Connexin43 Suppresses MFG-E8 While Inducing Contact Growth Inhibition of Glioma Cells

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ABSTRACT

Gap junction expression has been reported to control the growth of a variety of transformed cells. We undertook parallel analysis of connexins Cx32 and Cx43 in glioma cells, which revealed potential mechanisms underlying this phenomenon and led to several novel findings. Cx43, but not Cx32, suppressed C6 glioma cell growth. Paradoxically, Cx32 transfection resulted in several-fold more dye transfer than Cx43. However, Cx43 transfectants shared endogenous metabolites more efficiently than Cx32 transfectants. Interestingly, a significant portion of Cx43 permeants were incorporated into macromolecules more readily than those that transferred via Cx32. Cx43 induced contact inhibition of cell growth but in contrast to other reports, did not affect log phase growth rates. Cell death, senescence, or suppression of growth factor signaling was not involved because no significant alterations were seen in cell viability, telomerase, or mitogen-activated protein kinase activity. However, suppression of cell growth by Cx43 entailed the secretion of growth-regulatory factors. Most notably, a major component of conditioned medium that was affected by Cx43 was found to be MFG-E8 (milk fat globule epidermal growth factor 8), which is involved in cell anchorage and integrin signaling. These results indicate that Cx43 regulates cell growth by the modulation of extracellular growth factors including MFG-E8. Furthermore, the ability of a Cx to regulate cell growth may rely on its ability to mediate the intercellular transfer of endogenous metabolites but not artificial dyes.

INTRODUCTION

Gap junctions are the only channels that directly connect the cytoplasm of adjacent cells. They are formed by the multimeric aggregation of integral membrane proteins called Cxs, which contain four transmembrane domains, an intracellular loop and termini, and two extracellular loops that are involved in docking with Cxs of neighboring cells. There are over 13 mammalian Cx homologues, commonly named according to their predicted molecular weights, which enable selective interactions between family members, differential modes of regulation (1-3), and the formation of channels with different conductances (4, 5) and permeabilities to ions (6-8), fluorescent dyes (9), and metabolites (10, 11).

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The abbreviations used are: Cx, connexin; MAPK, mitogen-activated protein kinase; MFG-E8, milkfat globule epidermal growth factor 8; Dil, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; TRAP, telomere repeat amplification protocol; ERK, extracellular signal-regulated kinase; FPLC, fast protein liquid chromatography; AC0, α-carboxanolate; MALDI-TOF, matrix assisted desorption ionization-time of flight.
expression. MFG-E8 was the predominant extracellular factor af-

potentiated by alterations of extracellular factors in response to Cx43

Cx43 induced contact growth inhibition of these cells. This was

MAPK activity, or even log phase growth rates were detected. Instead,

signaling because no significant changes in cell viability, telomerase,

signals did not induce cell death, senescence, or suppress mitogenic

affected by Cx43 in these cells.

MATERIALS AND METHODS

Cell Maintenance and Evaluation of Gap Junctional Communication

and Growth. C6 cells transfected with Cx43 (clone 13), Cx32 (clone 3),

empty vector pLTR (clone 12), and parental cells (17, 19), as well as Cx43

transfectants (clone 13), subsequently transfected with the dominant-negative

Cx43 constructs Cx43L160 M (clone 6), Cx43A253V (clone 3), or empty

vector pReRSV (clone 1; Ref. 21), were maintained in DMEM + 10% FCS

at 37°C in 100% humidity.

Gap junctional communication was evaluated by the preloading method

with calcine (calcine acetoxymethyl ester; Molecular Probes, Eugene, OR) and

DiI (Di C18; Molecular Probes) as described previously (18). Calcine (623
daltons) is able to pass through gap junctions, whereas DiI, a stable, lipophilic
dye, does not pass to neighboring cells. Fluorescently labeled cells were mixed

and plated with nonlabeled cells at a 1:500 ratio such that they settled into

confuent monolayers. Calcine and DiI fluorescence was visualized through
green and red attenuating filters. Gap junctional communication was visualized

by microscopic examination and quantitated as the number of nonlabeled cells

receiving calcine from an individual labeled cell. Inhibition of dye transfer by

18a-carbenoxolone, a relatively specific and noncytotoxic gap junction

blocker (24), was used to verify the efficacy of this procedure.

