Connexin-protein interactions are believed to be critical for the regulation of gap junctional intercellular communication and for the function of gap junctions formed by these complexes. We have primarily used immunoprecipitation strategies to investigate whether connexin43 binds to selected signaling and cytoskeletal proteins and whether connexin43-protein binding is altered in cultured astrocytes exposed to chemical ischemia/hypoxia, a treatment that resembles ischemia in vivo. Chemical ischemia/hypoxia induced marked dephosphorylation of connexin43, which was accompanied by increased association of connexin43 with c-Src, ERK1/2, and mitogen-activated protein kinase phosphatase-1 and by decreased association between connexin43 and \( \beta \)-actin. Moreover, we found that endogenous c-Src in normal astrocytes exists primarily in the Triton X-100-soluble membrane fraction, distinct from the Triton-insoluble fraction, which contains gap junctions. After chemical ischemia/hypoxia, c-Src appeared in the Triton-insoluble fraction and was co-immunoprecipitated with connexin43, suggesting that chemical ischemia/hypoxia induced translocation of c-Src to the Triton-insoluble fraction and association with connexin43. Furthermore, the “dephosphorylated” form of connexin43 was immunoprecipitated by a phosphotyrosine antibody, suggesting tyrosine phosphorylation of connexin43 by c-Src. In addition, the association between connexin43 and c-Src was blocked by inhibition of connexin43 dephosphorylation, suggesting that the interaction between connexin43 and c-Src can be regulated by alterations in the phosphorylation state of connexin43. These results identify new binding partners for connexin43 and demonstrate that interactions between connexin43 and protein kinases and phosphatases are dynamically altered as a consequence of connexin43 phosphorylation.

Gap junctions in vertebrates are intercellular channels formed by proteins termed connexins and are permeable to second messenger molecules, metabolites, and ions. Connexin expression and gap junctional intercellular communication are critical for the regulation of cell locomotion, proliferation, and survival (1–4). To date several human diseases have been linked to connexin mutations (for reviews in Refs. 5 and 6).

Connexin43 (Cx43) is expressed in a variety of tissues including astrocytes in the nervous system. Phosphorylation is the primary form of posttranslational modification of Cx43, and alterations in Cx43 phosphorylation state have been correlated with changes in the strength of gap junctional coupling (7–11). Moreover, the regulation of gap junctional coupling by altering phosphorylation state of Cx43 is tissue- and kinase-specific (11–13). To date, phosphorylation sites of Cx43 for several protein kinases have been mapped, and a relationship between phosphorylation of these sites and gap junctional coupling state is being established (11). Thus, it is important and timely to investigate the nature of protein kinases and phosphatases interacting with Cx43 under particular physiological and pathological conditions.

The Src family kinases were among the first protein kinases known to interact with Cx43 and to regulate gap junctional coupling. For instance, v-Src expression in mammalian cells can induce phosphorylation at Tyr-247 and Tyr-265 of Cx43 and subsequent inhibition of gap junctional intercellular communication, which has been proposed to underlie the transformation of mammalian cells transfected with Rous sarcoma virus (11, 14). v-Src has also been shown to bind directly to Cx43 and to induce tyrosine phosphorylation both in vitro and in vivo (11, 14). c-Src, the cellular homolog of v-Src, is believed to interact with Cx43 in a similar manner. For example, agents such as lysophosphatidic acid and thrombin that are known to activate c-Src signaling pathways and exogenous overexpression of wild-type c-Src have each been shown to cause tyrosine phosphorylation of Cx43 and a reduction of gap junctional coupling (15–17). Moreover, exogenous wild-type c-Src has been shown to bind to endogenous Cx43 in cardiac myocytes and fibroblasts (16, 17). However, it has been controversial whether endogenous c-Src interacts with endogenous Cx43 and whether such interaction regulates gap junctional coupling (13, 16–18). In a recent study we found that c-Src association with Cx43 was markedly increased in astrocytes when intracellular pH was reduced, suggesting that the interaction between Cx43 and c-Src is a regulated process that may occur during acidosis (19). In this model the intracellular acidosis was induced by a collapse of transmembrane potassium concentration gradient in the presence of nigericin. This is an intriguing finding since we previously reported that both ischemia, which causes intercellular acidosis, and increased extracellular potassium concentration can induce dephosphorylation of Cx43 in astrocytes (20, 21). Thus, increased association between Cx43 and c-Src during intracellular acidosis may be
related to an alteration in the phosphorylation state of Cx43. In this study we tested this hypothesis in cultured cortical astrocytes, which express abundant Cx43 as their primary gap junction protein. Moreover, we and others have previously shown that dephosphorylation of Cx43 can be induced in rat astrocytes in vivo after ischemia and in vitro under the conditions described below (10, 22).

