

Characterization of the Mitogen-activated Protein Kinase Phosphorylation Sites on the Connexin-43 Gap Junction Protein*

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We have previously demonstrated that epidermal growth factor induced a rapid, transient decrease in gap junctional communication and increase in serine phosphorylation on the connexin-43 gap junction protein in T51B rat liver epithelial cells. The kinase(s) responsible for phosphorylation and specific serine targets in connexin-43 have not been identified. There are three consensus mitogen-activated protein (MAP) kinase serine phosphorylation sequences in the carboxyl-terminal tail of connexin-43 and purified MAP kinase phosphorylated connexin-43 *in vitro* on tryptic peptides that comigrated with a subset of peptides from connexin-43 phosphorylated *in vivo* in cells treated with epidermal growth factor. These data suggested that MAP kinase may phosphorylate connexin-43 directly *in vivo*. We have utilized a glutathione S-transferase fusion protein containing the cytoplasmic tail of connexin-43 to characterize MAP kinase phosphorylation. Site-directed mutagenesis, phosphotryptic peptide analysis, and peptide sequencing have confirmed that MAP kinase can phosphorylate connexin-43 at Ser²⁵⁵, Ser²⁷⁹, and Ser²⁸², which correspond to the consensus sites recognized earlier. Characterization of MAP kinase-mediated phosphorylation of connexin-43 has defined potential targets for phosphorylation *in vivo* following activation of the epidermal growth factor receptor and has provided the basis for studies of the effects of phosphorylation, at specific molecular sites, on the regulation of gap junctional communication.

Gap junctions are aqueous membrane channels that permit the exchange of small (<1000 Da) regulatory ions, molecules, and metabolites between cells. Gap junctional communication (GJC)¹ allows for synchrony in events such as contraction in

the uterus and myocardium and is believed to play an important role in regulating growth and differentiation (reviewed in 1–3). Gap junctions form between hexameric structures of connexin molecules (connexons) that interact with connexons in neighboring cells to form membrane pores (4, 5). Connexins are a conserved family of proteins with four membrane-spanning regions and with cytoplasmic amino and carboxyl termini, yielding one intracellular and two extracellular loops.

GJC is known to be regulated by posttranslational phosphorylation on connexin-43 (Cx43). Musil *et al.* (6) have demonstrated that a basal level of posttranslational phosphorylation on serine residues in Cx43 may be essential for functional assembly and activation of gap junctions. Up-regulation of GJC has been associated with increased levels of cyclic AMP and increased serine phosphorylation on Cx43 (7, 8). GJC and serine phosphorylation on Cx43 were also up-regulated in communication-deficient S180 mouse cells following transfection with liver cell adhesion molecule DNA (6). In contrast, disruption of GJC has been associated with increased tyrosine and/or serine phosphorylation on Cx43 (9–12). Down-regulation of GJC was associated with increased serine phosphorylation in cells expressing the *ras* oncogene (10) and in cells stimulated with epidermal growth factor (EGF; Refs. 13 and 14) and with increased tyrosine phosphorylation in *src* (15) and *fps* (16) transformed cells. Studies from this laboratory have demonstrated that Cx43 can serve as a direct substrate for the pp60^{src} tyrosine kinase (17), however, it is not known whether the *fps* kinase directly phosphorylates Cx43 or activates a downstream tyrosine kinase responsible for Cx43 phosphorylation (16). The possible increased serine phosphorylation on Cx43 in cells transformed by the *src* and *fps* oncogenes is presumably mediated by the activation of downstream serine/threonine kinases through signal transduction events.

A rapid and transient decrease in GJC, which correlated with an increase in serine phosphorylation on Cx43, was observed in T51B rat liver epithelial cells following stimulation of the epidermal growth factor receptor (EGFR; Refs. 13 and 14). The data suggested that the receptor tyrosine kinase activated a downstream serine/threonine kinase(s) that phosphorylated Cx43. The identity of this kinase(s) and the specific Cx43 sites phosphorylated have not been characterized. EGF stimulates pathways that lead to the activation of protein kinase C (PKC; Refs. 18 and 19). However, down-regulation of 12-*O*-tetradecanoylphorbol 13-acetate-sensitive forms of PKC did not prevent EGF-induced disruption of GJC or Cx43 phosphorylation (14). EGF also activates mitogen-activated protein kinase (MAPK) by signal transduction events that begin with ligand activation

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¹ The abbreviations used are: GJC, gap junctional communication; Cx43, connexin-43; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; GST-Cx43-CT, GST fusion protein containing the carboxyl-terminal tail of Cx43; PKC, protein kinase C; PBS, phosphate buffered saline; HPLC, high performance liquid chromatog-

raphy; PAGE, polyacrylamide gel electrophoresis; wt, wild type; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

of the EGFR and proceed through a sequence of protein-protein interactions coupled to a protein kinase cascade (20–22). MAPK was activated in EGF-treated T51B rat liver epithelial cells with kinetics that supported a potential role for MAPK in signal transduction events leading to Cx43 phosphorylation or perhaps in directly phosphorylating Cx43 (14). The cytoplasmic, carboxyl-terminal tail of Cx43 possesses three putative consensus MAPK phosphorylation sequences PX₁₋₂(S/T)P (23–25), underlined in Fig. 1. Furthermore, activated MAPK may phosphorylate Cx43 directly *in vivo*, since MAPK phosphorylated Cx43 *in vitro* on phosphotryptic peptides that comigrated with a subset of EGF-responsive phosphotryptic peptides obtained from Cx43 phosphorylated *in vivo* in EGF-stimulated cells (14).