Gap junctional transfer of endogenous metabolites between C6 cells trans-

fected with Cx32, Cx43, or empty vector was assayed by the capture protocol

(11, 25, 26) as described in method 2 of Goldberg et al. (25), except that

instead of donors being plated in the absence of receivers for quantitation,

transfer was quantitated based on donors and receivers from the same plates.

Briefly, donor cells were metabolically labeled overnight with D-[U- 3 H]glu-
cose and fluorescently labeled with DiI before being washed with PBS,

trypsinized, and plated with receiver cells at a 1:6.25 ratio such that they settled

into confluent monolayers. Cells were cocultured for 2–3 h, allowing them
to adhere to the plates and each other. Donors and receivers were

separated from each other by fluorescence-activated cell sorting, collected, and

lysed. Lysates were filtered through membranes with a Mf 

50,000 nominal molecular weight cutoff. Filtrates were further fractionated over a membrane

with a Mf 3,000 molecular weight cutoff. All retentates and filtrates were

assayed for radioactivity by scintillation counting. Radioactivity was calculated

case per cell for receivers and donors from the same plate. Rates

were then determined to ascertain the degree of transfer. The portion of

radioactive material within a given size range that was derived from incorpo-

rated transjunctional metabolites that transferred from donors to receivers was

calculated as the ratio of the radioactive material within the given size range in

donors over that of the same size range in receivers from the same plate. The

percentage of radioactive material within a given size range that was derived from transjunctional metabolites that transferred within 20 min of communi-
cation was calculated by dividing the portion of radioactive material within a
given size range that was derived from incorporated transjunctional metabo-
lites that transferred from donors to receivers within 20 min, by the portion of

radioactive material within the same size range that was derived from incor-

porated transjunctional metabolites that transferred from donors to receivers

within 2 h (steady state), and multiplying by 100.

For analysis of growth, 20,000 cells were plated in 1 ml of standard

medium/well on 12-well cluster plates and fed every 4 days. Cells were
counted by removing medium, suspending in 0.25% trypsin and 1 mM EDTA

in PBS, adding to Isotone (Coulter), and passing through a model ZM Coulter
counter.

Telomerase Assays. Telomerase extraction was performed on confluent

cells according to a 3-[3-cholamidopropyl]dimethylammonio]-1-propanosulfonate

detergent lysis protocol (27). Cells were washed twice with either PBS (C6 cells)

or TRAP washing buffer [10 mM HEPES-KOH (pH 7.5), 1.5 mM MgCl2,

1 mM KCl, and 1 mM EDTA]. HeLa cells, homogenized on ice for 30 s in 50 µl

of TRAP lysis buffer [1 mM Tris-HCl (pH 7.5), 1 mM MgCl2, 1 mM EGTA, 5

mM α-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glyc-

cerol] in 1.5 ml of microcentrifuge tubes with matching rotating pestles, left on

ice for 30 min, homogenized again for 30 s, and centrifuged at 4°C for 30 min

at 15,000 3 g. The supernatants were kept at 23°C until use.

Telomerase activity was assessed by the TRAP as described previously

(27). The reaction mixtures (50 µl) contained 20 mM Tris-HCl (pH 8.3), 63 mM

KCl, 1.5 mM MgCl2, 1 mM EGTA, 0.05% Tween 20, 25 µM of BSA, 50 µM

dexoyxynucleotides, triphosphates, 1 µg of T4gene32 protein (Boehringer-Mann-
heim), 0.1 µg of TS forward primer (5\’-AATCCGTGACAGAGATGT-3\’), 2

units of Taq polymerase (Boehringer-Mannheim), and 4 pCi of [α-32 P]dCTP

(3000 Ci/mmol, 10 µCi/µl). Sample extracts (5 µl) were added and the tubes

were kept at 23°C for 30 min to allow TS primer extension before addition of

0.1 µg of CX reverse primer (5\’-CCCTTAGGCTAA-3\’). A 30-cycle PCR (94°C

for 30 s, 50°C for 30 s, and 72°C for 90 s) was then performed in a Perkin-Elmer Thermo Cycler. Twenty-five µl of PCR reaction products were analyzed on 8% nondenaturing acrylamide gels. Gels were dried at 80°C, exposed to phosphor screens or X-ray film, and visualized on a Molecular Dynamics PhosphorImager or by autoradiography.

Evaluation of MAPK Levels. Total ERK1 and ERK2 protein levels were

evaluated by Western blot analysis of cell lysates with mouse monoclonal

antiserum directed against amino acids 219–358 of rat ERK2 (Transduction

Laboratories, Lexington, KY), which is pan-specific for ERK1 and ERK2.

