Regulation of Connexin-43 Gap Junctional Intercellular Communication by Mitogen-activated Protein Kinase*

(Received for publication, December 1, 1997, and in revised form, February 3, 1998)

Bonnie J. Warn-Cramer‡, G. Trevor Cottrell§, Janis M. Burt¶, and Alan F. Lau‡

From the ‡Molecular Carcinogenesis Section, Cancer Research Center of Hawaii, the §Department of Genetics and Molecular Biology, School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii 96813, and the ¶Department of Physiology, University of Arizona, Tucson, Arizona 85724

Activation of the Ras/Raf/mitogen-activated protein kinase kinase/mitogen-activated protein (MAP) kinase signaling cascade is initiated by activation of growth factor receptors and regulates many cellular events, including cell cycle control. Our previous studies suggested that the connexin-43 gap junction protein may be a target of activated MAP kinase and that MAP kinase may regulate connexin-43 function. We identified the sites of MAP kinase phosphorylation in in vitro studies as the consensus MAP kinase recognition sites in the cytoplasmic carboxyl tail of connexin-43, Ser255, Ser279, and Ser282. In this study, we demonstrate that activation of MAP kinase by ligand-induced activation of the epidermal growth factor (EGF) or lysophosphatidic acid receptors or by pervanadate-induced inhibition of tyrosine phosphatases results in increased phosphorylation on connexin-43. EGF and lysophosphatidic acid-induced phosphorylation on connexin-43 and the down-regulation of gap junctional communication in EGF-treated cells were blocked by a specific mitogen-activated protein kinase kinase inhibitor (PD98059) that prevented activation of MAP kinase. These studies confirm that connexin-43 is a MAP kinase substrate in vivo and that phosphorylation on Ser255, Ser279, and/or Ser282 initiates the down-regulation of gap junctional communication. Studies with connexin-43 mutants suggest that MAP kinase phosphorylation at one or more of the tandem Ser279/Ser282 sites is sufficient to disrupt gap junctional intercellular communication.

Connexin-43 (Cx43) is the 43-kDa member of a conserved family of membrane spanning gap junction proteins. The connexin proteins have intracellular amino and carboxyl termini and four membrane spanning regions that form two extracellular loops and one intracellular loop (2–4). There is considerable homology among the connexins with the greatest divergences in the intracellular loops and the carboxyl-terminal tails. Connexins are assembled into hexamers or connexons in the trans Golgi network prior to insertion into the plasma membrane (4). Connexons in one cell dock with connexons in adjacent cells through non-covalent interactions that involve the extracellular loops (5–7). An aqueous pore or channel (gap junction) forms and allows for the passive intercellular exchange of small molecular mass molecules, ions, and second messengers (<1000 Da).

The different connexin proteins form channels with unique functional properties including differences in the size and charge of the molecules and ions that can traverse the channel (2–4, 8). Channel gating can be modified by physiologic parameters (such as cellular pH and Ca2+ concentration (9–13)) and by pharmacological agents (such as 12-O-tetradecanoylphorbol-13-acetate and retinoic acid (14–17)). Cell-to-cell communication mediated through gap junctions is known to be essential to the synchronization of events such as contractions in the myocardium and uterus (18, 19) and is thought to play a critical role in regulating cell growth and differentiation (20–22). Down-regulation of gap junctional communication (GJC) has been noted in many tumor cells (20, 23, 24). Conversely, the up-regulation of GJC in cells deficient in communication has been associated with a decrease in cellular growth rates (25–28). Recently, a study by Martyn et al. (29) has reported that communication-deficient fibroblast cell lines isolated from Cx43 knock-out (K/O) mouse embryos display a subset of the properties of transformed cells, including increased growth rates. These data further advance the hypothesis that the loss of GJC is associated with the progression toward neoplastic transformation.

Some posttranslational phosphorylation on serine is required for the proper assembly of Cx43 gap junctions (4). However, the sites of phosphorylation and the kinases involved have not as yet been identified. Additional posttranslational phosphorylation on serine and/or on tyrosine has been associated with a decrease in GJC. Down-regulation of GJC in epidermal growth factor (EGF)-treated cells and cells transformed with v-ras has been associated with increased serine phosphorylation on Cx43 (30–33), whereas tyrosine phosphorylation on Cx43 has been associated with decreased GJC in cells transformed with the v-src or v-fps oncogene (34–37).

EGF induces disruption of GJC and increased serine phosphorylation on Cx43 in a rapid and transient manner that is not mediated by the activation of 12-O-tetradecanoylphorbol-13-acetate-sensitive isoforms of protein kinase C (30, 31). Mitogen-activated protein (MAP) kinase, another candidate serine/threonine kinase activated by the EGF receptor (38–40), has been demonstrated to phosphorylate a recombinant Cx43 preparation in vitro on phosphotryptic peptides that co-mi-
grated with phosphotryptic peptides obtained from EGF-treated cells, implicating a potential role for MAP kinase in Cx43 phosphorylation and the regulation of GJC (31).

We characterized MAP kinase-mediated phosphorylation on Cx43 utilizing a glutathione S-transferase (GST) fusion protein that contained the carboxyl-terminal cytoplasmic tail (CT) of Cx43 (aa Val236-Ile382). Site-directed mutagenesis, phosphotryptic peptide analysis, and peptide sequencing were used to identify the specific sites of serine phosphorylation on Cx43 (41). MAP kinase phosphorylated Cx43 in vitro on two tryptic peptides that contained the three MAP kinase consensus recognition sequences (tryptic peptide Ser244-Lys258, phosphorylated at Ser255, and tryptic peptide Tyr265-Lys287, phosphorylated at Ser279 and at Ser282). When we deleted the three consensus MAP kinase phosphorylation sites by altering the serine residues to alanine, MAP kinase still phosphorylated Cx43 in vitro on the Tyr265-Lys287 peptide at Ser272 and at Ser273. Ser273 fits the minimal recognition sequence for MAP kinase (serine residue followed by a carboxyl-terminal proline (42)). Phosphorylation at these alternate serine sites occurred only in the absence of the consensus MAP kinase phosphorylation sites. Taken together, our studies have supported the hypothesis that EGF-induced activation of the Ras/Raf/MEK (MAP kinase)/MAP kinase signal transduction pathway leads to MAP kinase-mediated phosphorylation on Cx43 at Ser255, Ser279, and Ser282 (Fig. 7). The data suggest that phosphorylation occurring at one or more of these MAP kinase phosphorylation sites may be sufficient to disrupt GJC.