Although it is known that GJC can be regulated by post-translational phosphorylation on connexin, little is known about the molecular sites in the connexin molecule that are critical for regulating intercellular communication through gap junctions. The increased phosphorylation on Cx43 at specific serine sites that follows activation of the EGFR may be the direct cause of the observed functional disruption of GJC. Thus, EGF-induced phosphorylation provides an excellent system for the identification of sites in Cx43 critical to GJC and for the characterization of the signal transduction events leading from the EGF receptor to the activation of downstream serine/threonine kinases that mediate Cx43 phosphorylation. The studies presented here were carried out to further delineate the role of MAPK in Cx43 phosphorylation by identifying specific MAPK serine phosphorylation sites on Cx43. We utilized a GST (glutathione *S*-transferase) fusion protein containing the cytoplasmic, carboxyl-terminal tail of Cx43 (GST-Cx43-CT; Ref. 17) as a MAPK substrate and have identified the sites of phosphorylation by deletion and site-directed mutagenesis, phosphotryptic peptide mapping, and peptide sequence analysis. The results of these studies support the concept that MAPK phosphorylates Cx43 *in vivo* in response to a signal transduced by the activated EGFR and define the phosphorylation sites in Cx43 that may be directly related to the functional disruption of GJC observed in EGF-treated cells.

MATERIALS AND METHODS

Cell Culture, Metabolic Labeling, and Cx43 Immunoprecipitation—T51B rat liver epithelial cells were cultured in Eagle's (BME) medium (Life Technologies, Inc.) supplemented with 10% bovine calf serum (HyClone Laboratories, Logan, UT) in a humidified, 5% CO₂ incubator at 37 °C. Confluent, quiescent monolayers were metabolically labeled with (³²P)_i (NEX-053, DuPont NEN) at 3 mCi/ml in phosphate-deficient medium for 3 h at 37 °C as described (14). During the last 30 min of the labeling period, cells were treated with 25 ng/ml EGF (U. S. Biochemical Corp.). At the end of the labeling period, cells were rinsed with phosphate-buffered saline (PBS), lysed in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 (Sigma), 0.1% sodium dodecyl sulfate (SDS), 10 mM Tris-HCl, pH 7.2) containing 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 160 μM Na₃VO₄, and Cx43 was immunoprecipitated from clarified lysate with rabbit antiserum directed against the carboxyl-terminal peptide of Cx43 (amino acids 368–382). Immunoprecipitates were resolved by SDS-PAGE on 12% acrylamide gels, and radiolabeled Cx43 was visualized by autoradiography.

Recombinant, Baculovirus-expressed Cx43—Full-length Cx43 was expressed in Sf9 insect cells infected with a recombinant baculovirus containing the rat Cx43 cDNA (17). Cx43 was isolated from Sf9 cell homogenates by detergent extraction of the membrane pellet and was immunoaffinity purified by incubation with a monoclonal antibody to Cx43, chemically coupled to protein G-Sepharose, as described (17).

GST-Cx43-CT Fusion Proteins—A GST fusion protein containing the cytoplasmic, carboxyl-terminal tail of Cx43 (amino acids 236–382, see Fig. 1) was generated by polymerase chain reaction amplification of nucleotides 907–1356 of the rat Cx43 cDNA and cloned into the *Bam*HI/*Eco*RI sites of the pGEX-KG expression vector as described (17). This construct is referred to as wild type (wt) GST-Cx43-CT. Deletion mutants were prepared by recombinant polymerase chain reaction (26)

using mutagenic oligonucleotide primers designed to delete nucleotides encoding amino acids 253–256 (PLSP) or amino acids 274–284 (PTA-PLSPMSPP) of Cx43 as described.² Underlined amino acids represent the consensus MAPK phosphorylation sequences (serine sites 255, 279, and 282). Deletion mutants were cloned into the pGEX 2TK expression vector (Pharmacia Biotech, Inc.). Serine site mutants of GST-Cx43-CT were prepared from double-stranded DNA (Chameleon site-directed mutagenesis kit, Stratagene) with oligonucleotide primers (Ransom Hill Biosciences, Inc.) designed to alter a serine residue to alanine: primer 5'-CTGGCCCACTGGCCCCATCAAAAGAC-3' was used to alter the AGC codon for Ser²⁵⁵ to GCC, a codon for alanine (mutant S255A), and primer 5'-GCTCCACTCGCGCCTATGGCTCCTCTGG-3' was used to alter the codons for Ser²⁷⁹ (TCG) and Ser²⁸² (TCT) to codons for alanine (GCG and GCT, respectively; double mutant S279A,S282A). Selection for mutants was provided by the simultaneous use of a selection primer (27) designed to eliminate a unique restriction site in the parental DNA (*Alu*NI at position 2617 or *Mlu*I at position 3647 in the pGEX-KG expression plasmid). The fidelity of all GST-Cx43-CT mutants was confirmed by DNA sequencing (28).

