

# A Mitosis-specific Phosphorylation of the Gap Junction Protein Connexin43 in Human Vascular Cells: Biochemical Characterization and Localization

Han-qing Xie,\* Dale W. Laird,‡ Tsg-Hui Chang,\* and Valerie W. Hu\*

\*Department of Biochemistry and Molecular Biology, The George Washington University, Washington, DC, 20037; and

‡Department of Anatomy and Cell Biology, McGill University, Montreal, Canada H3A-2B2

**Abstract.** Western blotting studies revealed that connexin43 (Cx43), one of the major gap junction proteins in human vascular endothelial cells, is posttranslationally modified during mitosis. This mitosis-specific modification results in a Cx43 species that migrates as a single protein band and was designated Cx43<sub>m</sub>. Cx43<sub>m</sub> was shown to be the result of additional Ser/Thr phosphorylation as indicated by: (a) the increased gel mobility induced by both alkaline phosphatase and the Ser/Thr-specific protein phosphatase-2A (PP2A) and (b) the removal of virtually all <sup>32</sup>P<sub>i</sub> from Cx43<sub>m</sub> by PP2A. Immunofluorescent confocal microscopy of mitotic

cells revealed that Cx43 is intracellularly located, while in nonmitotic cells Cx43 is located at regions of cell-cell contact. Dye coupling studies revealed that mitotic endothelial cells were uncoupled from each other and from nonmitotic cells. After cytokinesis, sister cells resumed cell coupling independent of de novo protein synthesis. The mitosis-specific phosphorylation of Cx43 correlates with the transient loss of gap junction intercellular communication and redistribution of Cx43, suggesting that a protein kinase that regulates gap junctions is active in M-phase.

**G**AP junctions are aqueous channels connecting adjacent cells through which small molecules with molecular weights of less than 1,000 can pass from one cell to contacting cells (Loewenstein, 1981). These channels are formed when oligomers of connexin proteins in each of the apposing plasma membranes become aligned, providing a continuous aqueous passageway between cells. Gap junctions are ubiquitously distributed in normal animal cells and thus have been thought to be a principal means of maintaining tissue homeostasis (Loewenstein, 1981). In addition to this function, these structures have been implicated in the control of cell growth, notably by their frequent absence or reduction in tumor or transformed cells (Loewenstein, 1979; Trosko et al., 1990) and by their corrective effects on cell proliferation when connexins are transfected into these cells (Eghbali et al., 1991; Mehta et al., 1991; Zhu et al., 1991; Rose et al., 1993). Regulation of gap junctions by growth and differentiation factors has also been extensively reported (Kamibayashi et al.,

1993; Meda et al., 1993; Trosko et al., 1993; Brissette et al., 1994; Cronier et al., 1994; Hu and Xie, 1994; Zhang and Thorgeirsson, 1994). In recent years, different connexins have been cloned and found to form a family of proteins with extensive homology (Beyer et al., 1990), and several of these have been identified as candidate tumor suppressor proteins (Eghbali et al., 1991; Lee et al., 1991; Mehta et al., 1991; Zhu et al., 1991; Bond et al., 1994; Mesnil et al., 1995; Hirschi et al., 1996).

The cell cycle is the basic unit of cell growth, and this process necessarily involves extensive regulation and coordination. Mitosis, in particular, is marked by dramatic morphological and biochemical changes in both nuclear and cytoplasmic elements. Several laboratories, including ours, have reported on changes in gap junction-mediated intercellular communication (GJIC)<sup>1</sup> during the cell cycle using various methods (Yee and Revel, 1978; Gordon et al., 1982; Dermietzel et al., 1987; Su et al., 1990; Xie et al., 1991; Stein et al., 1993). Furthermore, a number of studies have shown a reduction in GJIC between mitotic and nonmitotic cells (Goodall and Maro, 1986; Stein et al., 1992), although contradictory results indicate that at least some

Address all correspondence to Dr. Valerie Hu, Department of Biochemistry and Molecular Biology, George Washington University Medical Center, 2300 Eye St., N.W., Washington, DC 20037. Tel.: (202) 994-8431. Fax: (202) 994-8974.

Han-qing Xie's current address is Department of Psychiatry and Behavioral Neurobiology, University of Alabama at Birmingham, Birmingham, AL 35295-0017.

1. *Abbreviations used in this paper:* Cx43, connexin43; gap-FRAP, gap fluorescence redistribution after photobleaching (a dye coupling assay for gap junctions); GJIC, gap junction intercellular communication; HUVEC, human umbilical vein endothelial cells; PP2A, protein phosphatase-2A.

form of gap junction activity might persist between mitotic and nonmitotic cells (O'lague et al., 1970; Goodall and Maro, 1986). Although changes in the transcription and expression level of certain connexins during the cell cycle have been demonstrated (Dermietzel et al., 1987; Lee et al., 1992), no cell cycle-dependent posttranslational modification of connexin(s) has yet been identified.