Lysophosphatic acid (LPA) has also been reported to downregulate GJC and to increase serine phosphorylation on Cx43 (43). LPA binds to an 39-kDa membrane receptor and transmits a signal through interaction with a heterotrimeric G protein (Fig. 7), leading to the activation of Ras (44) and to signal transduction through the Ras/Raf/MEK/MAP kinase pathway (38–40). Partially purified MAP kinase from LPA-stimulated WB rat cells was shown to phosphorylate a Cx43 peptide, aa 247–260, that contained the Ser255 site (43). Zhao et al. (45) have reported that treating cells with pervanadate (a potent inhibitor of tyrosine phosphatases) was sufficient to trigger the activation of Raf, MEK, and MAP kinase (Fig. 7). Pervanadate dramatically increased the overall level of tyrosine phosphorylation on cellular proteins in the treated HeLa cells. Pervanadate has also been reported to down-regulate GJC and to increase phosphorylation on Cx43 in studies with hamster lung fibroblasts (46).

In the studies reported here, we have directly examined the role of MAP kinase in mediating the EGF-induced increased phosphorylation of Cx43 in vivo. We expressed wild type (wt) and mutant forms of Cx43 in communication-deficient cells and examined the effects of EGF, LPA, and pervanadate on Cx43 phosphorylation in the presence or absence of a specific MEK inhibitor. Compound PD98059 has been demonstrated to block MEK activation in many cells (47, 48) (Fig. 7) and thus prevent the MEK-mediated phosphorylation of MAP kinase at the Thr183 and Tyr265 sites that are essential for MAP kinase activity (49). Our studies have demonstrated that Cx43 is a target of the MAP kinase signaling pathway in vivo and that the activation of MAP kinase decreases Cx43-mediated junctional conductance and permeability. These studies demonstrate that phosphorylation on one or more of the MAP kinase consensus serine phosphorylation sites is responsible for the disruption of GJC.

MATERIALS AND METHODS

Cell Culture, Metabolic Labeling, and Cx43 Immunoprecipitation—The Cx43 knock-out (K/O) mouse cell line, n2/3 (29), human HeLa cells, and K/O or HeLa cells expressing exogenous Cx43 were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) with 10% fetal calf serum (HyClone) in a humidified 5% CO2 incubator at 37 °C as described (29). Confluent monolayers were metabolically labeled for 2–3 h with [35S]methionine (Amersham Pharmacia Biotech) at 100 μCi/ml in methionine-free basal medium Eagle’s medium supplemented with 4% calf serum with MEK inhibitor (5 μM STS, England Biolabs) or dimethyl sulfoxide (Me2SO) control was used to pretreat cells for 1 h prior to stimulation with EGF, LPA, or pervanadate as described below. Cells were treated with EGF, LPA, or pervanadate during the last 15–30 min of the labeling period. At the end of the labeling period, the monolayers were rinsed twice with cold phosphate-buffered saline (PBS) containing 10 mM NaF, 160 μM Na2VO4, and 1 μM phenylmethylsulfonyl fluoride (PMSF) and then frozen at –20 °C. Cells were lysed on ice with RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris (pH 7.2) containing phosphatase and protease inhibitors (10 mM NaF, 160 μM Na2VO4, and 1 mM PMSF). Cx43 was immunoprecipitated from clarified cell lysates with rabbit antisera directed against the carboxyl-terminal of Cx43 (aa 368–382) and activated protein A-Staphylococcus aureus (41). Controls for nonspecific immunoprecipitation were performed with high weight each cell lysate using non-immune rabbit serum. Immunoprecipitates were resolved on SDS-polyacrylamide gradient gels (7.5–15% acrylamide) and radiolabeled Cx43 was visualized by radioautography of the dried fluorographed gels. For phosphoamino acid analysis, cell monolayers were labeled with 35S-methionine (NECROBIO, NEX-053) at 1–2 mCi/ml in phosphate-free basal medium Eagle’s medium with 4% calf serum. Cx43 was immunoprecipitated from clarified cell lysates, resolved on gradient gels, and transferred to Immobilon-P membranes (Millipore). Phosphorylated Cx43 was visualized by radioautography, excised from the membrane, acid-hydrolyzed, lyophilized, and mixed with non-radioactive internal phosphoamino acid standards. Phosphoamino acids were resolved by two-dimensional electrophoresis at pH 1.9 and pH 3.5 on thin layer cellulose plates as described (50). Migration of the phosphoamino acid standards was visualized with ninhydrin prior to radioautography at –70 °C.

Expression of Exogenous Cx43—The EcoRI insert containing the G2 rat Cx43 gene with 5’ and 3’ non-coding sequences (51) was excised from Bluescript with BamHI and SalI and inserted into the pBABE retrovi vector (52). The pBABE-Cx43 DNA was transfected into the PE501 packaging cell line using LipofectAMINE (Life Technologies, Inc.) according to the manufacturer’s protocol. Virus from the PE501 cells was amplified by infecting a second packaging cell line, PA317. The PE501 and PA317 packaging cell lines were generally provided by Dr. Dusty Miller (53). Virus harvested from the PA317 cells was used to transfect K/O cells or HeLa cells. Infected cells were selected using puromycin (6 μg/ml for the K/O cells or 0.5 μg/ml for the HeLa cells) and subcloned by limiting dilution.

Mutant forms of Cx43 were prepared from double-stranded DNA using the Chameleon site-directed mutagenesis kit (Stratagene) with primers designed to alter a serine residue(s) to alanine or to aspartate (41). Selection for mutants was provided by the simultaneous use of a selection primer site in the to eliminate a restriction site in the plasmid DNA (54). The fidelity of the mutations was confirmed by sequencing the full-length Cx43 DNA.