GST fusion proteins were expressed in *Escherichia coli* (DH5α) by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 3 h at 37 °C. Cells were lysed by brief sonication on ice in PBS with 4 mM EDTA, 1 mM benzamidine, and 0.2 mM phenylmethylsulfonyl fluoride and then solubilized with 1% Triton X-100. GST fusion proteins were affinity purified from clarified cell lysates by a 2-h incubation at 4 °C with glutathione-Sepharose 4B-agarose beads (Sigma) followed by extensive washes with PBS (17).

In Vitro MAPK Assays—MAPK assays were carried out with GST-Cx43-CT fusion proteins immobilized on glutathione-Sepharose 4B beads (10 μl). The beads were washed three times with cold kinase buffer (20 mM HEPES, pH 7.4, 10 mM MgCl₂, and 1 mM dithiothreitol) and then incubated for 15 min at 30 °C in kinase buffer with the addition of purified MAPK (0.1–1 μl) and 10 μCi of [γ-³²P]ATP (Amersham Corp., 6000 Ci/mmol) per reaction. Full-length Cx43, isolated from baculovirus-infected Sf9 cells, was phosphorylated by MAPK in a 20-μl reaction mixture containing 0.2 μg of Cx43. Kinase reactions were terminated by the addition of 2 × SDS sample buffer and heating the sample tubes in boiling water. To phosphorylate preparative amounts of GST-Cx43-CT, 500 μl of beads was incubated in the same kinase buffer with 250 μCi of [γ-³²P]ATP and 1.5 μl of MAPK, and the addition of 0.16 μmol of unlabeled ATP. Two MAPK preparations were used in these studies: (i) activated, recombinant human MAPK produced in Sf9 cells coinfecting with Raf-1 and v-Ras (29), purified by HPLC (30) and used at 1 μl/reaction or 100 ng/reaction (kindly provided by Dr. Rikio Fukunaga, The Salk Institute, La Jolla, CA); and (ii) recombinant His-tagged rat MAPK (31) produced in bacteria and activated *in vitro* with an activated, His-tagged human MAPK kinase (MEK) preparation (32), used at 0.1 μl or 50 ng/reaction (generously provided by Drs. Natalie Ahn, University of Colorado, Boulder, CO and Melanie Cobb, University of Texas, Dallas, TX). The histidine tag on the bacterially expressed recombinant MAPK and MEK allowed for rapid and convenient purification of these kinases without significant loss of enzymatic activity. The His-tagged, activated rat MAPK preparation was used for all the *in vitro* phosphorylations in the figures presented in this paper. The deletion mutants of GST-Cx43-CT were phosphorylated with the activated human MAPK preparation from recombinant baculovirus-infected Sf9 cells.

Two-dimensional Phosphopeptide Mapping—Phosphotryptic peptide maps were prepared as described by Boyle *et al.* (33) and Kanemitsu and Lau (14). Briefly, MAPK-phosphorylated GST-Cx43-CT fusion proteins were resolved by SDS-PAGE and visualized by autoradiography. The protein bands were excised from the wet gel and extracted with a buffer containing 50 mM NH₄HCO₃, 0.1% SDS, and 5% β-mercaptoethanol. SDS was removed from the samples by precipitating the proteins with cold trichloroacetic acid in the presence of carrier protein (RNase A, 10–20 μg). Precipitated proteins were washed with ice-cold acetone, oxidized with performic acid at 0 °C, lyophilized to dryness, and then digested overnight at 37 °C with TPCK-treated trypsin (Worthington). Phosphotryptic peptides were resolved on thin-layer cellulose chromatography (TLC) plates (EM Scientific) by electrophoresis for 65 min at 1000 V in pH 1.9 buffer (2.5% formic acid (88%), 7.8% glacial acetic acid, v/v), followed by ascending liquid chromatography in the second dimension in isobutyric acid buffer (62.5% isobutyric acid, 1.9% *n*-butanol, 4.8% pyridine, 2.9% glacial acetic acid, v/v). Phosphorylated peptides

² M. Y. Kanemitsu, L. W. M. Loo, A. F. Lau, and W. Eckhart, manuscript in preparation.