**EXPERIMENTAL PROCEDURES**

**Reagents**—Dulbecco’s modification of Eagle’s medium and Hanks’ balanced salt solution were obtained from Cellgro (Herndon, VA), trypsin, fetal bovine serum, and antibiotics were from Invitrogen, and tissue culture dishes were obtained from Fisher. Sodium ioacetate (I-9148) and sodium cyanide (S-3296) were obtained from Sigma. Polyclonal antibodies against MKP-1 (M-18) and c-Src (SC-18) were obtained from Upstate Biotechnology Inc. (Lake Placid, NY); a monoclonal anti-phosphotyrosine antibody (05-321) were from Upstate Biotechnology Inc. (Lake Placid, NY); a monoclonal β-actin antibody was from Sigma. A polyclonal anti-p38 antibody was from Dako (Z0334, Carpinteria, CA). A well characterized affinity-purified polyclonal anti-Cx43 antibody termed 18A was also used in this study (10, 20, 23).

**Primary Culture of Mouse Cortical Astrocytes and Induction of Acidosis and Chemical Ischemia (CIH)**—Primary cortical astrocytes were obtained from postnatal day 2 mouse pups as described (10). Briefly, pups were sacrificed using isofluorane and decapitated, and brains were removed from the skull into cold phosphate-buffered saline solution. The cerebral cortices were dissected, cleared from leptomeninges, chopped into 0.5 mm³ pieces, digested in 0.25% trypsin solution for 30 min at 37 °C, triturated using flame-polished Pasteur pipettes, and centrifuged at 1200 x g for 10 min. The resulting supernatant was removed, the pellet was resuspended in Dulbecco’s modification of Eagle’s medium containing 1% fetal bovine serum, and cells were plated into 100-mm plastic Petri dishes (Fisher) and allowed to grow for 24 h before the culture medium was changed. All cultured cells were maintained in this medium, which was changed three times a week. In contrast to cultured rat astrocytes, mouse astrocytes prepared with this protocol were virtually free of microglia and oligodendrocytes based on the appearance and immunostaining using the glial fibrillary acidic protein antibody (data not shown). Astrocytes cultured for 3–4 weeks and passaged twice were used for this study.

**Intracellular acidosis and CIH were induced according to protocols described previously (10).** Briefly, intracellular acidosis of astrocytes was induced by 30 min of incubation in a 145 mM KCl solution containing 0.5 mM Lucifer yellow. After 2 min, Lucifer yellow solution was removed from the culture, which was subsequently washed in regular phosphate-buffered saline and fixed in 4% paraformaldehyde. The distance of spread of Lucifer yellow from the incision to neighboring cells was measured and analyzed using the Scion Image software.

**Immunoprecipitation and Western Blotting**—All procedures were performed at 4 °C unless otherwise noted. Cultured astrocytes were harvested into a RIPA buffer containing 1% Nonidet P-40, 10 mM sodium deoxycholate, protease inhibitors (P-8340, Sigma), 10 mM sodium fluoride, and 1 μM sodium orthovanadate (pH 7.4). The cell suspension was subsequently sonicated and centrifuged at 12,000 x g for 20 min. The supernatant was collected, and the protein concentration was determined using a Pierce BCA kit to ensure that similar amounts of protein samples were loaded for Western blotting analysis or for immunoprecipitation (IP). Moreover, the equivalency of protein loading in Western blots using whole cell lysates was further confirmed by Western blots of GAPDH using a monoclonal antibody against GAPDH (RD1-TRKSG4–6C5, Research Diagnostics Inc., shown in Fig. 1, A2). About 10% of sample was used for direct Western blotting analysis to determine the general profile of protein expression and the phosphorylation state of Cx43. The rest was used for protein co-IP studies. For protein co-IP, astrocyte lysates were preincubated with 40 μl of recombinant protein G-agarose (15920-010, Invitrogen) for 30 min on a cooled bench to remove free agarose beads. The supernatant was incubated with corresponding IP antibody overnight before a 1-h incubation with fresh agarose beads. This IP mixture was centrifuged, supernatant was removed, and the immunocomplex on agarose beads was washed six times using RIPA buffer before the addition of Laemmli sample buffer (24). Finally, samples were boiled for 5 min, loaded into precast gels (Bio-Rad) for protein electrophoresis on a mini-protein III system (Bio-Rad), and transferred to nitrocellulose membrane. Ten percent precast gels were used except when Western blotting was conducted for c-Src from Cx43 IP samples, in which 7.5% precast gels were used to enhance the separation between Src and IgG heavy chain bands.

Western blotting was conducted according to a protocol described previously (10). Briefly, membranes were blocked at 5% non-fat dry milk solution containing 0.2% Tween 20 and 5% skim milk for an hour before the overnight incubation with a primary antibody and subsequent horseradish peroxidase-conjugated secondary antibodies. The membranes were washed and incubated for 1 min in an ECL solution (Amersham Biosciences) before x-ray film exposure and development.