In this paper, we report on and characterize a mitosis-specific modification of one of the gap junction proteins (connexin43 [Cx43]) in vascular endothelial cells that had been suggested in an earlier study on cell cycle-dependent GJIC (Xie and Hu, 1994a). This mitosis-specific species of Cx43 was often localized to intracellular compartments in mitotic cells. Using a dye-transfer technique, we observed reduced coupling between mitotic cells and nonmitotic cells followed by recoupling of sister cells after cytokinesis.

## Materials and Methods

### Cell Culture and Mitotic Selection

Human umbilical vein endothelial cells (HUVEC), strain HX1, was isolated from a fresh human umbilical cord according to established procedures (Jaffe et al., 1973). HUVEC strain H101 was a generous gift from Dr. Thomas Maciag (American Red Cross, Rockville, MD). Human arterial endothelial cells were the kind gift of Dr. Boon Ooi (Veteran's Administration Medical Center, Washington, DC). All endothelial cells were cultured according to standard methods (Maier et al., 1990). Rat vascular smooth muscle cells were kindly provided by Dr. William Weglicki (Division of Experimental Medicine, The George Washington University Medical Center, Washington, DC). These cells were cultured in DME supplemented with 10% FBS.

Mitotic cells were obtained by collecting nocodazole- or colcemid-treated cells. A mitotic inhibitor, nocodazole at 0.4  $\mu\text{g/ml}$  (Sigma Chemical Co., St. Louis, MO) or colcemid at 0.1  $\mu\text{g/ml}$  (GIBCO BRL, Gaithersburg, MD), was added to cell culture 3 h after removal from a double thymidine block, which arrests cells at the G1/S boundary (Adams, 1990). 5 h later, floating and loosely attached mitotic cells were collected by gentle aspiration. Occasionally, mitotic cells were obtained after treating HUVEC with 0.5  $\mu\text{g/ml}$  nocodazole overnight without a prior thymidine block. Mitotic cells obtained by either procedure yielded the same results. By trypan blue staining, it was determined that the collected mitotic cells had a viability of 85–99%. When reseeded, >80% of the mitotic cells could adhere to culture dishes. Cells still adhering to the substratum after mitotic shakeoff or, alternatively, unsynchronized cells were dislodged with brief treatment of trypsin-EDTA and used as controls in all Cx43 analyses.

### Gap Fluorescence Redistribution after Photobleaching

Cell coupling assays were performed as previously described (Xie and Hu, 1994b) using gap fluorescence redistribution after photobleaching (gap-FRAP) techniques developed by Wade et al. (1986). Briefly, cells were stained at room temperature with 7.3  $\mu\text{g/ml}$  carboxyfluorescein diacetate for 15 min. Using an interactive laser cytometer (model ACAS 570; Meridian Instruments, Okemos, MI), selected cells were bleached with 8–14 50-ms pulses of a strong 488-nm laser beam. The bleached cells included rounded-up or dividing mitotic cells remaining in physical contact with nonmitotic cells after vigorously shaking the culture dish (Table I) or one of a pair of sister cells right after cytokinesis (see Fig. 4). Positive controls consisted of bleached nonmitotic cells that were attached to other nonmitotic cells, while negative controls were bleached cells that were not in contact with any other cells. The fluorescence in unbleached cells was also monitored over time and used to correct for background leakage or bleaching of dye during the course of the experiment. The time-dependent recovery of fluorescence after photobleaching was monitored for each cell by repeated laser scannings during the first 3 min after bleaching. Recovery rates represent the percentage of dye recovery/min relative to the initial (prebleach) fluorescence for the respective cells, as determined by the Cell-Cell Communication software provided by Meridian Instruments.

### Alkaline Phosphatase Digestion and Western Blots

Control and mitotic lysates were collected from human arterial endothelial cells as described earlier. Mitotic cell lysate buffer was exchanged for alkaline phosphatase digestion buffer (100 mM Tris, pH 8.0, 40 mM NaCl, 1 mM  $\text{MgCl}_2$ , and 0.1% SDS) by repeated centrifugation in a concentrator (model Centricon-30; Amicon, Danvers, MA). Briefly, lysis buffer was exchanged for digestion buffer by adding 1 ml of digestion buffer (3 $\times$ ) and concentrating the sample to  $\sim$ 0.2 ml by centrifugation (model J2-21; Beckman Instruments, Fullerton, CA) at 5,000 rpm for  $\sim$ 40 min. Samples were digested with 30 U of alkaline phosphatase (BMB, Molecular Biology grade; 20 U/ $\mu\text{l}$ ) for 20 h at 4°C in the presence or absence of 2.5 mM  $\text{NaVO}_4$  and 50 mM NaF. Finally, all samples were treated with gel cocktail buffer, boiled for 5 min, and resolved on a 10% SDS-polyacrylamide gel with an acrylamide/bis-acrylamide ratio of 30:0.8. Proteins were transferred to nitrocellulose and immunoblotted for Cx43 as described in Laird et al. (1995).