236 VK DR VK GR 244 SDPYHATTG(PLSP)*SK₂₅₈ DCGSPK
 265 YAYFNGCSS(PTAPLSPMSP)*GYK₂₈₇ LVTGDR
 NNSSCR NYNK QASEQNWANYSAEQNR
 MGQAGSTISNSHAQPFDFPDNDQNAK K
 VAAGHELQPLAIVDQRPSSR ASSR
 ASSRPRDDLEI₃₈₂--COOH

FIG. 1. Amino acid sequence of the cytoplasmic carboxyl-terminal tail of Cx43 in the GST fusion protein. The carboxyl-terminal tail of Cx43 in the GST-Cx43-CT fusion protein begins at Val²³⁶ of Cx43 on the cytoplasmic side of the membrane. Putative consensus MAPK phosphorylation sequences in Cx43 are underlined (serine sites Ser²⁵⁵, Ser²⁷⁹, and Ser²⁸², indicated by *). Predicted trypsin cleavage sites are indicated by *spaces* between the resulting tryptic peptides and the amino acids that are deleted in the GST-Cx43-CT deletion mutants are enclosed in *brackets* (amino acids 253–256 and 274–284). Peptide b extends from Tyr²⁶⁵ to Lys²⁸⁷.

were visualized by autoradiography at -70°C with the aid of an intensifying screen. MAPK-phosphorylated wt and deletion mutants of GST-Cx43-CT produced the same phosphorylation patterns when the kinase reactions were carried out in solution with proteins eluted from the glutathione-Sepharose beads (10 μg of fusion protein/reaction), indicating that phosphorylation was not hindered when the fusion proteins were immobilized on beads.

Protein Sequencing and Edman Degradation—Tryptic digests of preparative amounts of MAPK-phosphorylated wt GST-Cx43-CT were prepared as described, except that the carrier protein (RNase A) was omitted during trichloroacetic acid precipitation and a sequencing grade of TPCK-treated trypsin was used for digestions (Promega). Tryptic digests were fractionated by HPLC on a Bio-Rad Microsorb CB reverse phase column (5 μm , 300 \AA) using a water/acetonitrile/trifluoroacetic acid gradient that changed from 85.9/14/0.1 to 75.9/24/0.1 over 30 min. Fractions were collected every minute, and the radioactive fractions were characterized by their migration on two-dimensional phosphotryptic maps. The fraction that migrated with peptide b was identified and submitted for protein sequence analysis (University of Minnesota, St. Paul, MN) and Edman degradation (Ref. 34; University of Virginia, Charlottesville, VA).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out as described (35) on tryptic digests of MAPK-phosphorylated GST fusion proteins.

RESULTS

The *in Vitro* MAPK Phosphorylation Sites in Cx43 Are in the Cytoplasmic Carboxyl-terminal Tail—An earlier study demonstrated that numerous tryptic peptides were nominally phosphorylated in Cx43 isolated from control-treated T51B rat liver epithelial cells and that phosphorylation on these same peptides was markedly enhanced (~ 3 -fold) in cells treated with EGF (14). This study also demonstrated that a membrane preparation of full-length Cx43, isolated from recombinant baculovirus-infected Sf9 cells, was phosphorylated by purified MAPK *in vitro* on four tryptic peptides that comigrated with a subset of the EGF-responsive tryptic peptides obtained from Cx43 phosphorylated *in vivo* in EGF-treated cells (14). Three putative consensus MAPK phosphorylation sequences are present in the cytoplasmic, carboxyl-terminal tail of Cx43 (underlined in Fig. 1). We utilized a GST fusion protein, containing the carboxyl-terminal tail of Cx43 (Val²³⁶–Ile³⁸²), as a substrate to identify MAPK phosphorylation sites. Activated MAPK was prepared *in vitro* by phosphorylation with constitutively active MAPK kinase (MEK; Refs. 31 and 32) or purified by HPLC from recombinant baculovirus-infected Sf9 cells expressing activated human MAPK (29, 30). The two MAPK preparations yielded similar phosphorylation patterns for wt GST-Cx43-CT and for serine site mutants of GST-Cx43-CT (data not shown).

MAPK-phosphorylated full-length Cx43 and wt GST-

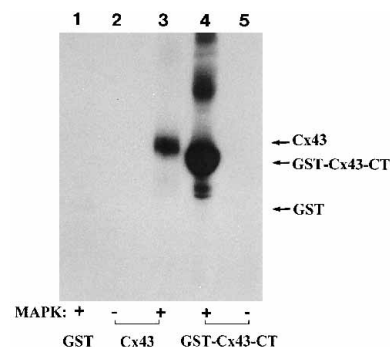


FIG. 2. Phosphorylation of full-length Cx43 and wt GST-Cx43-CT by MAPK. Wild type GST-Cx43-CT (10 μl of beads), GST alone (10 μl), and full-length Cx43 (0.2 μg) were incubated *in vitro* with [γ -³²P]ATP with or without MAPK and then subjected to SDS-PAGE (see “Materials and Methods”). Lane 1, GST alone (+ MAPK); lane 2, GST alone (no MAPK); lane 3, full-length Cx43 (+ MAPK); lane 4, wt GST-Cx43-CT (+ MAPK); lane 5, wt GST-Cx43-CT (no MAPK). The His-tagged, activated rat MAPK preparation was used for the *in vitro* phosphorylations presented in this and all other figures (see “Materials and Methods”).