Levels of ERK1 and ERK2 activity were evaluated with affinity-purified

antibody directed against tyrosine-phosphorylated amino acids 196–209 of

human ERK1 [DHTGFLTEYPVATWR; New England Biolabs, Beverly,

MA], which is specific for active phosphoforms of ERK1 and ERK2 because

phosphorylation of Y204 in the TXEYX consensus site contained in

this sequence is known to activate MAPK (28–30). Confluent cells were washed

once with PBS, lysed on the plates in SDS-PAGE sample buffer at a concen-

tration of 5,000 cells per µl, sonicated, boiled for 5 min, and centrifuged at

15,000 3 g for 5 min. The supernatants were stored at −80°C until resolution

by SDS-PAGE on 12% gels and transfer to Immobilon-P membranes (Milli-

pore). Antiserum was used according to the protocols suggested by the sup-

pliers and detected by chemiluminescence (ECL; Amersham).

Evaluation of Effects of Conditioned Media on Cell Growth. Serum-

free medium (DMEM) was conditioned for 24 h by 1 day postconfluent cells.

This medium was then concentrated to 100-fold by centrifugal filtration

through membranes (Amicon) with different molecular weight cutoffs to

obtain the fractions indicated in the text and related figures. Nontransfected C6

cells (20,000 cell/well) were plated in standard medium on multwell tissue

culture plates. After the cells settled (2–3 h), the medium was replaced with

media fortified with conditioned media fractions, as indicated in the text and

related figures. Fortified medium was created by adding concentrated condi-

tioned media such that equal concentrations were achieved (i.e., concentrated

conditioned medium was diluted 100-fold into standard medium). Cells were

cultured in the conditioned medium. Control cells were
grown in standard medium. Cells were counted by removing medium and

suspending in 0.25% trypsin and 1 mM EDTA in PBS, which was subsequently

added to Isotone (Coulter) and examined with a Coulter counter.

Analysis of Conditioned Media by FPLC, SDS-PAGE, and MALDI-

TOF Mass Spectrometry. Serum-free medium supplemented with 100

µCi/ml [35 S]Met and [35 S]Cys was conditioned and concentrated to obtain

proteins between Mf 50,000–100,000 as described above. The DMEM was

exchanged for 20 mM Tris (pH 7.5) by concentration over Centricon 50

membranes before the material was resolved by FPLC through an anion

exchange matrix (DEAE Sepharose; Pharmacia) with a 0–1 M gradient of

NaCl. Fractions were resolved by SDS-PAGE on 12% gels, which were
coomassie stained, dried, exposed to X-ray film (XAR-5 Kodak), and visual-

ized by autoradiography.

Bands were then excised from the gel, transferred to an acid-washed tube,
profiles were queried against the entire nr database by profound (32).4 formed on a perceptive voyager de-rp mass spectrometer in the linear mode applied to a MALDI sample plate, which was dried and washed with water to remove excess buffer salts. MALDI mass spectrometric analysis was performed on a perceptive voyager DE-RP mass spectrometer in the linear mode (31). Profiles were queried against the entire nr database by profound (32).4

Northern Blotting. Total cellular RNA was purified, resolved by gel electrophoresis (15 µg/lane), and blotted to nylon membranes as described previously (33). Blots were hybridized with [32P]dCTP-labeled (NEN Life Science Products) 2.0-kb, full-length mouse MFG-E8 cDNA (34) at 42°C in hybridization buffer (5× SSPE, 5× Denhardt’s solution, 50% formamide, 0.5% SDS, and 0.1 mg/ml salmon sperm DNA), washed at high stringency, and examined by autoradiography. Membranes were then stained with methylene blue (35). Bands were quantitated by image densitometry (ImageQuant; Molecular Dynamics).

RESULTS

Gap Junctional Dye Transfer Between Cells. Functional expression of CxS in C6 transfectants was initially assessed by the preloading assay to measure the spread of fluorescent dye to neighboring cells (18). As shown in Fig. 1A, Cx43 transfectants displayed significantly higher (>2-fold) levels of communication than control transfectants. However, Cx32 transfectants passed dye to several times as many cells as the Cx43 transfectants. In all cases, dye transfer was completely inhibited by ACO, a relatively specific and noncytotoxic gap junction blocker (24). Communication that occurred within 20 min (after removal for the blocker) displayed similar results as communication seen without ACO.