In other experiments, Western blotting for the detection of Cx43 was performed as previously described (Xie and Hu, 1994a,b). In essence, mitotic or control cells were lysed directly in the SDS gel-loading buffer. The lysates were boiled, sonicated, and centrifuged. 50  $\mu\text{g}$  of protein from each sample was fractionated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were sequentially incubated with mouse monoclonal anti-Cx43 antibody (Zymed Labs, S. San Francisco, CA), rabbit anti-mouse IgG antibody (Fisher Scientific, Pittsburgh, PA), and HRP-conjugated goat anti-rabbit IgG antiserum (Zymed Labs) before detection of the HRP-labeled bands with an enhanced chemiluminescence kit (Amersham Corp., Arlington Heights, IL). In some experiments, CT-360, a rabbit polyclonal antiserum against the carboxy terminus of Cx43 (Laird and Revel, 1990), was used in place of the anti-Cx43 mouse monoclonal antibody and secondary rabbit anti-mouse IgG antiserum.

### Immunoprecipitation of Cx43

For some experiments, Cx43 (either unlabeled or labeled with  $^{32}\text{P}_i$ ) was immunoprecipitated with CT-360. In these cases, equal amounts of the dialyzed solubilized protein from the different samples were incubated with the antiserum followed by immunoprecipitation with immobilized protein A (Pierce, Rockford, IL). After thorough washing of the protein A complexes with washing buffer I (50 mM Tris, 150 mM NaCl, 0.1 mM EDTA, and 0.5% Tween-20, pH 7.5), followed by washing with buffer II (100 mM Tris, 200 mM NaCl, 2 M urea, and 0.5% Tween-20, pH 7.5), and finally water, samples were mixed with 2 $\times$  SDS gel-loading buffer and analyzed by SDS-PAGE followed by Western blotting or autoradiography.

### Metabolic Labeling and Treatment with Protein Phosphatase-2A, a Protein (Ser/Thr) Phosphatase

Mitotic HUVEC were collected and labeled with [ $^{32}\text{P}$ ]orthophosphate (ICN Biomedicals, Costa Mesa, CA) at 1 mCi/ml for 2 h in phosphate-deficient DME containing 10% dialyzed FBS (dialyzed against 0.15 NaCl overnight) in the presence of 0.4  $\mu\text{g/ml}$  nocodazole. The cells were then lysed, dialyzed, and immunoprecipitated as described above. After washing with washing buffer I, the protein A complexes were washed twice with protein phosphatase-2A (PP2A) dilution buffer containing 20 mM Hepes, 1 mM DTT, 100  $\mu\text{g/ml}$  BSA, 50  $\mu\text{M}$  leupeptin, 1 mM  $\text{MnCl}_2$  (Scheidtmann et al., 1991). The immunoprecipitates were then mixed with either 0.3 U PP2A (Upstate Biotechnology Inc., Lake Placid, NY) or dilution buffer alone. The mixtures (50  $\mu\text{l}$ ) were incubated at 30°C for 1 h. The phosphatase reaction was terminated by addition of SDS gel-loading buffer. Samples were then analyzed by SDS-PAGE and autoradiography. Similarly, immunoprecipitated Cx43 from unlabeled mitotic cells was treated with PP2A + or – phosphatase inhibitors (2 mM vanadate [Sigma Chemical Co.] and 1  $\mu\text{M}$  okadaic acid [Calbiochem]) and analyzed by Western blotting.

### Immunofluorescence Studies

Mitotic cells were obtained as previously described. The harvested cells were then fixed in 100% ice-cold ethanol for 30 min before rehydration with PBS. The anchored nonmitotic cells remaining on glass coverslips in the dish after the mitotic shakeoff were also fixed with ethanol and rehydrated. Mitotic cells were attached to Cell-Tak-coated glass coverslips by centrifuging at 1,500 g for 5 min. Both mitotic and nonmitotic cells were immunolabeled with the mouse monoclonal anti-Cx43 antibody or an antiserum (CT-360) generated against the carboxy terminus of Cx43 (Laird

and Revel, 1990). Both immunological reagents produced similar results, and only results obtained with the commercial anti-Cx43 antibody are shown in this report. Antibody binding to Cx43 was detected by incubating the cells in goat anti-mouse antibody conjugated to rhodamine.

Immunolabeled cells were analyzed on a confocal microscope (model 410 LSM; Carl Zeiss, Inc., Thornwood, NY). In the case of mitotic cells, the cell thickness in the Z dimension was determined and a confocal optical slice (~1  $\mu$ m thick) for Cx43 was taken at or near the center of the cell. Nonconfocal transmitted light images of the mitotic cells were taken at the same Z settings. In some cases, three to five confocal optical slices of Cx43 labeling were combined to more accurately illustrate the spatial localization of a broader representation of Cx43 in mitotic cells. Finally, single optical images of Cx43 in nonmitotic cells were collected. All images were printed on a high resolution printer (model 8300; Kodak, Inc., Rochester, NY).