**Triton X-100-soluble and -insoluble Fractionation**—Confluent cultures of astrocytes were incubated in 1% Triton X-100 solution containing 0.3 mM sodium iodoacetate and 1 mM sodium cyanide at pH 7.4, 7.1, 6.8, and 6.5. For induction of CIH, astrocytes were incubated for 30 min in Hanks’ balanced salt solution containing 0.3 mM sodium iodoacetate and 1 mM sodium cyanide at pH 7.4 using a Nikon fluorescence microscope equipped with a Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) or an Olympus Fluoview500 confocal system (Melville, NY). Immunofluorescence images were acquired using a Nikon fluorescence microscope equipped with a Spot CCD camera (Diagnostic Instruments Inc., Sterling Heights, MI) or an Olympus Fluoview500 confocal system (Melville, NY). Western blots were quantified by measuring the relative density of protein bands recognized by a particular antibody using Scion Image software (Frederick, MD). A comparison between the values of control (set at 1 unless otherwise specified) and CIH samples was made and analyzed using the Student’s t test. p values ≤0.05 were considered statistically significant between two groups.

**RESULTS**

**Induction of Cx43 Dephosphorylation by Intracellular Acidification**—Because it has been shown that intracellular acidosis can enhance the binding of c-Src to Cx43 (19), we first used Western blotting to examine whether intracellular acidosis caused alterations in the phosphorylation state of Cx43. In non-stressed cells serine/threonine phosphorylation of Cx43 resulted in decreased mobility on SDS-PAGE, and slower migrating bands usually appearing as a doublet that are commonly referred to as P1 and P2 (7–9). The fastest migrating form is usually referred to as P0 and represents non-phosphorylated Cx43. In astrocytes, the majority of Cx43 protein is phosphorylated and migrates at about the 43-kDa level, whereas the non-phosphorylated or dephosphorylated form migrates at the 41-kDa level (10, 20, 25, 27). Under certain experimental interventions, the 41-kDa band increases with a
the relationship between changes in Cx43 phosphorylation condition rarely seen in living cells. Thus, we further examined of the transmembrane potassium concentration gradient, a

\[ A1 \]

Equivalent sample loading in control cells (lane 1). This band corresponds to the 41-kDa form of precipitate from astrocytes treated with low pH (lane 2) but not from control cells (lane 3). This band corresponds to the 41-kDa form of Cx43 in cell lysate from control (lane 3) and low pH-treated astrocytes (lane 4).

proportional loss in P2 and P1, and this has been used as indication of dephosphorylation of Cx43 at serine/threonine residues (7, 10, 20, 22, 26, 28). In this study dephosphorylated Cx43 refers to the 41-kDa isoform of Cx43 that has reduced content of phosphate groups even though the exact phosphate content is not yet well defined. In cultured astrocytes this Cx43 isoform can be selectively recognized by antibody Zy13 (10).

As shown in Fig. 1A, when astrocytes were subjected to 30-min treatments, causing graded reduction of intracellular pH from 7.4 to 6.5, there was a gradual reduction of the 43-kDa phosphorylated form and a concomitant increase of the 41-kDa non-phosphorylated form, suggesting that intracellular acido-
sis caused dephosphorylation of Cx43. Consistent with our previous finding, c-Src antibody did not pull down significant amounts of Cx43 when astrocytes were maintained at neutral pH (Fig. 1B, lane 1). However, an intense Cx43 band was detected when astrocytes were held at pH 6.5 for 30 min (Fig. 1B, lane 2). Surprisingly, this Cx43 band migrated at the 41-

\[ A2 \]

kDa level, corresponding to the dephosphorylated form of Cx43. Because the 41-kDa form of Cx43 is also present in neutral pH samples but is apparently not associated with c-Src, this result suggests that intracellular acidosis induced a selective binding of c-Src to dephosphorylated Cx43.

In this experiment astrocytes were exposed to solutions containing 145 mM KCl and nigericin, which causes a total collapse of the transmembrane potassium concentration gradient, a condition rarely seen in living cells. Thus, we further examined the relationship between changes in Cx43 phosphorylation state and Cx43/c-Src binding in astrocytes using an in vitro ischemia model, CIH, in which cell metabolism is inhibited by the addition of iodoacetate and cyanide. We and others have previously reported that CIH causes massive dephosphorylation of Cx43 in rat astrocytes (10, 22).