Effects of Cx Expression on Cell Growth. Growth curves revealed a significant inhibition of cell growth by Cx43, but not Cx32, consistent with previous studies on these cells (17, 19). However, in contrast to previous reports (17, 36), as shown in Fig. 2A, all cells showed similar log phase growth rates. Instead, growth suppression resulted from an induction of contact inhibition of growth, reflected in a 2-fold decrease in cell saturation densities of the Cx43 transfected cells compared with Cx32 or control transfectants (Fig. 2B).

Gap Junctional Transfer of Endogenous Metabolites between Cells. Interestingly, although Cx32 transfectants showed no growth inhibition, they were much better coupled than the Cx43 transfectants when assayed by fluorescent dye transfer (Fig. 1A). This presented an apparent paradox with respect to gap junctional communication and growth control. However, transfer of a particular dye may not accurately represent the communication potential of a specific channel for functionally relevant metabolites. To address this situation, we used the capture approach to examine the transfer of endogenous metabolites through these channels. We used glucose, which is the major food source for these and many other cells, to label a wide range of metabolites, thereby allowing the detection of endogenous transjunctional molecules in a nonbiased manner (11, 25, 26).

As shown in Fig. 1B, no significant difference in the ability of metabolites derived from glucose to travel through gap junctions composed of Cx43 or Cx32 was seen. Transfer of these metabolites was blocked by ACO. However, examination of molecules that transferred within 20 min of communication (after ACO removal) revealed that Cx43 transfectants were much better coupled with respect to metabolites than Cx32 cells. This was in stark contrast to communication measured by fluorescent dye shown in Fig. 1A.

3 Internet address: http://prowl.rockefeller.edu/cgibin/ProFound.
These molecules are natural permeants that may underlie the effects of gap junctions in cells, in this case, cell growth control. As a first step in investigating potential function, we sought to determine the fate of these transjunctional compounds. This was done by measuring the radioactivity of size fractionated radioactive material derived from transjunctional metabolites that transferred from donors to receivers (as described in “Materials and Methods”). As shown in Fig. 1B, this suggested that a significant portion of the transjunctional metabolites in these cells were used anabolically by the receivers.

However, Cx32 and Cx43 may mediate the transfer of compounds with different fates with respect to incorporation into macromolecules. As shown in Fig. 1C, about 22–35% of the radioactive material of M_r <50,000 in control and Cx32 transfectants coupled for up to ~2 h was derived from metabolites that transferred from donors to receivers within 20 min of communication. In contrast, approximately twice this amount, 72–78%, of the material within this size range was derived from transjunctional metabolites that transferred within 20 min between Cx43 transfectants. Although ~34% of labeled material M_r >50,000 in Cx32 receivers was derived from metabolites that transferred within 20 min, 50–57% of this material was derived from transjunctional precursors that passed within 20 min between control or Cx43 transfectants. This suggests that similar molecules passed through the channels made from endogenous or exogenous Cx43 in the control and Cx43 transfectants, respectively, whereas Cx32 mediated the transfer of compounds that were not as readily incorporated into macromolecules M_r >50,000. This effect was significant, with 57% of the total variance accounted for by the Cx phenotype (P < 0.05) by ANOVA.

**Effect of Cx43 on Telomerase Activity.** The Cx-induced contact growth inhibition described above could have been attributable to either inhibition of cell division or induction of cell death. One indication of changes in cell senescence that accompanies neoplastic transformation is the activity of telomerase, proposed to be responsible for the chromosome maintenance that is necessary for immortalization of transformed cells (37). This activity was measured by the TRAP in Cx43 and control transfectants. On the basis of the relative intensities of the ladder of bands obtained (representing amplified telomeric sequences), shown in Fig. 3A, the expression of Cx43 was not accompanied by any decreased telomerase activity. This suggested that the decreased cell saturation density associated with Cx-mediated growth suppression was not associated with enhanced cell senescence. There was also no morphological evidence for increased levels of necrotic cell death or detachment of cells from the culture dishes at higher densities. Therefore, we chose to examine the effects of these Cxs on the inhibition of mitogenic pathways.