Cx43-CT were excised from SDS-PAGE gels (see Fig. 2), eluted, and subjected to tryptic digestion as described under “Materials and Methods.” Multiple phosphopeptides were observed on two-dimensional tryptic analysis of Cx43 (Fig. 3A) and wt GST-Cx43-CT (Fig. 3B). Peptides a–d are labeled according to the corresponding EGF-responsive phosphopeptides of MAPK-phosphorylated full-length Cx43 in a previous study (14). Phosphotryptic peptides of wt GST-Cx43-CT comigrated (Fig. 3D) with a subset of the tryptic peptides of Cx43 phosphorylated *in vivo* when T51B rat cells were treated with EGF (Fig. 3C) and also comigrated with phosphopeptides obtained from full-length Cx43 phosphorylated by MAPK *in vitro* (data not shown). Control reactions, performed in the absence of MAPK, failed to phosphorylate full-length Cx43 or wt GST-Cx43-CT, indicating that endogenous kinases, capable of phosphorylating these substrates, were not present in these preparations (Fig. 2, lanes 2 and 5). The GST portion of the fusion protein was not significantly phosphorylated by MAPK ($\sim 0.13\%$ and 0.35% of wt GST-Cx43-CT in two experiments, see Fig. 2, lane 1) and autophosphorylation was not detected in the kinase alone control reaction (data not shown). These data demonstrated that the major MAPK target sites in Cx43 are located in the cytoplasmic, carboxyl-terminal tail (Val²³⁶–Ile³⁸²) and that GST-Cx43-CT is a suitable substrate to characterize *in vitro* MAPK phosphorylation of Cx43.

Identification of Tryptic Peptides of GST-Cx43-CT Containing MAPK Phosphorylation Sites—MAPK is a proline-directed serine/threonine kinase (23–25). To identify tryptic peptides in Cx43 phosphorylated by MAPK, we utilized GST-Cx43-CT mutants with deletions of the proline-rich consensus MAPK phosphorylation sequences (Fig. 1, *enclosed* amino acids). The deletion of Pro²⁵³–Pro²⁵⁶ ($\Delta 253$ – 256) or Pro²⁷⁴–Pro²⁸⁴ ($\Delta 274$ – 284) was expected to alter the mobility of the peptides containing these amino acids (predicted tryptic peptides Ser²⁴⁴–Lys²⁵⁸ and Tyr²⁶⁵–Lys²⁸⁷, respectively). These mutant peptides are smaller and have a different amino acid composition than those in wt GST-Cx43-CT and, thus, will migrate differently on two-dimensional analysis. The phosphopeptide map of the Cx43 $\Delta 253$ – 256 mutant contained phosphopeptides b and c; however, phosphopeptides d and e were absent (data not shown; refer to wt GST-Cx43-CT for phosphopeptide labeling, Fig. 3B). This indicated that the Ser²⁴⁴–Lys²⁵⁸ tryptic peptide contained a site phosphorylated by MAPK in wt GST-Cx43-CT. Furthermore, it was clear that phosphopeptides d and e are related, since both disappeared when the Ser²⁵⁵ consensus sequence