## Results

### Cx43 Protein Is Modified during Mitosis

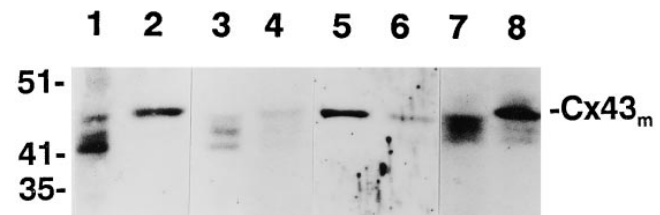
Western blot analyses with a monoclonal antibody against Cx43 revealed a higher relative molecular mass isoform of Cx43 in mitotic cells (Fig. 1, lanes 2, 4, 5, 6, and 8). This unique species, designated Cx43<sub>m</sub>, was estimated at 47–48  $M_r$  and was not restricted to a single cell type inasmuch as two strains of HUVEC (strains HX1 and H101; Fig. 1, lanes 5 and 6, respectively) as well as rat vascular smooth muscle cells (Fig. 1, lanes 7 and 8) exhibited this mitosis-specific modification. As shown in Fig. 1, mitotic cells contained predominantly one Cx43 species of higher relative molecular mass, while control cells contained up to three species of lower relative molecular mass. This mobility shift is unlikely to be the direct result of treatment with a specific mitotic inhibitor in that both colcemid (lane 2) and nocodazole (lane 4) arrest gave rise to Cx43 bands of similar mobility, and unsynchronized control cells that were treated for a few hours with nocodazole contained only the faster mobility forms of Cx43 (data not shown). Moreover, the mobility shift of Cx43 observed in mitotic cells is unrelated to a general rounding-up effect per se since the control cells were detached from the substratum with trypsin (causing the cells to round-up) before being lysed with the SDS gel-loading buffer.

### Cx43 Is Additionally Phosphorylated during Mitosis

As shown in Fig. 2 (lane *M* + *AP*), alkaline phosphatase treatment eliminated the gel shift of Cx43 seen in mitotic cells (lane *M*), suggesting that the mitosis-specific modification of Cx43 is a phosphorylation phenomenon. Furthermore, the phosphatase-mediated conversion of Cx43<sub>m</sub> to a Cx43 species of lower relative molecular mass was inhibited by phosphatase inhibitors (lane *M* + *AP* + *I*). Nonmitotic cells (lane *A*) had the well-characterized pattern of Cx43 species at relative molecular masses lower than Cx43<sub>m</sub>.

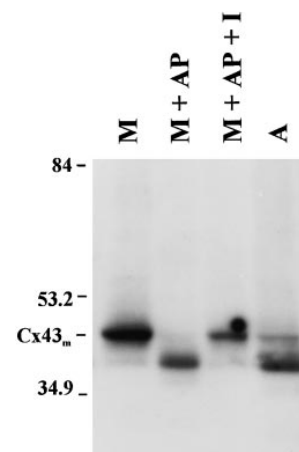
### Mitosis-specific Phosphorylation of Cx43 Is on Ser/Thr

Serine phosphorylation has been shown to correlate with gap junction assembly (Musil et al., 1990; Musil and Goodenough, 1991) as well as with 12-*O*-tetradecanoylphorbol-13-acetate and EGF stimulation (Brissette et al., 1991; Lau et al., 1992; Warn-Cramer et al., 1996). In addition to serine phosphorylation, Cx43 can be modified on tyrosine residue(s) by *src*, which has been shown to inhibit GJIC (Crow et al., 1990; Filson et al., 1990; Swenson et al., 1990).

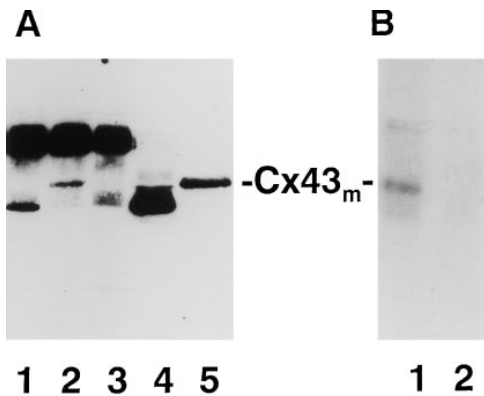


**Figure 1.** Western blot analyses revealed that Cx43 in mitotic cells from HUVEC strains HX1 and H101 and rat vascular smooth muscle cells had slower mobility, with a relative molecular mass of 47–48. Lanes 1 and 2, control nonmitotic and colcemid-arrested mitotic HX1 cells, respectively; lanes 3 and 4, control nonmitotic and nocodazole-arrested mitotic HX1 cells, respectively; lanes 5 and 6, mitotic HX1 and H101 HUVEC cells, respectively; lanes 7 and 8, nonmitotic and mitotic rat vascular smooth muscle cells, respectively.