The K/O cell clone expressing wt Cx43 used in this study (clone G3) has been characterized previously ((29), termed clone 3R2). A second wt clone was also used for the GJC and junctional conductance studies (clone C9). The K/O cell clone G11, expressing the S255A, S279A, S282A mutant was used for most of these studies. Unless otherwise noted all data reported are for this clone. However, some results were also obtained with a second S255A, S279A, S282A Cx43 mutant clone, H5. The HeLa cell clones expressing wt Cx43 or the S255D Cx43 mutant used in this study were clones A7–A12 and C2-A5, respectively.

Immunofluorescence Microscopy—Clones stably expressing wt Cx43 or mutant Cx43 were initially screened for membrane-localized protein in punctate gap junctions by immunofluorescence microscopy. Cells were grown overnight on glass coverslips, rinsed with PBS, and fixed in ice-cold 100% methanol for 30 min at –20 °C. Cell membranes were permeabilized with 1% Triton X-100 in PBS for 10 min, rinsed twice with PBS, and blocked with 1% bovine serum albumin (BSA) in PBS. The coverslips were then incubated with rabbit anti-Cx43 at 1:1000 dilutions in 1% BSA/PBS for 1 h at room temperature, rinsed twice with PBS and twice with 1% BSA/PBS, and then incubated with goat anti-rabbit fluorescein isothiocyanate conjugate (Sigma), 1:70 for 1 h in the dark. Coverslips were rinsed three times with PBS, mounted on glass slides with 50% glycerol, 50% PBS containing 0.1% phenylendiamine (Sigma), and visualized using a Zeiss Axiosplan Universal microscope equipped with epifluorescence.
Measurement of GJC by Dye Transfer—The membrane-impermeant fluorescent dye, Lucifer Yellow (Sigma), was used to characterize cell clones for GJC (transfer of dye from a microinjected cell to neighboring cells) and to monitor GJC in treated cells. Single cells were microinjected with 10% (w/v) Lucifer Yellow dye (457 Da) in 330 mM LiCl with a glass micropipette (Flaming-Brown P800/PC micropipette puller, Sutter Instrument Co.) using an Eppendorf micromanipulator and Eppendorf pneumatic injector. Dye transfer to neighboring cells was determined after ~3 min using a Zeiss Axiosvert 10 phase contrast inverted microscope equipped with epifluorescence. Approximately 85% of the K/O cells expressing wt Cx43 or the S255A,S279A,S282A Cx43 mutant communicated to neighboring cells, and all injections were used to determine the mean value for GJC. Approximately 60% of the HeLa cells expressing wt Cx43 or the S255D Cx43 mutant communicated. Only those injections that resulted in dye transfer to at least one other cell were used to determine the mean value for GJC. Dye transfer was determined in the treated cells with EGF ~25–30 min after the addition of EGF in injections over ~15–30 min.

Measurement of Functional Conductance—C9 cells expressing wt Cx43 or G3 prior to stimulation with the S255A,S279A,S282A Cx43 mutant were trypsinized and replated in culture medium on glass coverslips. After 0.5 h the cells were removed from the incubator and maintained at room temperature in a 5% CO2 in air environment until used for electrophysiological study. Coverslips were mounted in a perfusion chamber, pairs of cells visualized, and patch type microelectrodes lowered onto the surface of each cell. After establishment of the whole cell recording mode, macroscopic junctional conductance was continuously monitored, as described previously (29), during exposure of the cells to 0 or 100 ng/ml EGF.

Cell Treatment Protocols—Confluent 60-mm monolayers were treated with 100 ng/ml recombinant human EGF (U. S. Biological Corp. and Amersham Pharmacia Biotech) in conditioned medium (or phosphate-deficient or methionine-free media for metabolically labeled cells) for 15 min at 37 °C. Cells were treated with 50 μM LPA (Sigma, 5 μM stock in PBS/1% fatty acid-free BSA) in 1 ml of medium for 15 min at 37 °C. Pervanadate was prepared freshly for each experiment as a 1:1 mix of 100 mM H2O2 and 100 mM Na3VO4, incubated at room temperature for 10–20 min prior to addition to the monolayers in 1 ml of medium (final concentration 100 μM pervanadate). Cells were incubated with pervanadate for 20 min at 37 °C. To block MEK activation, cells were treated with 50 μM PD98059 (50 μM stock in Me2SO) for 1 h at 37 °C prior to stimulation with EGF, LPA, or pervanadate. Controls for these experiments were treated with 0.1% Me2SO. Following treatment, cells were used for GJC measurements or the monolayers were rinsed twice with cold PBS containing 10 mM NaF, 160 μM Na3VO4, 1 mM PMSF and stored frozen. Metabolically labeled cells were treated with the MEK inhibitor or Me2SO, and EGF, LPA, or pervanadate during the last part of the labeling period.

In vitro MAP Kinase Assays—Confluent monolayers were pretreated with MEK inhibitor or Me2SO (control) for 1 h at 37 °C and then stimulated with EGF. At the end of the treatment time, the monolayers were rinsed twice with cold PBS with phosphatase and protease inhibitors (10 mM NaF, 160 μM Na3VO4, and 1 mM PMSF) and frozen at −70 °C. In vitro MAP kinase assays were carried out with clarified cell lysates (in RIPA buffer with 10 mM NaF, 100 μM Na3VO4, and 1 mM PMSF) for 30 min at 37 °C. Reactions contained 7.5 μl of the cell lysate (2.5 μl of lysate for a 60-mm plate) and 15 μl kinase reaction buffer. Final concentrations in the reaction were 20 mM HEPES (pH 7.4), 10 mM MgCl2, 1 mM dithiothreitol, 20 μl unlabeled ATP, 0.5 mg/ml myelin basic protein (MBP, Sigma) as a MAP kinase substrate, and 1 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech). Reactions were incubated at 30 °C for 20 min and stopped by addition of 7.5 μl of 4X SDS sample buffer and placing the samples in boiling water. Proteins were resolved on 12% SDS-polyacrylamide gels and stained with Coomassie Blue. The gels were dried and radiographed at −70 °C.