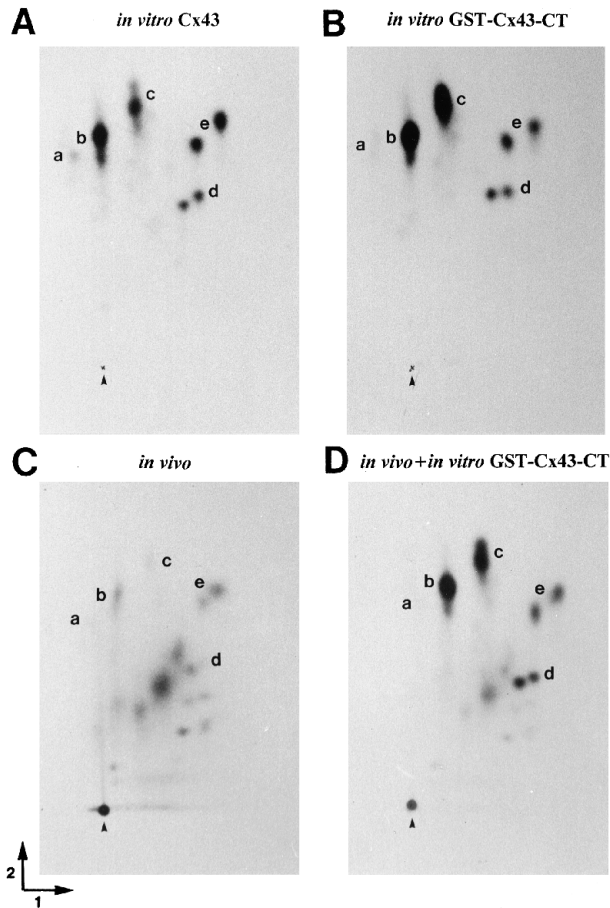


FIG. 3. Two-dimensional phosphotryptic peptide maps of ^{32}P -labeled Cx43. Panel A, recombinant full-length Cx43 (isolated from baculovirus infected Sf9 cells) phosphorylated by MAPK *in vitro*; panel B, wt GST-Cx43-CT phosphorylated by MAPK *in vitro*; panel C, Cx43 isolated from ^{32}P -labeled EGF-treated T51B rat liver epithelial cells; panel D, mix of *in vivo* labeled Cx43 from EGF-treated cells with *in vitro* MAPK-phosphorylated wt GST-Cx43-CT. The origins of sample application are marked with arrowheads, and the directions of migration in both dimensions are indicated by the arrows in the lower left corner of the figure. Phosphopeptides a–e are indicated.

was deleted. Tryptic phosphopeptide analysis of the MAPK-phosphorylated Cx43 $\Delta 274$ – 284 mutant produced a phosphopeptide map where phosphopeptides b and c were absent, but peptides d and e were present (data not shown). Therefore, the deleted amino acids (PTAPLSPMSPP) contain a site(s) phosphorylated by MAPK in wt GST-Cx43-CT. A synthetic peptide containing this deleted region and corresponding to the carboxyl-terminal 17 amino acids of the Tyr²⁶⁵–Lys²⁸⁷ tryptic peptide (NH₂-CSSPTAPLSPMSPPGYK-COOH) was also phosphorylated by MAPK, confirming the presence of a phosphorylation site(s) in the Tyr²⁶⁵–Lys²⁸⁷ peptide (data not shown). Phosphopeptides b and c are also related, since both disappeared in phosphotryptic maps of the $\Delta 274$ – 284 mutant (*i.e.* both contain the predicted tryptic peptide Tyr²⁶⁵–Lys²⁸⁷). These studies with Cx43 deletion mutants indicated that MAPK phosphorylated Cx43 on two tryptic peptides that contain the consensus MAPK phosphorylation sequences (peptides Ser²⁴⁴–Lys²⁵⁸ and Tyr²⁶⁵–Lys²⁸⁷).

We focused on the identification of peptide b because it is a major phosphopeptide on two-dimensional tryptic maps of MAPK-phosphorylated full-length Cx43 (Fig. 3A) and wt GST-Cx43-CT (Fig. 3B) and is also phosphorylated in Cx43 labeled *in vivo* in EGF-treated cells (Fig. 3C). Peptide b was isolated by HPLC from tryptic digests of MAPK-phosphorylated wt GST-Cx43-CT and submitted for amino acid sequence analysis.

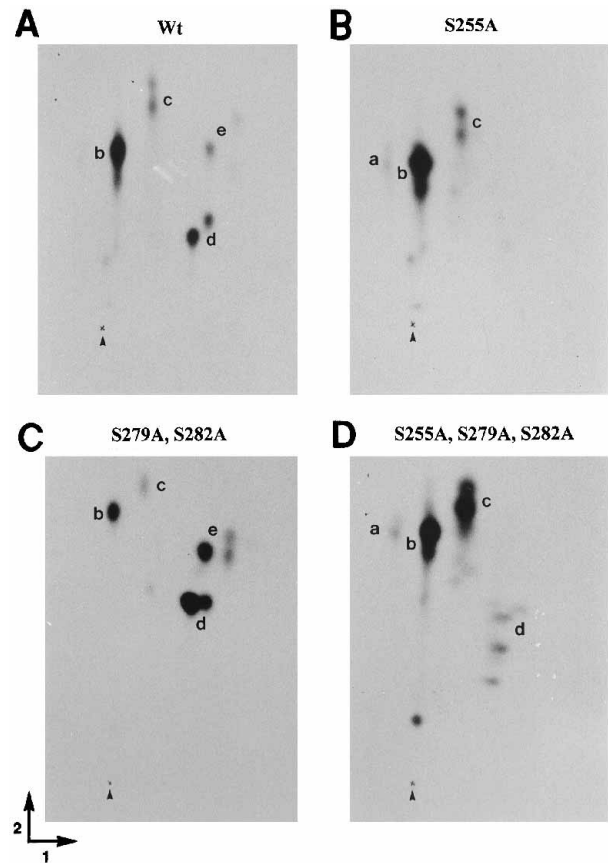


FIG. 4. Two-dimensional phosphotryptic peptide maps of MAPK-phosphorylated serine site mutants of GST-Cx43-CT. Panel A, wt GST-Cx43-CT; panel B, GST-Cx43-CT S255A mutant; panel C, GST-Cx43-CT S279A,S282A double mutant; panel D, GST-Cx43-CT S255A,S279A,S282A triple mutant. The sample origins are marked with arrowheads, and the directions of migration in both dimensions are indicated by the arrows in the lower left corner of the figure.