Because *src* can be activated by a key initiator of mitosis, M-phase-promoting factor (Morgan et al., 1989; Shenoy et al., 1989), the possibility of Cx43 being phosphorylated by *src* on tyrosine residue(s) during mitosis was investigated by Western analyses of immunoprecipitated, unlabeled Cx43 protein with an anti-tyrosine phosphate antiserum. Although both mitotic and nonmitotic Cx43 were precipitated by the polyclonal anti-Cx43 antiserum CT-360, neither form of Cx43 reacted with the polyclonal anti-tyrosine phosphate antiserum on Western blots (data not shown). Absence of phosphotyrosine was further corroborated by the observation that the Ser/Thr-specific protein phosphatase PP2A, like alkaline phosphatase, was able to shift the Cx43<sub>m</sub> species (Fig. 3 A, lane 2) to the species of lowest relative molecular mass (highest mobility) (Fig. 3 A, lane 1) as well as remove virtually all <sup>32</sup>P-labeled phosphate from immunoprecipitated Cx43<sub>m</sub> (Fig. 3 B, lane 2). In comparison to Cx43 from nonmitotic cells, both immunoprecipitated and in whole cell lysate (Fig. 3 A, lanes 3 and 4, respectively), the Cx43<sub>m</sub> species (both immunoprecipitated and in whole cell lysate) appears to have a slightly higher relative molecular mass (Fig. 3 A, lanes 2 and 5, respectively), consistent with Cx43 banding patterns seen in Fig. 1. Furthermore, phosphoamino acid analysis by thin layer electrophoresis of <sup>32</sup>P-labeled mitotic Cx43 hydrolyzed by treatment with HCl revealed the presence



**Figure 2.** The mitotic form of Cx43 is alkaline phosphatase sensitive. Control human arterial endothelial cell lysates (*A*) or mitotic cell lysates (*M*) were treated with alkaline phosphatase (*AP*) in the presence or absence of inhibitors (*I*). Samples were resolved by SDS-PAGE and immunoblotted for Cx43. Note that the mitotic form of Cx43 (Cx43<sub>m</sub>) was specifically sensitive to alkaline phosphatase.



**Figure 3.** Western blot (A) and autoradiogram (B) of immunoprecipitated Cx43<sub>m</sub> after PP2A digestion. (A) Cx43 was immunoprecipitated from human arterial endothelial cells using polyclonal rabbit anti-Cx43 antiserum (CT-360). The immunoprecipitates from mitotic cells were treated for 1 h at 30°C with PP2A (0.25 U/μl), in the absence (lane 1) or presence (lane 2) of phosphatase inhibitors, and analyzed by Western blotting. A nonmitotic sample was also immunoprecipitated for comparison (lane 3). Also shown on the Western blot are whole cell lysates from nonmitotic (lane 4) and mitotic (lane 5) cells. The broad band above the Cx43 protein is the immunoprecipitating rabbit antibody that is recognized by the HRP-conjugated goat anti-rabbit IgG antiserum. (B) Mitotic HUVEC cells were labeled with [<sup>32</sup>P]orthophosphate for 2 h before immunoprecipitation. The immunoprecipitates were treated either with PP2A or with dilution buffer only, under conditions similar to those described above. Samples were analyzed by SDS-PAGE and autoradiography. Lane 1, sample treated with dilution buffer only; lane 2, sample treated with PP2A, which removes virtually all of the <sup>32</sup>P label from the immunoprecipitated Cx43<sub>m</sub>.

of only phosphoserine (data not shown). Taken together, these data suggest that the phosphorylated form of Cx43 unique to mitotic cells arises most likely by phosphorylation of a serine residue(s), although limits in the sensitivity of the techniques used might not have detected minor phosphorylation of tyrosine or threonine. However, additional serine phosphorylation has also been observed in mitotic Rat-1 fibroblasts, either transformed with *v-src* or overexpressing *c-src*, (Lau, A., personal communication).

### Mitotic Cells Are Transiently Uncoupled from Nonmitotic Cells

The coupling of mitotic HUVEC with nonmitotic cells was assessed through gap-FRAP studies that involved monitoring the flow of fluorescent dye from one cell into an attached photobleached cell. Gap-FRAP was conducted on rounded-up mitotic cells or dividing cells (doublets) that remained attached to neighboring or underlying nonmitotic cells after culture dishes had been vigorously shaken. Table I summarizes the fluorescence recovery data obtained for mitotic and control cells. The data show that the mean recovery rate for bleached mitotic cells in contact with nonmitotic cells is substantially lower than that of bleached nonmitotic cells and is similar to the mean recovery rate of bleached isolated cells that lacked any neighboring cell contacts (negative controls).

Colcemid-arrested mitotic HUVEC underwent cytokinesis within 2 h after resuspension in warm culture me-

**Table I. Comparison of Recovery Rates for Photobleached Mitotic and Nonmitotic Cells**

Cell	Recovery rate* (Percentage of initial F/min)	SEM
Nonmitotic	6.251 (7) <sup>‡</sup>	0.882
Mitotic	0.766 (7)	0.254
Isolated	0.452 (5)	0.139

\*Corrected for background leakage or bleaching.

<sup>‡</sup>Numbers in parentheses correspond to number of cells analyzed.