Immunoblotting—Confluent monolayers in 35-mm dishes were treated with 0.1% Me2SO or MEK inhibitor (50 μM) for 1 h and then treated with or without EGF (100 ng/ml) for 2 min. At the end of the treatment period, the monolayers were rinsed with cold PBS and frozen at −70°C. Cells were lysed in 100 μl of hot SDS sample buffer, heated in boiling water, and centrifuged for 30 min in an Eppendorf microcentrifuge. The supernatant proteins (30 μl) were resolved on 12% acrylamide SDS-polyacrylamide gels and transferred to Immobilon (Millipore). The membrane was blocked with 5% BSA in PBS, 0.1% Tween and incubated with an antibody specific for the active, phosphorylated form of MAP kinase (Promega) or with an antibody to p42 MAP kinase (Santa Cruz). Immunoblots were developed with a peroxidase-conjugated secondary antibody using the ECL chemiluminescence system (Amersham Pharmacia Biotech).

RESULTS

Previous studies implicated MAP kinase in mediating the EGF-induced phosphorylation of Cx43 and the accompanying disruption of GJC (30, 31, 41). The goal of the present study was to determine whether MAP kinase is directly responsible for these EGF-induced effects on Cx43 in vivo. Our experimental approach to this problem was to analyze cells expressing wt or phosphorylation site mutants of Cx43 treated with different agonists to activate MAP kinase and/or with a specific MEK inhibitor to block the activation of MAP kinase.

Phosphorylation Site Mutants of Cx43 Are Localized in Punctate Gap Junction Plaques—We prepared a Cx43 mutant that lacked the three consensus MAP kinase phosphorylation sites (serine to alanine mutations, S255A, S279A, S282A, termed here as S255A,S279A,S282A). This triple phosphorylation site mutant and wt Cx43 were expressed in the non-communicating Cx43 K/O cells. We also expressed wt Cx43 and an aspartate mutant of Cx43 (S255D) in HeLa cells. Membrane-localized Cx43 was apparent in punctate gap junction plaques as demonstrated by immunofluorescence microscopy in cells expressing wt Cx43 (Fig. 1, panels B and C, in the G3 and C9 K/O cell clones and panel G, in the HeLa cells), the S255A,S279A,S282A Cx43 mutant (panels D and E, in the K/O cells, clones G11 and H5, respectively; panel F, parental HeLa cells; panel G, wt Cx43 expressed in the HeLa cells; panel H, the S255D Cx43 mutant expressed in the HeLa cells. Some cytoplasmic staining for Cx43 was also observed (indicated by arrows).

Effective phosphorylation of Cx43 requires that the site be phosphorylated by MEK. The S255A,S279A,S282A Cx43 mutant did not show any staining (Fig. 1, panel C, and panel G). S255A was not phosphorylated in the S255A,S279A,S282A Cx43 mutant, but the S279A and S282A sites were phosphorylated. The S255D Cx43 mutant showed cytoplasmic staining, which could be due to the high background staining of the S255D mutant, as indicated by arrows. The parental K/O cells (panel A) and HeLa cells (panel F) do not express the Cx43 protein.

EGF-induced Increased Phosphorylation of Cx43 Is Dependent on the Activation of MAP Kinase—We determined the effects of EGF on the phosphorylation of wt Cx43 in the K/O cells expressing wt Cx43 or the S255A,S279A,S282A Cx43 mutant. Cells were metabolically labeled with [35S]methionine to radio-label all isoforms of the Cx43 protein. We (34) and others (4, 16) have previously shown that Cx43 exhibits a multiple banding pattern on SDS-PAGE where the non-phosphorylated Cx43 (NP) migrates most rapidly and the phosphorylated isoforms (P1 and P2) migrate more slowly. Compound PD98059 has been reported to specifically block the activation of MEK (MAP kinase kinase, Fig. 7) and thus prevent the activation of MAP
kinase in many cells (47, 48). The MEK inhibitor in Me₆SO (or Me₂SO alone) was used to pretreat some monolayers prior to the addition of EGF to examine the role of MAP kinase in the stimulated cells.

Cx43 obtained from unstimulated cells expressing wt Cx43 (Fig. 2A, lane 2) migrated by SDS-PAGE primarily in the non-phosphorylated form (NP) with some Cx43 migrating more slowly as a phosphorylated species (P1). When the cells were treated with EGF, most of the Cx43 migrated as phosphorylated isoforms consisting primarily of the P1 form as well as the slowest migrating P2 form (Fig. 2A, lane 4). In cells pretreated with the PD98059 MEK inhibitor the increase in the P1- and P2-phosphorylated isoforms was not observed after EGF stimulation, and Cx43 was primarily present in the non-phosphorylated form (NP) with some Cx43 migrating more slowly (Fig. 2A, lanes 1, 3 and 5). Thus, the PD98059 MEK inhibitor appeared to block the EGF-induced increased phosphorylation of Cx43.

EGF treatment of the S255A,S279A,S282A Cx43 mutant also resulted in increased phosphorylation of Cx43 (see Fig. 2B, compare lane 4 with lane 2). The increase in the P1 and P2 isoforms of Cx43 was prevented in the cells pretreated with the MEK inhibitor (Fig. 2B, lane 6), indicating that increased phosphorylation of the S255A,S279A,S282A Cx43 mutant was also dependent upon the activation of MAP kinase. The EGF-induced increased phosphorylation of Cx43 in the S255A,S279A,S282A mutant was also demonstrated with Cx43 immunoprecipitated from [³²P]labeled cells, on Western blots of whole cell lysates from unlabeled cells, and using a second clone (H5) expressing the S255A,S279A,S282A mutant in the K/O cells (data not shown).