Induction of Cx43 Dephosphorylation by CIH—Similar to what has been reported in rat astrocytes, 30 min CIH of mouse astrocytes caused changes in Cx43 mobility on SDS-PAGE (Fig. 2A). Anti-Cx43 antibody 18A revealed an evident shift of the phosphorylated forms to the dephosphorylated form (Fig. 2A). Western blot probed with monoclonal anti-Cx43 antibody Zy13 showing the selective detection of a single band at 41 kDa and the increase of this band in the CIH sample (second lane) compared with the control sample (first lane). B, Western blot probed with monoclonal anti-Cx43 antibody Zy13 showing the selective detection of a single band at 41 kDa and the increase of this band in the CIH sample (second lane) compared with the control (first lane). C, Cx43 immunostaining showing alteration of Cx43 antibody recognition in astrocytes after CIH. Antibody 18A detected abundant Cx43 at the plasma membrane and in the cytoplasm of control (Cont) astrocytes (C1). The immunodetection of antibody 18A was greatly reduced in CIH cells (C2). In contrast, antibody Zy13 produced very little staining in control astrocytes (C3) but displayed extensive staining of Cx43 at both plasma membrane and cytoplasm in CIH cells (C4). D, scrape loading of Lucifer yellow showing reduced a spread of Lucifer yellow to neighboring cells after CIH treat-

\[ B \]

ment (compare D2 with D1). In summary, 30-min of CIH treatment reduced gap junctional coupling by about 60% (D3). Scale bars, 25 μm.

* p < 0.001.
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FIG. 3. Western blots showing Cx43 and c-Src protein levels in control (Cont) and CIH astrocytes and the association between Cx43 and c-Src after CIH treatment. A, Western blot (WB) probed with anti-Cx43 antibody 18A showing a shift of Cx43 from the 43-kDa form to the 41-kDa form after CIH. B, Western blotting showing the virtually unchanged protein level of c-Src after CIH. C, detection of c-Src in proteins immunoprecipitated by antibody Cx43NT1. An intense protein band was detected at 66 kDa in CIH samples with very little signal in the control (Fig. 3C). Similar results were obtained when Cx43 was immunoprecipitated using anti-Cx43 antibodies Cx43CT1 and Zy13. Reciprocally, when c-Src was immunoprecipitated, Cx43 antibody detected a protein band at 41 kDa in CIH samples, but there was virtually no detection in controls (Fig. 3D). These results suggest that CIH induced the selective association between c-Src and the rapidly migrating and presumably dephosphorylated form of Cx43, similar to the change occurring during intracellular acidosis described above.

Tyrosine Phosphorylation of Dephosphorylated Cx43—c-Src is a non-receptor tyrosine kinase that has been shown to phosphorylate Cx43 at tyrosine residues when overexpressed in mammalian cells (16, 17). The selective association of c-Src with the 41-kDa form of Cx43 raises the possibility that Cx43 in astrocytes may be phosphorylated at tyrosine residues after CIH. To test this possibility, we immunoprecipitated proteins containing phosphotyrosine using a well characterized phosphotyrosine antibody and probed the membrane with a Cx43 antibody. Consistent with previous findings in several other cell types, there were only faint bands detected at 43 kDa in control astrocyte samples and none at the 41-kDa level (Fig. 4A, lane 3), suggesting that even though the 43-kDa forms of Cx43 were phosphorylated at serine/threonine residues, they contain very little phosphotyrosine in normal astrocytes and that the 41-kDa form does not appear to be phosphorylated (Fig. 4A, lane 3). In contrast, an intense band was detected at 41 kDa in CIH samples, corresponding to dephosphorylated Cx43 (Fig. 4A, lane 4). Moreover, an apparent 100% increase in the phosphotyrosine content in Cx43 in CIH samples was revealed by comparing the intensities of the 43-kDa band in control samples and the 41-kDa band of the CIH samples (Fig. 4B). These results suggest that the dephosphorylated Cx43 is phosphorylated at tyrosine residues during CIH, perhaps by c-Src. Therefore, the dephosphorylated Cx43 in essence is a Cx43 isoform containing abundant phosphotyrosines and a reduced level of phosphoserine/threonines.

Translocation of Endogenous c-Src during CIH—Although wild type c-Src was shown to bind to Cx43 in vivo when it was overexpressed (16, 17), whether endogenous c-Src binds to Cx43 in normal cells has been controversial. The virtual absence of association between endogenous c-Src and Cx43 in co-IPs from control astrocytes suggests that Cx43 is not readily accessible to endogenous c-Src, perhaps because these two proteins exist in different subcellular compartments. We tested this possibility by taking advantage of the insolubility of gap junctions in Triton X-100, thereby distinguishing this compartment biochemically from Triton X-soluble subcellular compartments.