Clear sequence data that corresponded to the predicted tryptic peptide beginning at Tyr²⁶⁵ (see Fig. 1) was obtained through at least the first 15 amino acids of this peptide. The identity of peptide b is consistent with the absence of this phosphopeptide in tryptic maps of the Cx43 $\Delta 274$ – 284 deletion mutant and with MAPK phosphorylation on the synthetic peptide that corresponded to Cys²⁷¹–Lys²⁸⁷. Peptide b contains two tandem consensus MAPK phosphorylation sequences (underlined in Fig. 1; Ser²⁷⁹ and Ser²⁸²).

Site-directed Mutagenesis of the Consensus MAPK Sites—To identify the MAPK phosphorylation targets in wt GST-Cx43-CT, we prepared mutants with one or more consensus MAPK phosphorylation site(s) altered from serine to alanine. Two-dimensional tryptic phosphopeptide maps of phosphorylated serine site mutants are shown in Fig. 4. Eliminating the Ser²⁵⁵ consensus site (mutant S255A, panel B) produced a tryptic map with phosphopeptides d and e missing (compare with wt GST-Cx43-CT in panel A), confirming that Ser²⁵⁵ is a MAPK phosphorylation site in wt GST-Cx43-CT. Eliminating either the Ser²⁷⁹ or the Ser²⁸² consensus site (mutants S279A or S282A) resulted in phosphopeptide maps indistinguishable from that of wt GST-Cx43-CT (data not shown). This is consistent with the possibility that MAPK phosphorylates peptide b at either or both of these sites in wt GST-Cx43-CT, since the Tyr²⁶⁵–Lys²⁸⁷ peptide is phosphorylated when either consensus MAPK site is eliminated. Since peptides b and c migrated diagonally in relation to each other (see panels A and B), they may represent phosphoisomers (33) or different degrees of phosphorylation on

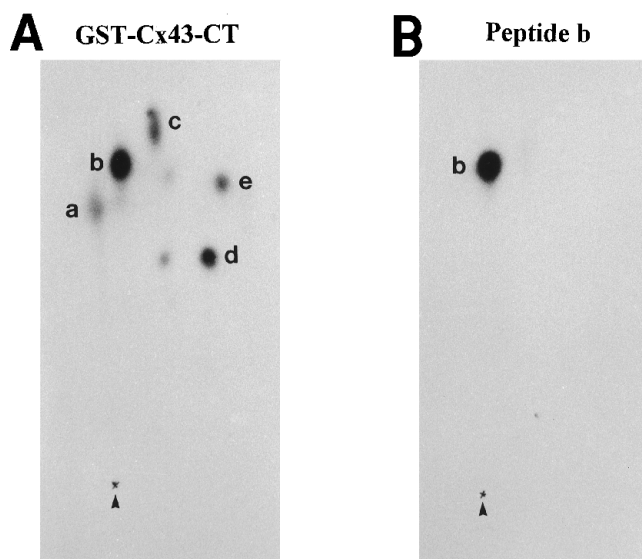
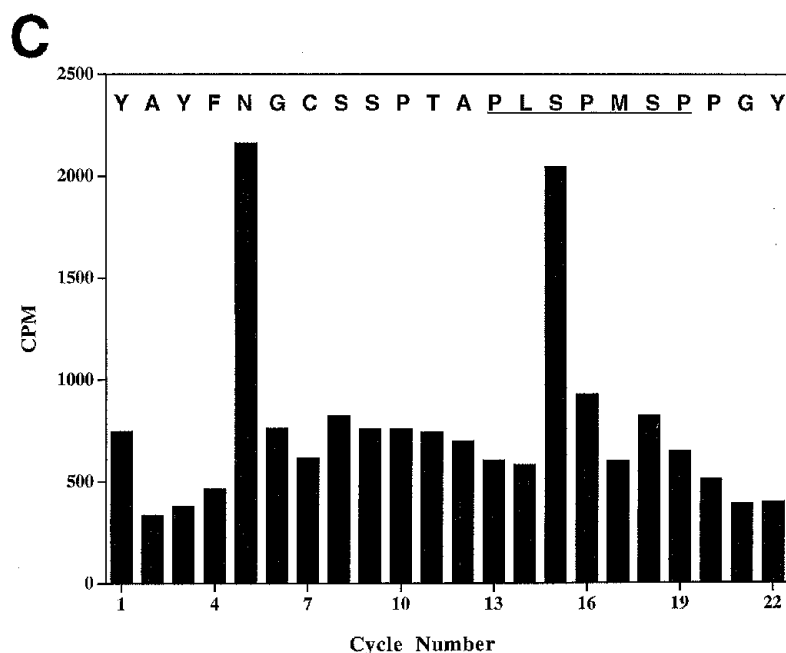