The MEK Inhibitor Blocks Activation of MAP Kinase in EGF-treated Cells—MAP kinase is activated through a signal transduction pathway initiated by ligand-induced activation of the EGF receptor (38–40). We examined the effect of the PD98059 MEK inhibitor on the activation of MAP kinase in EGF-treated cells in an in vitro kinase assay (panels A and B) and on immunoblots of whole cell lysates utilizing an antibody specific for the activated form of MAP kinase (panel C). Panel A, time course of MAP kinase activation in cells treated with 100 ng/ml EGF: top row, K/O cells expressing the S255A,S279A,S282A (S255,279,282A) Cx43 mutant (clone H5); bottom row, HeLa cells expressing wt Cx43. Panel B, effect of pretreating the cells with the MEK inhibitor for 1 h prior to EGF stimulation: top row, K/O cells expressing the S255A,S279A,S282A (S255,279,282A) Cx43 mutant (clone G11); bottom row, HeLa cells expressing wt Cx43. Panel C, whole cell lysates from treated K/O cells expressing wt Cx43 (clone G3) were subjected to SDS-PAGE, transferred to Immobilon, and probed with an antibody specific for the activated form of MAP kinase (top row). The blot was reprobed with an antibody to p42 MAP kinase to visualize total MAP kinase in the cell lysates (bottom row). Min refers to the time of EGF treatment in minutes. MekInh refers to the PD98059 MEK inhibitor.

To provide further evidence that the MEK inhibitor interfered with MAP kinase activation, whole cell lysates from K/O cells expressing wt Cx43 (clone G3) untreated or treated with EGF in the presence or absence of the MEK inhibitor were subjected to SDS-PAGE and transferred to Immobilon. Immuno blot analysis with an antibody that specifically recognizes the activated, phosphorylated form of MAP kinase clearly demonstrated MAP kinase activation at 2 min after EGF stimulation and that the MEK inhibitor interfered with MAP kinase activation (Fig. 3C, upper panel). The blot was reprobed with an antibody to the p42 MAP kinase (Fig. 3C, lower panel) to visualize total MAP kinase in the cell lysates.

LPA- and Pervanadate-induced Increased Phosphorylation of Cx43—LPA and pervanadate have also been reported to induce increased phosphorylation on Cx43 and down-regulate GJC (43, 46). In addition, MAP kinase was activated by LPA treatment in WB rat liver cells (43) and by pervanadate in HeLa cells (45). We were interested in determining the effects of LPA and pervanadate on Cx43 phosphorylation in our cells (Fig. 4). Both LPA and pervanadate stimulated increased phosphorylation of Cx43 in cells expressing wt Cx43 as shown by increases in the more phosphorylated isoforms (P1 and P2) of Cx43 (see Fig. 4A, lane 4 for LPA-treated K/O cells and Fig. 4C, lane 4 for...
pervanadate-treated HeLa cells). LPA and pervanadate also induced increased phosphorylation of Cx43 in the K/O cells expressing the S255A,S279A,S282A Cx43 mutant (see Fig. 4B, lane 4 for LPA and Fig. 4D, lane 4 for pervanadate).

The increase in Cx43 phosphorylation was blocked in the LPA-treated cells by pretreatment with the MEK inhibitor (see Fig. 4A, lane 6 for wt Cx43 in the K/O cells and Fig. 4B, lane 6 for the S255A,S279A,S282A Cx43 mutant, in the K/O cells). Pervanadate appeared to induce a greater increase in the phosphorylation of Cx43 than either EGF or LPA treatments, since most of the Cx43 migrated in the highly phosphorylated P2 isoform. Pretreating cells with the MEK inhibitor appeared to have little effect on the pervanadate-induced increased phosphorylation of Cx43 (see Fig. 4C, lane 6 for wt Cx43 in the HeLa cells and Fig. 4D, lane 6 for the S255A,S279A,S282A Cx43 mutant in the K/O cells).

EGF-induced Disruption of GJC Is Dependent on the Activation of MAP Kinase—We determined the effects of EGF treatment on GJC in the K/O cells expressing wt Cx43 and in K/O cells expressing the S255A,S279A,S282A Cx43 mutant (Table I). The G3 clone expressing wt Cx43 in the K/O cells (see Fig. 1B) communicates to 4–5 neighboring cells as described previously (Ref. 29; termed clone 3R2). The C9 clone expressing wt Cx43 in the K/O cells (see Fig. 1B) communicates to 2 neighbors (Fig. 1C). The G11 clone expressing the S255A,S279A,S282A Cx43 mutant in the K/O cells (Fig. 1D) communicates to 4 neighbors and the H5 clone (Fig. 1E) to 2 cells. EGF treatment induced an ∼38–40% decrease in GJC in the K/O cells expressing wt Cx43 for the G3 and C9 clones, respectively, and an ∼22% decrease in GJC in the HeLa cells expressing wt Cx43 (see Fig. 5A and Table I). However, EGF appeared to have no effect on GJC in the G11 S255A,S279A,S282A Cx43 mutant that lacks the consensus MAP kinase phosphorylation sites. Pretreatment with the MEK inhibitor prevented the EGF-induced decrease in GJC in the K/O cells expressing wt Cx43, clone C9 (n = 3), and in the HeLa cells expressing wt Cx43 (n = 3, see Fig. 5B).

Dual whole cell voltage clamp was used to obtain a more quantitative assessment of the effects of EGF on GJC in the wt Cx43 and S255A,S279A,S282A Cx43 mutant expressing cells. Mean junctional conductance in the C9, wt Cx43 expressing cells was 22.42 ± 2.75 nanosiemens, n = 14. Comparable levels of coupling were observed in the G11 S255A,S279A,S282A Cx43 mutant expressing cells, 18.93 ± 2.07 nanosiemens, n = 14. EGF induced a significant decrease in junctional conductance within minutes in the wt Cx43 expressing cells (Fig. 6A, closed circles) but had little effect on the S255A,S279A,S282A Cx43 mutant expressing cells (Fig. 6B, closed circles). These data demonstrated that despite ongoing phosphorylation of the mutated connexin protein, elimination of the main MAP kinase consensus sites interfered with EGF-stimulated down-regulation of junctional function.

