Analysis. Total protein extracted from cell cultures was prepared by lysing a confluent 100-mm Petri plate with 900 ml of SDS sample buffer [125 mM Tris (pH 6.8), 1.0% (w/v) SDS, and 0.1% (w/v) bromophenol blue], 10% (v/v) glycerol, and 4.0% (v/v) β-mercaptoethanol (Sigma). After shearing the genomic DNA through 22-gauge and 27-gauge needles (Fisher Scientific), the lysates were stored at −20°C. Crude membrane and cytoplasmic samples were prepared by lysing a confluent plate of cells with 1 ml of 10 mM Tris-HCl (pH 7.4) containing 2 ml 10x zepelin, 50 mM EDTA, 2% (w/v) NaCl, and 0.5% NP-40. The membrane and nuclear were separated from the cytoplasm by centrifugation at 30,000 × g for 60 min at 4°C (42). The cytoplasmic fraction was denatured with one-fifth volume of 5× SDS sample buffer, whereas the pellet was dissolved in SDS sample buffer and passed through the same needles as mentioned above. Both samples were stored at −20°C.

For protein extraction from tumors, 100 mg of tissue was minced on ice and filtered sequentially through a 70-μm nylon mesh, followed by a 25-μm nylon mesh. A small portion was aliquoted for protein determination. The dispersed cells were concentrated and partially purified by centrifugation. The pellet was dissolved in SDS sample buffer to a concentration of 100 μg per 50 μl. Protein determination was performed using Bio-Rad protein assay (Bio-Rad). Separation of proteins was by SDS-PAGE according to Laemmli (43). Approximately 50 μl of each cell protein lysate (5 × 10^6 cells) and tumor-derived protein sample was loaded into each lane. One lane on each gel contained 10 mg of molecular weight standard markers (Rainbow markers; Amersham). The samples were dehydrated for 1 min at 95°C prior to loading. To assure approximately equal loading of protein per lane, parallel gels were stained with 0.05% (w/v) Coomassie Brilliant Blue (Bio-Rad) in 10% (v/v) acetic acid (BDH) and 25% (v/v) isopropanol (BDH) and destained in 10% (w/v) acetic acid and 40% (v/v) methanol to visualize the amount of protein on the gel. For Western blotting, the gel was transferred to nitrocellulose filter (0.22-μm pore; Bio-Rad) as described (44). The membrane was blocked in 5% (w/v) NFDM in PBS for 1 h at room temperature, washed twice for 10 min each with PBS, incubated with primary antibody overnight at 4°C in PBS with 1% NFDM (5 ml), washed three times for 15 min each with PBS, incubated 1 h with biotinylated goat antirabbit or antimouse secondary antibody in PBS with 1% NFDM at room temperature (10 ml), and then washed three times for 15 min each at PBS. The immunoreaction was visualized with the alkaline phosphatase-ABC reaction kit (Dimension Laboratories).

Intracellular Injection of Dye. Dye injection experiments were repeated on two different occasions using established protocols (45). Cells were entered by applying a small vibrational force with the microelectrode pushed up against the surface membrane. An appearance of the membrane potential indicated entry into the cell. A continuous train of hyperpolarizing pulses of current (2–6 nanammps for 200 ms duration at 1 pulse/s) was used to inject the fluorescent CF dye (10 μM in distilled water, pH 7; Eastman Kodak Co.). Cells were injected for 1 min and monitored for 3–5 min. Only those cells that maintained a stable membrane range of −40 to −60 mV during the injection period were analyzed (45). The transfer of dye was recorded with a real time counter videocassette recorder (Sony model TVO-1000) through a modified RCA silicon-intensified target television camera. Pictures were taken from the screen of the monitor.

Scrape Loading. Cells were scrape-loaded according to El-Fouly et al. (46). A razor blade was drawn across a newly confluent cell layer in a 60-mm plate that was covered in PBS containing 0.1% (w/v) CF (M, 356,000; Kodak) and 0.1% (w/v) Dsoxhodamine (M, 4,000; Sigma Chemical Co.). The cells were incubated in the dye for 2 min and then rinsed three times in PBS. The cells were then examined for fluorescent dye transfer after 10 min using a Zeiss Axioshott fluorescent microscope and photographed as mentioned earlier. Clones were assessed in the absence or presence of 100 μM Zn²⁺.

Scrape-loading experiments were quantified using a modified digital image analysis (Mocha for Windows), as reported previously (17). Briefly, the number of Dsorhodamine-labeled cells were counted along 600 μm of the scrape. Then the number of CF-labeled cells along the same area of the scrape was counted. The ratio of these cell numbers provided a relative comparison of inducible gap junctional coupling.

In Vitro Growth Analysis. Cells from confluent cultures were seeded at 3.0 × 10⁵ cells/60-mm plate. Heavy metal ions were added at time of planting to induce Cx43 expression at 100 and 1.0 μM for Zn²⁺ and Cd²⁺, respectively. At selected time points after plating, triplicate plates were disassociated into a single-cell suspension with 0.25% trypsin (Life Technologies, Inc.) and 1 μM EDTA (BDH) in PBS (pH 7.4) and counted in a Coulter counter.

In Vitro Growth Analysis. Single-cell suspensions were washed once at room temperature and resuspended at 1 × 10⁶ cells/ml in serum-free DMEM. An inoculum of 1 × 10⁶ cells was injected s.c. into the dorsum of nude/nude mice (Harlan-Sprague-Dawley). The mice were then maintained on normal lab chow. Tumors were examined, and their volumes were determined using a Vernier caliper every 3.5 days over a 3-week period. At the end of 3 weeks, the mice were sacrificed, the tumors were removed, and their wet weights were determined. Various portions of the tumors were used for immuno-}

Statistical Analysis. Statistical analysis involving testing for significance of differences for quantitative observations were performed using Statgraphic Statistical Graphic System, Version 3 (1988). All sets of data were subjected to an one-way ANOVA with a Scheffe range test to determine significant difference between any two sets. Subsequently, the sets of data were subjected to the Student’s t test to determine if a significant difference exists between the individual samples.

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References