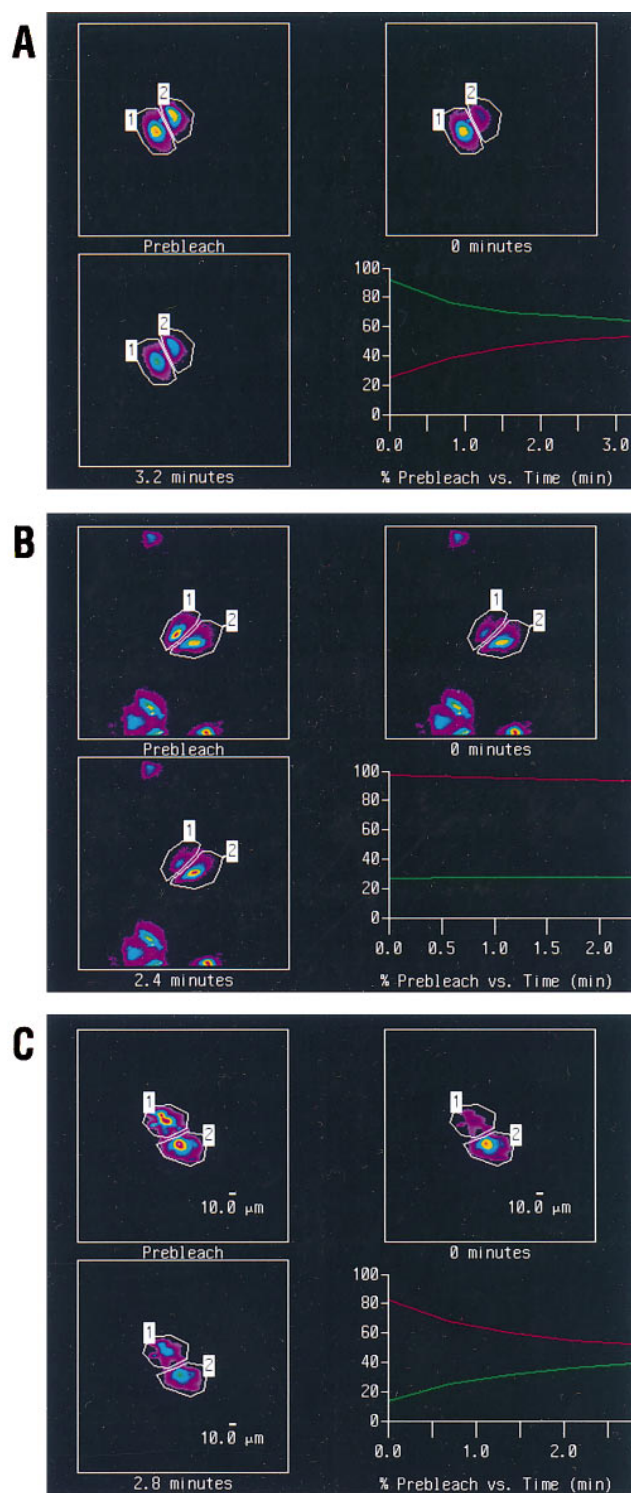
dium and attachment to the substratum. Microscopic observation suggested that the process of cytokinesis started immediately after attachment. Gap-FRAP analyses conducted on these newly divided cells show that the siblings resumed GJIC shortly after cytokinesis (Fig. 4 A). The characteristic kinetics of dye recovery (shown by the lower curve in Fig. 4 A) coupled with the observed suppression of dye recovery by 0.1% octanol, a gap junction inhibitor (Fig. 4 B), argue against the involvement of remaining cytoplasmic bridges between sister cells. On the other hand, recovery of GJIC could not be blocked when the mitotic cells were replated in the presence of cycloheximide (100 μg/ml) (Fig. 4 C), suggesting that de novo protein synthesis is not necessary for the reestablishment of functional gap junctions.

### Redistribution of Cx43 Protein during Mitosis

To determine the distribution pattern of Cx43 during mitosis, mitotic cells were immunolabeled for Cx43 and analyzed on a confocal microscope. The transmitted light images (Fig. 5, A and C) of mitotic cells in comparison with the corresponding confocal immunofluorescent images (Fig. 5, B and D) show that Cx43 was often localized to intracellular compartments when optical sections were taken through the center of the cell. Moreover, optical slice reconstructions revealed a substantial amount of redistributed Cx43 in mitotic cells (Fig. 5 E, arrows) as well as an overall increase in intracellular cytoplasmic staining. Conversely, nonmitotic cells that remained on the substrate after mitotic shake-off showed the typical distribution of Cx43, principally at locations of cell-cell contact (Fig. 5 F).

### Discussion

In this study we have identified and characterized a mitosis-specific species of Cx43 (Cx43<sub>m</sub>) in mitotic vascular endothelial and smooth muscle cells. Cx43<sub>m</sub> runs at a higher relative molecular mass on SDS-PAGE gels than at least two other well-characterized species of Cx43 that correspond to the unphosphorylated form of Cx43 and the Cx43(P<sub>1</sub>) species (Musil et al., 1990; Laird et al., 1991). However, the Cx43<sub>m</sub> species of Cx43 has only a slightly higher relative molecular mass than Cx43(P<sub>2</sub>) (Musil et al., 1990) and is similar in relative molecular mass to an EGF-induced Cx43 species (Lau et al., 1992). It is doubtful that Cx43<sub>m</sub> is directly related to the EGF-induced species of Cx43 or to any other reported phosphorylated species of Cx43 that are inducible by 12-O-tetradecanoylphorbol-13-acetate (Oh et al., 1991; Berthoud et al., 1992; Moreno et al., 1994), but this remains to be seen.