As shown in Fig. 5, Triton-soluble and -insoluble membrane fractions are indicated by the respective presence and absence of the soluble protein GAPDH (Fig. 5A). Moreover, the equivalency of control and CIH protein samples used for Western blotting and co-IP was also indicated by the virtual identical intensity of the GAPDH band. In control astrocytes, only the rapidly migrating 41-kDa form of Cx43 was present in the Triton-soluble fraction (Fig. 5B, first lane), whereas the Triton-
insoluble fraction contained only the more slowly migrating form of Cx43 (Fig. 5B, second lane). After the CIH treatment, the relative amount of 41-kDa Cx43 was increased somewhat in the Triton-soluble fraction, suggesting CIH and/or the accompanying dephosphorylation increased solubility of Cx43 in astrocytes (Fig. 5B, third lane). Strikingly, the Triton-insoluble fraction of CIH samples contained primarily the 41-kDa form of Cx43 even though a trace amount of the more slowly migrating form was also present (Fig. 5B, fourth lane), suggesting that a large portion of the dephosphorylated Cx43 maintained its Triton insolubility. When membrane fractions of control astrocytes were immunoprecipitated with a c-Src antibody, it appeared that endogenous c-Src was mostly present in the soluble fraction (Fig. 5C, first lane) and virtually absent from the insoluble fraction (Fig. 5C, second lane), which is consistent with observations that have been described in different cell types (30, 31). However, c-Src emerged in the Triton-insoluble fraction after CIH (Fig. 5C, fourth lane), suggesting the translocation of a portion of endogenous c-Src from Triton-soluble subcellular compartments to insoluble compartments. Interestingly, Cx43 was only co-immunoprecipitated with c-Src in the Triton-insoluble fraction after CIH (Fig. 5D, fourth lane), suggesting that Cx43 induced a translocation of c-Src to gap junctions and binding to junctional Cx43 given that the majority of the 41-kDa form of Cx43 was located at the plasma membrane (Fig. 2B).

Correlation of Binding of c-Src and β-Actin to Cx43 with Cx43 Phosphorylation State—The consistent occurrence of Cx43 dephosphorylation during intracellular acidosis and CIH and the selective association between c-Src and the 41-kDa form of Cx43 indicate that removal of phosphate groups from serine and/or threonine residues may facilitate the binding of c-Src to Cx43. To test this possibility, we took advantage of the finding that the phosphorylation-dependent shift can be completely inhibited by the addition of okadaic acid and FK506, inhibitors of types 1, 2A, and 2B protein phosphatases (10). As shown in Fig. 6A, the Cx43 profile was similar to the control (Fig. 6, A1, first lane) when CIH was co-applied with both phosphatase inhibitors (Fig. 6A1, lane 3), suggesting the complete inhibition of Cx43 dephosphorylation by these compounds. In Western blots of proteins immunoprecipitated by the c-Src antibody, Cx43 was virtually absent in the astrocyte sample treated with both CIH and phosphatase inhibitors (Fig. 6, A2, third lane) even though it was clearly present in the sample treated with CIH alone (Fig. 6, A2, second lane). These results suggest that dephosphorylation is most likely required for the binding of c-Src and Cx43. Similar results were obtained using a different pair of similar phosphatase inhibitors calyculin A and cyclosporine A (data not shown). Moreover, the acidosis-induced association between c-Src and Cx43 was also blocked by the addition of these phosphatase inhibitors (Fig. 6B). These results suggest that removal of phosphate groups from serine/threonine residues may be required for Cx43 to be accessible to endogenous c-Src.

Next, we asked whether changes in Cx43 dephosphorylation also affected the binding of β-actin to Cx43, an interaction suggested previously based on the co-localization of actin and Cx43 immunostaining (32). In contrast to c-Src/Cx43 binding, a strong signal was detected by a β-actin antibody in proteins immunoprecipitated by antibody 18A in control astrocytes (Fig. 6, C2, first lane), suggesting that β-actin is associated with Cx43 in normal astrocytes. CIH treatment caused an apparent reduction in Cx43/β-actin binding (Fig. 6, C2, second lane). Moreover, the β-actin/Cx43 association was preserved when

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**FIG. 4.** Western blots (WB) showing the detection of Cx43 in proteins immunoprecipitated with a phosphotyrosine (p-tyr) antibody. Very little signal was detected in control (Cont; lane 3 in A). However, an intense band was detected in the CIH sample (lane 4 in A), and this band corresponds to the dephosphorylated Cx43 shown in normal Western blots of cell lysates from control (lane 1) and CIH astrocytes (lane 2). The intensity of the 43-kDa band in control samples was about 50% that of the 41-kDa band in the CIH samples (B). *, p < 0.05.