**Effects of Cx Expression on Mitogenic Signal Transduction.** MAPK represents a key element of the serum-induced mitogenic signal transduction cascade (28). Thus, as a first step in determining the influence of gap junctional communication on mitogenesis, effects of growth-inhibitory Cx expression on MAPK levels and activity were examined. The procedure used two immunological reagents. One antisera was pan-specific for ERK1 and ERK2, whereas the other was specific for active phosphoforms of these proteins. In this way, both total and active levels of MAPK were determined from 1-day postconfluent cultures by Western blot analysis. Growth curves were obtained from cells grown in parallel to confirm that specific Cx-mediated growth inhibition was evident. No significant changes in total MAPK levels were associated with Cx43-mediated growth inhibition of these cells (Fig. 3B). If anything, a slight increase in active MAPK may appear to have accompanied Cx43 expression in C6 cells. However, this was not consistent nor statistically significant upon repeated analysis. Preliminary evidence also suggests that levels of MAPK kinase 1/2, MAPK kinase kinase, signal transducers and activators of transcription 3, and cyclic AMP-responsive element binding protein were not significantly affected by Cx-mediated growth inhibition of these cells (data not shown).

**Examination of Secreted Factors Modulated by Cx43.** Although no overt effects on mitogenic signaling or processes related to cell

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**Fig. 2. Effects of Cx expression on cell growth. A.** growth curves. Twenty thousand cells were plated on 12-well cluster plates, fed every 4 days, and counted at the time points indicated. Each data point represents the mean of three wells, each counted three times; bars, SE. **B, cell saturation densities shown as cells/cm^2^. *, growth significantly different from control cells (P < 0.001 by t test).**

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**Fig. 3. Effects of growth-inhibitory Cx expression on telomerase and MAPK activity.** A, telomerase activity. Cell extracts, standardized for cell number, were examined for telomerase activity by the TRAP assay as described in “Materials and Methods.” Telomerase activity is indicated by intensity and size of species produced by the amplification reactions. No decrease in band profiles or intensities accompanied Cx43-mediated growth suppression. Arrow, positions of primer-dimer migration. **B, MAPK activity. Levels of total (top) and active (bottom) forms of ERK1 and ERK2 were evaluated in cells from 1 day postconfluent cultures (75,000 cells/lane) by Western blot analysis (see text). Positions of ERK1 and ERK2 are indicated. The apparent increase in active MAPK levels is not statistically significant or completely reproducible. Bars, SE.
senescence were evident, earlier reports have linked growth suppression of C6 cells by Cx43 to the production of a diffusible growth-inhibitory factor (36). Because this might provide insight into the intracellular processes that may be affected by gap junctions, we sought to further characterize this activity. Using a series of size exclusion filters (with \( M_r 3,000, 10,000, 30,000, 50,000, \) and \( 100,000 \) nominal molecular weight cutoffs), it was found that the growth-inhibitory activity consistently associated with fractions containing material \( M_r >50,000 \) and \( M_r <100,000 \) (Fig. 4). Parallel fractions of media from control transfectants showed no such activity. The size range of the active fraction suggested that the soluble factor was likely to be a complex polypeptide. Consistent with this, the activity of this fraction was refractive to nuclease but sensitive to heat treatment (95°C for 5 min; data not shown).

After purification by size, components in the conditioned media were resolved by charge over a DEAE anion exchange matrix. FPLC elution profiles of 14 ml of the \( M_r 50,000–100,000 \) fraction of conditioned media from Cx43 and control transfectants yielded one major absorbance peak that eluted at \( \sim 470 \text{ mM NaCl} \) (Fig. 5A). However, a minor peak and a shoulder that eluted at about 550 and 700 mM, respectively, were increased in Cx43-coupled cells. In contrast, a major peak eluting at \( \sim 900 \text{ mM NaCl} \) (arrow in Fig. 5A) was suppressed by Cx43.

Examination of these FPLC fractions by SDS-PAGE (Fig. 5B) identified polypeptides that were differentially induced (arrowheads) or suppressed (arrows) by Cx43. These bands were metabolically labeled and, therefore, represented direct products of the cells, as opposed to exogenous components of the media. The major difference was seen as a 60,000 extracellular compound that was selectively suppressed by Cx43 (arrow in Fig. 5B). Resolution of this peptide from other components in the conditioned medium enabled its identification by MALDI-TOF peptide mass mapping of trypsin-generated fragments eluted from the gel (32). As shown in Fig. 6, with \( >50\% \) coverage, this peptide mass mapping revealed the protein to be rat MFG-E8 (38).