A Negative Charge at Position 255 Did Not Prevent GJC or EGF-induced Disruption of GJC—Disruption of GJC in EGF-treated cells could be due to the presence of a negatively charged phosphate group at one or more of the three MAP kinase serine phosphorylation sites, i.e., at Ser255 (on the Tyr265-Lys287 tryptic peptide), and/or at Ser279, and/or Ser282 (on the Tyr265-Lys287 tryptic peptide). To mimic the effect of a negatively charged phosphate group at the Ser255 position, we prepared a serine to aspartate Cx43 mutant (S255D) and expressed this mutant in HeLa cells. The S255D Cx43 protein was expressed in punctate gap junctions (see Fig. 1H) and the cells communicated to 7 neighboring cells (Fig. 5A and Table I). Thus, the presence of a negative charge at position 255 in Cx43 did not prevent GJC as determined by the transfer of Lucifer Yellow dye. EGF treatment induced an increase in serine phosphorylation of Cx43 (data not shown) and an ∼31%
Junctional conductances were measured as described under “Materials and Methods” for the wt Cx43 K/O cell clone C9 (panel A) and the mutant S255A,S279A, S282A (S255,279,282A) Cx43 K/O cell clone G11 (panel B). Percent of initial junctional conductance was calculated for each cell pair, and the mean ± S.E. for each time point was calculated. Data for each time point could not be obtained from all cell pairs. The initial n value and n values at subsequent points where n changes are noted in the figure.

**TABLE I**

| Clone          | Number of cells communicating<sup>a</sup> | % GJC<sup>b</sup> | GJC EGFG/control |
|----------------|------------------------------------------|------------------|------------------|
| K/O wt G3      | 4.5 ± 0.5<sup>c</sup>(10)                | 2.9 ± 0.6 (6)    | 64               |
| K/O wt C9      | 10.4 ± 1.0 (14)                           | 6.2 ± 0.2 (2)    | 60               |
| K/O S255,279,282A G11 | 3.6 ± 0.4 (18)                           | 3.5 ± 0.5 (13)   | 97               |
| HeLa wt A7A12  | 5.0 ± 0.4 (15)                            | 3.9 ± 0.5 (5)    | 78               |
| HeLa S255D C2A5 | 6.7 ± 1.1 (10)                            | 4.6 ± 0.6 (6)    | 69               |

<sup>a</sup> GJC was determined by the transfer of Lucifer Yellow dye as described under “Materials and Methods.” Cells were treated with 100 ng/ml EGF for 30 min.

<sup>b</sup> Values are reported as the mean ± S.E.

<sup>c</sup> The number of plates injected (n) is indicated in brackets, ~9 cells injected per plate.

fall in GJC in the HeLa cells expressing the S255D Cx43 mutant (Fig. 5A and Table I).

**DISCUSSION**

Our earlier studies demonstrated that MAP kinase phosphorylated full-length Cx43 and a GST fusion protein containing the cytoplasmic carboxyl tail (CT) of Cx43 (aa Val236-Ile382) on tryptic peptides that co-migrated with a subset of the phosphotryptic peptides obtained from Cx43 in EGF-treated cells (31, 41). Our efforts to obtain high resolution tryptic maps of the Cx43 isolated from the EGF-treated cells expressing the S255A,S279A,S282A Cx43 mutant were thwarted due to the relatively low levels of the Cx43 protein expressed in these cells. However, we were able to distinguish a phosphotryptic peptide that migrated with the characteristics of the doubly phosphorylated Tyr<sup>265</sup>-Lys<sup>287</sup> tryptic peptide at the Ser<sup>272</sup> and Ser<sup>273</sup> alternate MAP kinase sites that were identified in our earlier in vitro study (41). It is important to note that phosphorylation at these alternate sites in vitro occurred only in the absence of the consensus MAP kinase phosphorylation sites.

Our efforts to obtain high resolution tryptic maps of the Cx43 isolated from the EGF-treated cells expressing the S255A,S279A,S282A Cx43 mutant (Fig. 2B), since this mutant lacks the 3 consensus MAP kinase phosphorylation sites. In this instance, the increased phosphorylation was also dependent on the activation of MAP kinase. Phosphorylation most likely occurs on the Tyr<sup>265</sup>-Lys<sup>287</sup> tryptic peptide at the Ser<sup>272</sup> and Ser<sup>273</sup> alternate MAP kinase sites that were identified in our earlier in vitro study (41). It is important to note that phosphorylation at these alternate sites in vitro occurred only in the absence of the consensus MAP kinase phosphorylation sites.

Although PD98059 prevents MEK activation in many cells, MEK activation is not completely blocked in some cells following a strong stimulus, allowing catalytic activation of significant amounts of MAP kinase (48). We were fortunate with the −/−/3 K/O cells that the PD98059 MEK inhibitor successfully blocked MAP kinase activation (see Fig. 3B for the S255A,S279A,S282A Cx43 mutant in the K/O cells and Fig. 3C for wt Cx43 in the K/O cells and thus blocked the increased phosphorylation on Cx43 induced by EGF treatment (see Fig. 2A, wt Cx43 and Fig. 2B, S255A,S279A,A282A Cx43 mutant in the K/O cells). Compound PD98059 also substantially reduced the activation of MAP kinase in the EGF-treated HeLa cells expressing wt Cx43 (Fig. 3B).

LPA also stimulated increased phosphorylation of wt Cx43 and the S255A,S279A,S282A Cx43 mutant expressed in the K/O cells (Fig. 4). Increased phosphorylation required the activation of MAP kinase, since it was blocked in cells pretreated with the MEK inhibitor. His et al. (43) suggested that LPA-induced increased phosphorylation on Cx43 might occur through the activation of MAP kinase. Our data support the role of MAP kinase in mediating the increased serine phosphorylation on Cx43 observed in LPA-treated cells. The LPA-induced, MAP kinase-dependent phosphorylation on the S255A,S279A,S282A Cx43 mutant presumably occurred at the Ser<sup>272</sup> and Ser<sup>273</sup> alternate MAP kinase sites.

Taken together, these data provide strong evidence that
MAP kinase is the serine/threonine kinase responsible for the EGF- and LPA-induced increased serine phosphorylation on Cx43. Phosphorylation appears to occur at the MAP kinase consensus phosphorylation sites, Ser255, Ser279, and Ser282 in cells expressing wt Cx43.