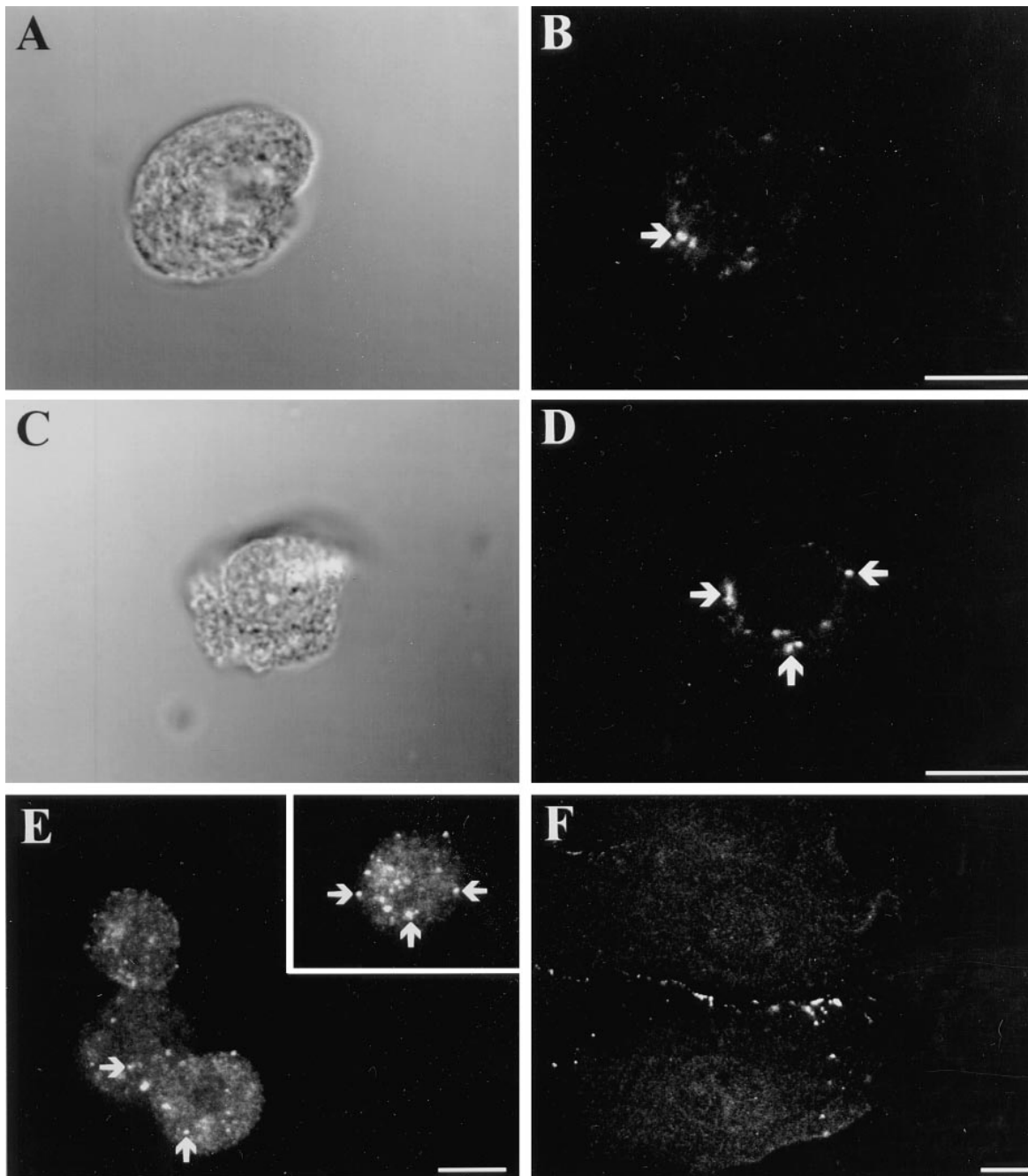
**Figure 4.** A representative set of gap-FRAP experiments examining the resumption of GJIC after cytokinesis. One of a pair of sister cells (HX1) was photobleached in each experiment. Cells were scanned at 0.8-min intervals after bleaching. Pseudocolor fluorescence images of the cells are shown at three time points: before bleaching, immediately after bleaching, and at  $\sim 3$  min after bleaching. The lower curves in the % prebleach fluorescence vs. time graphs depict the time-dependent fluorescence changes in the respective photobleached cells. In coupled cell pairs, a decrease in fluorescence in the unbleached cell is also observed (*a* and *c*, upper curves) as expected, as dye is transferred to the pho-

Although the kinase responsible for the mitosis-specific phosphorylation of Cx43 on serine is unknown, a consensus site for phosphorylation by p34<sup>cdc2</sup> (the kinase component of M-phase-promoting factor) has been identified on Cx43 (Kanemitsu and Lau, 1993). The generation of Cx43<sub>m</sub> cannot be attributed to the process of rounding up and lifting off from the substratum since trypsinized nonmitotic cells do not contain this form of the Cx43 protein. Furthermore, this high relative molecular mass species could not be induced by the short term addition of mitotic inhibitors to nonmitotic cells, thus ruling out a simple drug effect. While we cannot eliminate the possibility that Cx43<sub>m</sub> is solely a product of cells in metaphase arrest, the presence of this predominant species of Cx43 in M-phase cells suggests that alterations in gap junction activity/assembly/distribution might be specifically coordinated with the process of cell division and growth.