Northern blotting was used to verify repression of this protein by Cx43 at the RNA level. As seen in Fig. 7, MFG-E8 mRNA was suppressed to \( \sim 50\% \) of the levels seen in either Cx32, empty vector, or nontransfected cells. As stated in the introduction, the dominant-negative Cx43 mutants L160M and A253V both ablate growth regulation by wild-type Cx43. Northern blot analysis in Fig. 7 reveals that, along with abrating growth suppression, these mutants also ablate MFG-E8 suppression by wild-type Cx43. This observation is consistent with MFG-E8 repression playing a functional role in Cx43 induction of contact growth inhibition. Suppression of MFG-E8 by Cx43 carries some interesting implications, as discussed below.

**DISCUSSION**

**Growth Rate versus Contact Growth Inhibition.** To our knowledge, this report contains the first direct comparison of the effects of Cxs and Cx32 on cell growth. Transfection of C6 glioma cells with Cxs has created one of the earliest and most comprehensively studied systems to investigate the effects of gap junctions on cell growth (15, 17). In line with previous reports (15, 17, 19, 36), Cx43 suppressed C6 cells in vitro, whereas Cx32 had no effect. However, in contrast to earlier reports (17), growth rate was not affected in the course of Cx-mediated cell growth inhibition. Instead, Cx43 induced contact growth inhibition, which resulted in a 2-fold decrease in cell saturation density. This discrepancy between our results and those of others may be explained by our use of cultures seeded at low density to more precisely define growth rates before cells were able to touch each other. In addition, our use of parallel growth curves to simultaneously examine the effects of Cxs on these cells circumvented possibilities of serum or other components in the media, aging, or somehow altering their ability to support cell growth between experiments. We saw no evidence of cell death or cells being released from the plate throughout these studies. This is in line with our earlier findings that Src-transformed cells remain viable during their reversion to a normal phenotype by Cx43-mediated interactions with neighboring nontransformed cells (35).

Because Cx-mediated cell growth suppression occurred at the level of contact growth inhibition, we assayed mechanisms at cell confluence that may have been involved. Telomerase activity was not decreased by Cx43, which was not surprising because, as stated above, cell death did not accompany growth suppression. In the absence of cell death or senescence, attention was turned to mitogenic signaling through the MAPK pathway. MAPK is both necessary and sufficient for cell transformation and is a key element of serum induced growth (28, 39). However, we found no significant alterations in MAPK levels to accompany Cx-mediated growth inhibition.

**Gap Junctional Communication.** Despite a large number of investigations, mechanisms underlying the ability of Cxs to suppress transformed cell growth have not been defined. Even the most basic...
questions regarding this have gone unanswered. The only bona fide function of Cxs is to allow cells to share small hydrophilic molecules (1–3). Rather than decrease growth rates, Cx43-mediated growth suppression resulted from increased contact growth inhibition of these cells. This observation is consistent with the formation of gap junctions and intercellular communication networks being required for cell growth inhibition by Cxs.

However, as discussed in the “Introduction,” a role for gap junc-

Fig. 5. Resolution of factors in conditioned media by FPLC and SDS-PAGE. Serum-free medium supplemented with [35S]Met and [35S]Cys was conditioned by LTR and Cx43-transfected C6 cells, as indicated, and concentrated to obtain proteins between $M_r$ 50,000 and $M_r$ 100,000 as described above. A, FPLC. Components within this fraction were then resolved by FPLC. A large absorbance peak unaffected by Cx43 eluted at about 470 mM NaCl. However, Cx43 increased the presence of a shoulder at 550 mM and a minor peak at 700 mM (arrowhead), while decreasing a major peak at ~900 mM (arrow). B, SDS-PAGE. Each fraction of eluted material was examined by SDS-PAGE and autoradiography to find polypeptides that were differentially induced (arrowheads) or suppressed (arrows) by Cx43. The number of each lane corresponds to the fraction numbers in A that eluted from the column as indicated on the x-axis. As described in the text, these bands were metabolically labeled and, therefore, represented direct products of the cells, as opposed to exogenous components of the media. In particular, a $M_r$ 60,000 protein was suppressed by Cx43 and eluted at 900 mM, which is indicated by an arrow in fraction (Lane) 8. The migration of molecular weight markers are indicated in thousands. These results were completely reproducible ($n$ = 4).