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**FIG. 5.** Western blotting analysis of membrane fractionations showing the association between c-Src and Cx43 in the Triton X-100-insoluble membrane fraction from CIH astrocytes. A, Western blot (WB) of GAPDH showing that the Triton X-100-insoluble (Tx-Ins) fraction does not contain appreciable amounts of this protein. Tx-S, Triton X-100-soluble fraction. Cont, control. B, Western blot of Cx43 showing the emergence of the 41-kDa form of Cx43 in the Triton X-100-insoluble membrane fraction after CIH. C, Western blotting of proteins in both the Triton-soluble and -insoluble membrane fractions immunoprecipitated by a c-Src antibody showing the appearance of c-Src in the Triton-insoluble fraction of CIH astrocytes. D, Cx43 Western blot of proteins in both Triton-soluble and -insoluble membrane fractions immunoprecipitated by a c-Src antibody showing the presence of the 41-kDa form of Cx43 in Triton-insoluble fraction of CIH astrocytes.
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MAP kinases have been shown to phosphorylate Cx43 (MAP) Kinase ERK1/2 and MAP Kinase Phosphatase-1 (MKP-1) in a different manner from c-Src/Cx43. Acidosis and CIH induced the shift of Cx43 from the 43-kDa form to the 41-kDa form, both of which were immunoprecipitated by anti-Cx43 antibody 18A. CIH treatment induced the shift of Cx43 from the 43-kDa form to the 41-kDa form, both of which were immunoprecipitated by anti-Cx43 antibody 18A. Cx43 dephosphorylation was prevented by the administration of OA and FK506. Cx43 shifted from the 43-kDa form to the 41-kDa form by CIH. The shift of Cx43 from 43 to 41 kDa was induced by CIH (Fig. 7B, first and second lanes). Furthermore, it appears that the 44-kDa ERK2 band was more prominent than the 42-kDa ERK1 band in the CIH-treated sample. MKP-1 was detected in the Cx43 immunocomplex from CIH-treated astrocytes (last lane) but not in control even though CIH did not cause a marked change in the total protein level of MKP-1.

**FIG. 6. Western blots (WB) showing the regulation of binding of c-Src and β-actin to Cx43 by alterations in the phosphorylation state of Cx43.** The shift of Cx43 from 43 to 41 kDa was induced by CIH (A1) or intracellular acidosis (B1), which was completely inhibited by the administration of okadaic acid (OA) and FK506. The co-IP of Cx43 and c-Src induced by CIH (second lane in A2) or intracellular acidosis (second lane in B2) was abolished by the administration of OA and FK506. C1, Cx43 Western blot of immunoprecipitate using anti-Cx43 antibody 18A showing the shift of 43-kDa forms of Cx43 to 41-kDa form after CIH and its inhibition by OA and FK506. C2, β-actin WB of immunoprecipitate using antibody 18A showing the alterations in the amount of β-actin co-immunoprecipitated with Cx43. An intense band was detected in control and in astrocytes treated with both CIH and phosphatase inhibitors. Only a faint band was detected in astrocytes subjected to CIH or exposed to phosphatase inhibitors.

Cx43 dephosphorylation was prevented by the administration of phosphatase inhibitors (Fig. 6, C2, third lane). We conclude that β-actin/Cx43 binding is also affected by Cx43 phosphorylation state albeit in a different manner from c-Src/Cx43.

**FIG. 7. Western blots (WB) showing the co-immunoprecipitation of ERK1/2 and MKP-1 with Cx43 in astrocyte samples treated with CIH.** A, CIH treatment induced the shift of Cx43 from the 43-kDa form to the 41-kDa form, both of which were immunoprecipitated by anti-Cx43 antibody 18A. **B**, no evident change in total protein level of ERK1/2 was detected in astrocytes after CIH. However, the presence of ERKs was increased in the Cx43 immunocomplex from CIH-treated astrocytes. Note that the 44-kDa ERK2 band was more prominent than the 42-kDa ERK1 band in the CIH-treated sample. C, MKP-1 was detected in the Cx43 immunocomplex from CIH-treated astrocytes (last lane) but not in control even though CIH did not cause a marked change in the total protein level of MKP-1.