Gap-FRAP analyses of GJIC between mitotic and nonmitotic cells revealed the absence of coupling between these cells. This result is similar to that of some previously published studies (Goodall and Maro, 1986; Stein et al., 1992, 1993). The lack of dye coupling correlates with the phosphorylation of Cx43 protein during mitosis, suggesting that at least one consequence of the mitosis-specific modification might be the closure or disassembly of gap junctions between mitotic and nonmitotic cells, although the cellular mechanism of this downregulation is not known. Also unknown is the extent to which cell-to-cell transfer of material is inhibited, as electrical coupling has been shown to persist between mitotic and nonmitotic cells (O'lague et al., 1970; Goodall and Maro, 1986), even in the absence of dye coupling.

Gap-FRAP analyses of sister cells after cytokinesis indicate that cell coupling can resume in the absence of de novo protein synthesis. This result suggests several possible interpretations: (*a*) Cx43<sub>m</sub>, which is the predominant Cx43 species in mitotic cells, can be recycled into functional gap junctions; (*b*) a previously synthesized minor portion of nonmitotic Cx43 (residing in the endoplasmic reticulum or Golgi apparatus) is incorporated into the plasma membrane and new gap junctions are assembled; or (*c*) other endothelial connexins are responsible for cell coupling after division. To date, no clear evidence has been presented that demonstrates that connexins can recycle. On the other hand, intracellular reservoirs of Cx43 are well documented, as Cx43 has been localized to the Golgi apparatus of cardiomyocytes (Laird et al., 1993) and mammary tumor cells (Laird et al., 1995). Furthermore, in mammary tumor cells, the ER/Golgi store of Cx43 can be used to assemble gap junctions in the absence of protein synthesis (Laird et al., 1995). In other studies, Moskalewski et al.

photobleached cell. (*A*) Gap-FRAP on a pair of sister cells shows that the siblings resumed GJIC after cytokinesis ( $n = 10$ ). (*B*) Octanol inhibited the gap junction-mediated dye transfer between sister cells after cytokinesis. Octanol (0.1%) was added immediately before gap-FRAP analyses ( $n = 5$ ). (*C*) Gap-FRAP on a pair of sister cells shows that the resumption of gap junctions between sister cells could not be blocked by cycloheximide treatment. Mitotic cells were reseeded in culture dishes for 3 h in the presence of 100  $\mu\text{g/ml}$  cycloheximide ( $n = 5$ ).



**Figure 5.** Spatial distribution of Cx43 in mitotic and nonmitotic HX1 cells. Transmitted light images (*A* and *C*) of mitotic cells immunolabeled for Cx43 (*B* and *D*) show the spatial distribution of Cx43. Note the punctate labeling of Cx43 in intracellular compartments (*arrows*). (*E*) Reconstructed image of Cx43 in mitotic HX1 cells. Mitotic cells immunolabeled for Cx43 were optically sectioned and three images collected at 1- $\mu$ m intervals were superimposed to illustrate a succession of Cx43-positive cellular locations (*arrows*). The mitotic cell in the insert is a reconstruction of five images collected at 1- $\mu$ m intervals. (*F*) A confocal microscopic image for Cx43 in nonmitotic HX1 cells revealed punctate labeling at sites of cell-cell apposition with little intracellular labeling. Bars, 10  $\mu$ m.

(1994) showed that Cx43 gap junctions could assemble after cytokinesis even if protein trafficking was blocked with brefeldin A, suggesting that Cx43 in compartments that are more distal to the *trans*-Golgi was being used for gap junction assembly. The possibility that other connexin molecules (e.g., Cx40, Beyer et al., 1992; or Cx37, Reed et al., 1993) take part in cell coupling immediately after cytokinesis cannot be excluded, and it will be interesting to see if

any of these connexins undergo a similar mitosis-specific modification.

Immunofluorescence analyses indicate that Cx43 is indeed redistributed in mitotic cells and appears to be concentrated in intracellular structures. While some Cx43 is located near the plasma membrane, it is not clear if this protein still resides on the cell surface or if it is in the early stages of internalization. This apparent internalization of



junctional protein is one possible mechanism by which cell coupling between mitotic cells and nonmitotic cells could be reduced, although changes in gating, or alternate mechanisms, cannot be excluded. In contrast, Cx43 in anchored nonmitotic cells is observed at the contact regions between cells with limited localization in cytoplasmic compartments. Thus, the mitosis-specific phosphorylation of Cx43 may signal processes that lead to internalization and eventual degradation of Cx43 and/or closure of gap junction channels between mitotic and nonmitotic cells.

In summary, the identification of a mitosis-specific phosphorylation of Cx43 is the first demonstration of a cell cycle-related change in the posttranslational modification of a connexin protein. This phase-dependent modification is associated with the downregulation of GJIC between mitotic and nonmitotic cells as well as with a redistribution of Cx43 in mitotic cells. This relationship further emphasizes the coordination of molecular and cellular events that accompany mitosis.

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