Fig. 6. Examination of a Cx43 suppressed peptide by MALDI-TOF mass spectroscopy. A secreted peptide that was suppressed by Cx43 (arrow in fraction 8 of Fig. 5B) was excised from the gel, digested with trypsin, and identified to be MFG-E8 by MALDI-TOF mass spectroscopy of the resulting fragments. Predicted trypsin-generated fragments of rat MFG-E8 between $M_r$ 1000 and $M_r$ 3000 are listed, with those detected indicated by asterisks and annotated on the profile. The position of the fragments is given as residues numbered after removal of the amino terminal signal peptide. Only 1 fragment $M_r$ >3000 ($M_r$ 4,526 from residues 1–43) would be expected from the digest that was not detected. The x-axis and y-axis of the profile are molecular weights and intensity of signal, respectively. Fragments generated by one or two missed cleavage sites are prefixed by a and b, respectively. *, position of the internal standard.
tional communication in Cx-mediated cell growth inhibition has been disputed. This claim has arisen from reports that the ability of Cx26 and Cx43 to normalize HeLa and C6 cell growth does not always correlate with their ability to transfer fluorescent dye (20, 21, 40). At first, the data reported here supported this contention, because Cx32 transfectants were better coupled than Cx43 transfectants when assayed by fluorescent dye transfer. However, use of the capture technique revealed that, in contrast to synthetic dyes, the Cx43 transfectants shared endogenous metabolites more efficiently than Cx32 transfectants. In support of this, we have shown recently that ATP and glutamate may travel 160 or 29 (respectively) times more efficiently through channels made of Cx43 than through channels made from Cx32 (11). We propose that the data reported here are consistent with gap junctional communication, rather than other unknown Cx-mediated interactions, underlying the ability of a given Cx to mediate growth suppression of a particular cell type.

Tracking the fates of transjunctional metabolites revealed that a significant portion of Cx43 permeants were incorporated into macromolecules more readily than those that transferred via Cx32. This is also consistent with preferential transfer of ATP and glutamate through Cx43, which could be used for RNA and protein production by receptor cells. However, the significance of this finding in terms of signals that mediate growth suppression is not clear. In the absence of specific growth regulatory transjunctional metabolites, we sought to define their targets and mechanisms as described below.

**Conditioned Media and Distinct Mechanisms.** As reported previously (36), growth-inhibitory activity was found in conditioned medium from Cx43-transfected C6 cells. These factors could present a handle to mechanisms by which gap junctions, or more specifically Cx43, can suppress the growth of particular cells (in this case, glioma). As a step in this direction, we resolved components in the conditioned media of Cx43 and control transfectants to find those that may play a part in this. Although actual growth-inhibitory factors induced by Cx43 were not identified here, direct and unbiased analysis revealed that suppression of MFG-E8 represented a major effect of Cx43 in these cells. This corroborates earlier work indicating that Cx43 modulates expression of extracellular proteins such as insulin-like growth factor binding protein 4 (41). However, our results are unique in that the proteins identified by this direct approach represented the major component of the extracellular solution that was altered by Cx43 (i.e., the peak containing MFG-E8 in fraction 8 of Fig. 5).

Cx43-transfected C6 cells that were secondarily transfected with dominant-negative Cx43 constructs were used to further define relationships between Cx43, MFG-E8, and glioma cell growth. These constructs restore tumorigenicity of C6 cells in the face of Cx43 expression. The mutant Cx43L160M, in which lysine 160 is changed to Met, is analogous to the Cx32V139M CMTX mutant. In addition to ablating growth regulation of C6 cells by Cx43 (21), the Cx43L160M construct also ablates Cx26 growth suppression of HeLa cells (40). Another mutant, Cx43A253V, represents a human polymorphism and has been found in metastatic meningiomas (42). Interestingly, although both constructs ablate growth regulation by wild-type Cx43, only Cx43L160M blocks dye transfer between these cells. In contrast, the Cx43A253V mutant increases tumorigenicity of the cells without affecting their ability to share Lucifer Yellow (21). However, both of these mutants elevated MFG-E8 expression in the Cx43 transfectants. This result is consistent with MFG-E8 suppression playing an important role in Cx43-mediated growth suppression of these cells.

In addition to providing a potential handle to mechanisms by which Cx43 inhibits glioma cell growth, this finding may also shed light on reasons behind the cell type dependence of specific Cxs to regulate cell growth. For example, if suppression of MFG-E8 is functionally related to Cx43 growth suppression, HeLa cells would not be susceptible because they do not produce significant levels of MFG-E8 (43). This seems to be the case because Cx43 does not suppress HeLa cell...
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