The MEK inhibitor appeared to only partially block the pervanadate-induced increased phosphorylation on Cx43. Pervanadate initiated a greater increase in Cx43 phosphorylation, and most of the Cx43 migrated in the more phosphorylated P2 isoform on SDS-PAGE (see Fig. 4C, lane 6 for wt Cx43 in the HeLa cells and Fig. 4D, lane 6 for the S255A,S279A,S282A Cx43 mutant in the K/O cells). Pervanadate is a potent inhibitor of tyrosine phosphatases and has been reported to increase the overall level of tyrosine phosphorylation on cellular proteins ~200-fold in HeLa cells (100 μM pervanadate) as compared with the phosphorylation that was observed in the HeLa cells treated with EGF (50 ng/ml (45)). Furthermore, pervanadate induced a sustained activation of MAP kinase in the HeLa cells compared with the more transient activation of MAP kinase induced by EGF treatment (45). We thought that pervanadate treatment might induce tyrosine phosphorylation on Cx43 as well as serine phosphorylation, and the MEK inhibitor would not prevent tyrosine phosphorylation. We subjected Cx43 isolated from 32P-labeled cells expressing the S255A,S279A,S282A Cx43 mutant to phosphoamino acid analysis. Phosphotyrosine was detected in Cx43 isolated from the pervanadate-treated cells but not in Cx43 isolated from untreated cells or EGF-treated cells (data not shown). Mikalsen et al. (55) have also recently confirmed that pervanadate induced tyrosine phosphorylation on Cx43 in hamster embryo fibroblasts as had been suggested in their earlier study (46).

Some phosphothreonine was also detected in Cx43 isolated from our lysates from the pervanadate-treated K/O cells expressing the S255A,S279A,S282A Cx43 mutant probed with an antibody to phosphotyrosine to detect activated MAP kinase (data not shown). These data support a role for MAP kinase in mediating increased serine phosphorylation on Cx43 and the participation of additional kinase(s) in mediating the tyrosine and threonine phosphorylation on Cx43 in the pervanadate-treated cells. The kinase(s) responsible for the pervanadate-induced tyrosine phosphorylation on Cx43 and the role that such a kinase(s) may have in the regulation of Cx43 function under basal conditions is unknown.

Earlier studies correlated the decrease in GJC with the increase in serine phosphorylation of Cx43 in EGF-treated T51B rat liver epithelial cells (30, 31). However, there has been no direct evidence that the EGF-induced phosphorylation of Cx43 is responsible for the disruption of GJC. Our data demonstrated that activation of MAP kinase was essential for the EGF-induced decrease in GJC. EGF decreased GJC by ~36–40% in the K/O cells expressing wt Cx43 and ~22% in the HeLa cells expressing wt Cx43 (Fig. 5A and Table I). Junctional conductance was decreased ~55% in the C9 wt Cx43 expressing K/O cells. The MEK inhibitor blocked the EGF-induced decrease in GJC in the HeLa cells and K/O cells expressing wt Cx43 compared with cells treated with the MEK inhibitor alone (Fig. 5B). These results indicated the likelihood that phosphorylation of Cx43 by MAP kinase not only regulates Cx43 function but, in addition, phosphorylation on one or more of the MAP kinase consensus serine phosphorylation sites is sufficient to diminish the passage of molecules through Cx43 gap junction channels. The slight stimulation of GJC induced by the MEK inhibitor in the HeLa cells may be due to inhibition of a basal level of MAP kinase activation in these cells which could contribute to the regulation of Cx43 in unstimulated cells.

EGF had no effect on GJC in the K/O cells expressing the S255A,S279A,S282A Cx43 mutant (Fig. 5A and Table I) indicating that this Cx43 triple phosphorylation site mutant is resistant to the EGF-induced disruption of GJC. EGF did in-
Regulation of Cx43 Function by MAP Kinase

In summary, our studies have demonstrated that Cx43 GJC is regulated in vitro by the EGF-induced activation of a signal transduction pathway that leads to the activation of MAP kinase and that MAP kinase-mediated phosphorylation on Cx43 at the Ser279 and/or Ser282 site(s) is sufficient to disrupt GJC (Fig. 7). We are currently preparing additional Cx43 mutants to determine which of these serine phosphorylation sites is critical for the MAP kinase-mediated disruption of GJC.

Acknowledgments—We thank Kendra Martyn for help in establishing the retroviral expression system and Kendra Martyn and Rui Lin for helpful discussions during the course of these studies.

REFERENCES

1. Warn-Cramer, B. J., and Lau, A. F. (1997) in Proceedings of the 1997 International Gap Junctional Conference, (Werner, R., ed) pp. 276–280, 108 Press, Amsterdam.

2. Khor, N. M., and Gilula, N. B. (1996) Cell 84, 381–388.

3. Goodenough, D. A., Goliger, J. A., and Paul, D. L. (1996) Annu. Rev. Biochem. 65, 475–502.

4. Musil, L. S. (1994) in Molecular Mechanisms of Epithelial Cell Junctions: From Development to Disease (Citi, S., ed) pp. 173–184, R. G. Landes Company, Austin, TX.

5. Grisham, S., Goodenough, D. A., and Sosinsky, G. E. (1995) J. Membr. Biol. 146, 15–28.

6. Meyer, R. A., Laird, D. W., Revel, J. P., and Johnson, R. G. (1992) J. Cell Biol. 119, 179–189.

7. Dahl, G. Werner, R., Levine, E., and Randahl-Diehl, C. (1992) Biophys. J. 62, 172–182.

8. Veenstra, R. D. (1996) J. Bioenerg. Biomembr. 28, 327–337.

9. Turin, L., and Warner, A. (1977) Nature 270, 56–57.

10. Liu, S., Taffet, S., Stoner, L., Delmar, M., Vullano, M. L., and Jalfie, J. (1993) Biophys. J. 64, 1422–1433.

11. Morley, G. E., Taffet, S. M., and Delmar, M. (1996) Biophys. J. 70, 1299–1302.

12. Laumenstein, W. R. (1988) Physiol. Rev. 68, 829–911.

13. Spray, D. C., and Bennett, M. V. L. (1985) Annu. Rev. Physiol. 47, 281–303.

14. Reynolds, J. K., Lampe, P. D., and Johnson, R. G. (1992) Exp. Cell Res. 198, 337–342.

15. Oh, S. Y., Gruppen, C. C., and Murray, A. W. A. (1991) Biochem. Biophys. Acta 1094, 234–245.

16. Bienholm, V. M., Ledbetter, M. L. S., Hertzberg, E. L., and Saez, J. C. (1992) Eur. J. Cell Biol. 57, 40–50.

17. Mehta, P. P., Bertram, J. S., and Loewenstein, W. R. (1996) Cell 84, 187–196.

18. Page, E., and Manijanath, C. K. (1986) in The Heart and Cardiovascular System (Pozzard, H. A., ed) pp. 573–600, Raven Press, Ltd, New York.

19. Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1990) J. Membr. Biol. 116, 187–194.

20. Yamasaki, H. (1990) Carcinogenesis 11, 1051–1058.

21. Loewenstein, W. R., and Rose, B. (1992) Semin. Cell Biol. 3, 59–79.

22. Holtz-Wagenblatt, A., and Shalloway, D. (1993) Crit. Rev. Oncog. 4, 541–548.

23. Mesnil, M., and Yamasaki, H. (1994) Mol. Carcinogen. 1, 14–17.

24. Yamasaki, H., and Naus, C. C. G. (1996) Carcinogenesis 17, 1199–1213.

25. Mehta, P. P., Holtz-Wagenblatt, A., Rose, B., Shalloway, D., and Loewenstein, W. R. (1991) J. Membr. Biol. 124, 207–225.

26. Zalc, D., Kidder, G. M., Caveney, S., and Naus, C. C. G. (1992) Proc Natl. Acad. Sci. U. S. A. 89, 10218–10221.

27. Chen, S.-C., Pelletier, D. B., Ao, P., and Boynton, A. L. (1995) Cell Growth Diff. 6, 681–690.

28. Hiroshi, K. K., Xu, C.-E., Tetsukato, T., and Sager, R. (1996) Cell Growth Diff. 7, 861–870.

29. Martyn, K. D., Kurata, W. E., Warn-Cramer, B. J., Burt, J. M., Tenbroek, E., and Lau, A. F. (1997) Cell Growth Diff. 8, 1015–1027.

30. Lau, A. F., Kanemitsu, M. Y., Kurata, W. E., Danesh, S., and Boynton, A. L. (1992) Mol. Biol. Cell 3, 865–874.

31. Yamashita, M., and Yamasaki, H. (1993) Mol. Biol. Cell 4, 837–848.

32. Dotto, G. P., El-Fouly, M. H., Nelson, C., and Troeko, J. E. (1989) Oncogene 4, 637–641.

33. El-Fouly, M., Troeko, J. E., Chang, C. C., and Warren, S. T. (1989) Mol. Carcinogen. 2, 131–135.

34. Crow, D. S., Beyer, E. C., Paul, D. L., Kobe, S. S., and Lau, A. F. (1990) Mol. Cell. Biol. 10, 1754–1763.

35. Crow, D. S., Kurata, W. E., and Lau, A. F. (1992) Oncogene 7, 999–1003.

36. Filson, A. J., Azarina, R., Beyer, E. C., Loewenstein, W. R., and Brugge, J. S. (1990) Cell Growth Diff. 1, 661–668.

37. Kurata, W. E., and Lau, A. F. (1994) Oncogene 9, 329–335.

38. Ahn, N. G. (1990) Mol. Cell. Biochem. 127/128, 201–209.

39. Malarkey, R., Belham, C. M., Paul, A., Graham, A., McLees, A., Scott, P. H., and Flevin, R. (1990) Biochem. J. 309, 361–375.

40. Segur, R., and Krebs, E. G. (1995) FASEB J. 9, 726–735.

41. Warn-Cramer, B. J., Lampe, P. D., Kurata, W. E., Kanemitsu, M. Y., Loo, L. W. M., Eckhart, W., and Lau, A. F. (1996) J. Biol. Chem. 271, 3779–3786.

42. Clark-Lewis, I., Sanghra, J. S., and Pelech, S. L. (1991) J. Biol. Chem. 266, 15180–15184.

43. Hii, C. S. T., Oh, S.-Y., Schmidt, S. A., Clark, K. J., and Murray, A. W. (1994) Biochem. J. 303, 475–479.

44. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moolenaar, W. H. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1257–1261.

45. Zhao, Z., Tan, Z., Diltz, C. D., You, M., and Fischer, E. H. (1996) J. Biol. Chem. 271, 22255–22255.
46. Husoy, T., Mikalsen, S.-O., and Sanner, T. (1993) *Carcinogenesis* **14**, 2257–2265
47. Dudley, D. T., Pang, L., Decker, S. J., Bridges, A. J., and Saltiel, A. R. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7686–7689
48. Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T., and Saltiel, A. R. (1995) *J. Biol. Chem.* **270**, 27489–27494
49. Robbins, D. J., Zhen, E., Owaki, H., Vanderbilt, C. A., Ebert, D., Geppert, T. D., and Cobb, M. H. (1993) *J. Biol. Chem.* **268**, 5097–5106
50. Kamps, M. P., and Sefton, B. M. (1989) *Anal. Biochem.* **176**, 22–27
51. Beyer, E. C., Paul, D. L., and Goodenough, D. A. (1987) *J. Cell Biol.* **105**, 2621–2629
52. Morgenstern, J. P., and Land, H. (1990) *Nucleic Acids Res.* **18**, 3587–3596
53. Miller, A. D., Miller, D. G., Garcia, J. V., and Kynch, C. M. (1993) *Methods Enzymol.* **217**, 581–599
54. Deng, W. P., and Nickoloff, J. A. (1992) *Anal. Biochem.* **200**, 81–88
55. Mikalsen, O., Kasihou, O., Husoy, T., and Sanner, T. (1997) *International Gap Junction Conference*, Key Largo, FL, Werner, R., Conference Chair, University of Miami (Abstr. 88) p. 78
56. Swenson, K. I., Piwnica-Worms, H. P., McNamee, H., and Paul, D. L. (1990) *Cell Regul.* **1**, 989–1002