**DISCUSSION**

In this study we have demonstrated that intracellular acidosis and CIH-induced changes in Cx43 phosphorylation state, translocation of endogenous c-Src to gap junctions, and binding of c-Src to the rapidly migrating dephosphorylated form of Cx43. Moreover, we found that the association between c-Src and Cx43 is accompanied by increased tyrosine phosphorylation of dephosphorylated Cx43, which may contribute to the down-regulation of gap junctional coupling in astrocytes during acidosis and CIH. CIH also facilitated the association of ERK1/2 and MKP-1 with Cx43 and reduced the association of c-Src to the rapidly migrating dephosphorylated form of Cx43. The role of Cx43 phosphorylation in the regulation of gap junctional coupling has been intensively investigated. Under
physiological conditions, Cx43 usually exists in phosphorylated forms at gap junctions (7–10, 26, 27, 36). Moreover, phosphorylation of Cx43 was previously shown to be critical for gap junction assembly and acquisition of Triton insolubility (8, 36). However, dephosphorylated Cx43 has occasionally been found in apparently disintegrating plasma membranes of astrocytes, and dephosphorylation of Cx43 has been shown to precede the onset of massive internalization of gap junctions in astrocytes after ischemia (20). Thus, it has been a concern that gap junctions composed of dephosphorylated Cx43 might retain structural and biochemical characteristics of gap junctions in normal cells even though the channels formed by this protein are likely to be closed (10, 22). Previously, we found that dephosphorylation of Cx43 can be induced in the brain by mild ischemia and in the spinal cord by stimulation of peripheral nerves (20, 27), neither of which is believed to cause significant neural injury. Moreover, by taking advantage of the selective recognition of dephosphorylated Cx43 by a monoclonal anti-Cx43 antibody, we were able to locate dephosphorylated Cx43 in morphologically intact gap junctions between astrocytes, suggesting that gap junctions formed by dephosphorylated Cx43 are structurally indistinguishable from those formed by phosphorylated Cx43 (10, 20, 27). In the current study we found that the majority of dephosphorylated Cx43 was present in the Triton X-100-insoluble membrane fraction, suggesting that gap junctions formed of dephosphorylated Cx43 retain critical biochemical characteristics of intact gap junctions. As Cx43 phosphorylation occurs primarily at serine/threonine residues in normal cells, these results indicate that serine/threonine phosphorylation of Cx43 is not necessary for its Triton insolubility or the stability of gap junctions.

The shift of Cx43 mobility on SDS-PAGE appears to result from changes of phosphorylation state at serine/threonine sites but not tyrosine residues. For instance, when all phosphate groups are removed from Cx43 by alkaline phosphatase treatment, Cx43 migrates at 41 kDa and co-migrates with non-phosphorylated naïve Cx43 protein (7–10). Moreover, mobility of wild-type Cx43 on Western blots is similar to that of Cx43 mutants containing tyrosine-phenylalanine mutations after v-src transfection, which has been shown to phosphorylate tyrosines in Cx43 (37). Thus, the shift of Cx43 mobility on SDS-PAGE indicates alterations in the phosphorylation of serine/threonine residues but not of tyrosine sites. This offers a valid interpretation for the observation that the presumptive dephosphorylated 41-kDa form of Cx43 was co-immunoprecipitated by the anti-phosphotyrosine antibody. These results indicate that dephosphorylated Cx43 at 41 kDa may not be identical to newly synthesized non-phosphorylated Cx43, as it is apparently the preferential target of endogenous c-Src and may contain phosphorylated tyrosine residues.

Binding of Endogenous c-Src to Cx43—When wild-type c-Src or a constitutively active c-Src mutant was overexpressed in fibroblasts, it was found to bind to Cx43, to phosphorylate Cx43 at tyrosine residues, and to close gap junctions, suggesting that endogenous Cx43 was capable of interacting with wild-type c-Src (16, 17). This is consistent with the observation of interaction between endogenous c-Src with Cx43 in cardiac myocytes during cardiomyopathy and in astrocytes during acidosis (19, 38). However, the interaction between endogenous c-Src and endogenous Cx43 has not been observed in several studies using non-stressed cells (17, 18). Consistent with these previous studies, our immunoprecipitation and membrane fractionation data suggest that endogenous c-Src does not appreciably interact with Cx43 under normal physiological conditions probably due to localization within distinct membrane compartments, which is consistent with findings suggesting that endogenous c-Src is predominantly located within the Triton-soluble membrane fraction (30, 31). Apparently, endogenous c-Src does not play an active role in the regulation of intercellular coupling through gap junctions formed by Cx43 under physiological conditions. However, c-Src can be translocated from the Triton-soluble membrane fraction to the -insoluble fraction containing gap junctions during CIH and perhaps also acidosis and bind to Cx43. Although direct evidence is not yet available, c-Src/Cx43 binding during CIH and acidosis likely contributes to the increased tyrosine phosphorylation of Cx43 and the closure of gap junction channels that has been shown to result from tyrosine phosphorylation of Cx43.

Binding of ERK1/2 and MKP-1 to Cx43—MAP kinases can interact with Cx43, increasing its phosphorylation state and reducing gap junctional coupling when cells are stimulated with growth factors (11). However, it has not been clear whether MAP kinases are involved in the regulation of gap junctional coupling in unstimulated cells given that MAP kinases are not responsible for the basal phosphorylation state of Cx43 (13) that seems to be required for gap junction assembly (8, 36). Our results suggest that MAP kinases ERK1/2 do not interact appreciably with Cx43 in control astrocytes but do bind strongly to Cx43 during CIH. Although one might expect that kinase binding would be associated with increased phosphorylation, this finding of serine/threonine dephosphorylation of Cx43 indicates that ERKs may be inactivated by MKP-1 that was also immunoprecipitated in CIH samples by a Cx43 antibody. It is not yet clear whether MKP-1 is directly associated with Cx43 or indirectly through binding to ERKs during CIH, as described above. To date, MAP kinases are the only known MKP-1 substrates (33, 39). The association between MKP-1 and Cx43 during CIH nevertheless raises the interesting possibilities that Cx43 may be a novel substrate of MKP-1 and that MKP-1 may be a novel protein phosphatase interacting with Cx43.

Cx43-Protein Interactions and Phosphorylation State of Cx43—Our findings indicate that serine/threonine-dephosphorylated Cx43 is the preferential target of c-Src during CIH and acidosis. However, this does not exclude the possibility that phosphorylated Cx43 can be a binding partner for c-Src especially when this kinase is ectopically expressed at high levels. Indeed, in studies in which exogenous wild-type c-Src was expressed, both co-localization and association between wild-type c-Src and Cx43 were observed in the absence of gross changes in the phosphorylation state of Cx43 (16, 17). These findings suggest that phosphorylated Cx43 is accessible to a high concentration of wild-type c-Src and that alteration of the phosphorylation state of Cx43 is not the only mechanism for the regulation of Cx43/c-Src binding. In a recent study we found that the direct binding of c-Src to Cx43 was also regulated by pH in vitro (19). Taken together, these observations indicate that the interaction between c-Src and Cx43 is controlled by multiple mechanisms, each modulating c-Src/Cx43 binding affinity. Based on detailed biochemical studies, it has been proposed that c-Src/Cx43 interaction involves sequential binding of the SH3 domain to the P274-P284 proline-rich region of Cx43, phosphorylation of Tyr-265, and binding of SH2 to this phosphorylation site (37). We presume that a similar binding sequence occurs for c-Src. However, the molecular mechanisms of exogenous and endogenous Src/Cx43 binding may differ for reasons discussed above. Nevertheless, the elimination of c-Src/Cx43 binding by inhibition of Cx43 dephosphorylation suggests that dephosphorylation of Cx43 is critical for the interaction between endogenous c-Src and Cx43 and that serine/threonine phosphorylation and tyrosine phosphorylation may be reciprocally regulated. Moreover, our observation that
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β-actin/Cx43 binding was also affected by the phosphorylation state of Cx43 indicates that alterations in Cx43 phosphorylation state may be an important regulatory factor in Cx43-protein interactions.

Functional Significance of Cx43 Dephosphorylation and Cx43 Interaction with Binding Proteins—Gap junctional intercellular communication in astrocytes is believed to be important for the homeostasis and glucose metabolism of the central nervous system under physiological conditions and is critical for neuronal survival during ischemia (4). Dephosphorylation of Cx43 appears to be a common mechanism for the regulation of gap junctional communication during increased neuronal activity and ischemia. We and others have previously demonstrated that Cx43 undergoes dephosphorylation at gap junctions in brain and heart during ischemia and in spinal cord during intense peripheral nerve activation (10, 20, 27). Moreover, we have shown that inhibition of Cx43 dephosphorylation contributes to the preservation of gap junctional coupling in astrocytes during CIH (10). The finding of Cx43 association to c-Src and Cx43 phosphorylation at tyrosine residues provides further insight into the regulation of gap junctional coupling, as tyrosine phosphorylation of Cx43 could contribute to the down-regulation of gap junctional coupling in astrocytes during ischemia and CIH. Because it has been shown that the removal of c-Src exerts a neuroprotective effect (40), it would be interesting to examine whether c-Src/Cx43 interaction and resulting tyrosine phosphorylation of Cx43 are critical events in neuronal cell death.

Recently we and others have demonstrated that Cx43 expression is involved in the regulation of cell proliferation and migration in a variety of cell types (1, 3, 41). Interestingly, the role of Cx43 in the regulation of these cellular activities appears not to be dependent on the gap junction channels that it forms but, rather, is associated with the interaction of Cx43 with other proteins (3, 40). The association of Cx43 with β-actin is particularly interesting due to the critical role of actin in cell migration. Thus, it is conceivable that the interaction between Cx43 and β-actin would affect the migratory behavior of astrocytes that are known to reorganize stress fibers and gap junctions and become reactive and motile. Indeed, we have found that wild-type astrocytes migrate more rapidly than Cx43 knock-out astrocytes. Studies are under way to more thoroughly characterize the interaction between Cx43 and β-actin and the significance of this interaction in the regulation of astrocyte locomotion.

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² W. Li and D. C. Spray, unpublished observation.
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