FIG. 5. **Identification of the MAPK phosphorylation sites in peptide b isolated from wt GST-Cx43-CT.** A tryptic digest of preparative amounts of MAPK-phosphorylated wt GST-Cx43-CT (panel A) was fractionated by HPLC and the fraction containing peptide b (panel B) was subjected to Edman degradation. The ^{32}P released at each cycle was determined by Cerenkov counting and is shown in panel C. The partial amino acid sequence of the Tyr²⁶⁵–Lys²⁸⁷ peptide b is shown at the top of panel C. The overlapping consensus MAPK phosphorylation sequences are underlined.



the same peptide (peptide b may represent doubly phosphorylated peptide, and peptide c represent singly phosphorylated peptide). The addition of a negatively charged phosphate group retards peptide mobility in both dimensions on two-dimensional analysis. However, peptide b (presumably doubly phosphorylated) was present in the tryptic maps of the S279A and S282A single site mutants, suggesting that at least one additional site on the Tyr²⁶⁵–Lys²⁸⁷ peptide may be phosphorylated. It is not clear from these data whether this other site is a preferred site or an alternate MAPK phosphorylation site that is phosphorylated in the absence of one of the primary consensus sites.

To clarify this issue, two-dimensional phosphotryptic maps of GST-Cx43-CT double or triple mutants with consensus serine sites altered to alanine were prepared. Eliminating both consensus MAPK sites in peptide b (mutant S279A,S282A, Fig. 4C) left the Ser²⁵⁵ site intact and peptides d and e were phosphorylated. However, surprisingly, phosphopeptides b and c were both present in this tryptic map, suggesting the presence of two additional phosphorylation sites in the Tyr²⁶⁵–Lys²⁸⁷ peptide that are phosphorylated by MAPK in the absence of the

MAPK consensus sites. Results from the phosphorylation of the Cx43 triple mutant S255A,S279A,S282A (all three consensus MAPK serine sites altered to alanine, Fig. 4D) are consistent with these data. Peptides d and e were not phosphorylated; however, peptide b was phosphorylated, indicating the presence of two phosphorylation sites on peptide b, in addition to the two consensus MAPK sites. Eliminating both the Ser²⁵⁵ and Ser²⁷⁹ sites (mutant S255A,S279A) produced a peptide map consistent with the data presented previously; peptides d and e were absent (elimination of Ser²⁵⁵) and peptides b and c were present (consistent with phosphorylation of peptide b at Ser²⁸² and at an additional site; data not shown).

Identification of the Preferred Sites of Phosphorylation by MAPK on Peptide b—The data obtained from two-dimensional phosphotryptic peptide maps of the serine site mutants suggested that MAPK phosphorylates GST-Cx43-CT at the consensus MAPK sites in the Tyr²⁶⁵–Lys²⁸⁷ peptide, but may also phosphorylate this peptide at alternate sites when the consensus sites are absent. To unequivocally identify the preferred sites of MAPK phosphorylation in peptide b, we submitted peptide b (Fig. 5B), purified by HPLC from tryptic digests of wt

GST-Cx43-CT (Fig. 5A), to Edman degradation. Each cycle was monitored for the release of ^{32}P by Cerenkov counting. Three major peaks of radioactivity were identified at cycles 5, 15, and 18 (Fig. 5C). The peaks at positions 15 and 18 correspond to Ser²⁷⁹ and Ser²⁸² in the Tyr²⁶⁵-Lys²⁸⁷ peptide (peptide b), indicating that the consensus MAPK sites are the preferred phosphorylation sites in wt GST-Cx43-CT. The Ser²⁸² site appears to be phosphorylated to a lesser extent than the Ser²⁷⁹ site, even accounting for reduced yields with repetitive cycles (~85% recovery at each cycle). Possible explanations for this include: 1) increased β -elimination of phosphate at Ser²⁸² relative to Ser²⁷⁹; 2) some endogenous phosphorylation on Ser²⁸² in wt GST-Cx43-CT, decreasing the MAPK-mediated phosphorylation at Ser²⁸² in *in vitro* kinase reactions; and 3) small amounts of peptide c in the fraction submitted for Edman degradation may contribute unequally to the phosphorylation data (peptide c may be phosphorylated primarily at the Ser²⁷⁹ site).

The peak at cycle 5, Asn²⁶⁹ in the Tyr²⁶⁵-Lys²⁸⁷ peptide, presumably is due to a contaminating phosphopeptide. Although no evidence of a contaminating peptide (other than small amounts of peptide c) was obtained on two-dimensional peptide maps of the sample submitted to Edman degradation (Fig. 5B), it was possible that a contaminating peptide was undetected because it comigrated with peptide b. It is unlikely that the contaminating peptide originated from the carboxyl terminus of Cx43 because none of its predicted tryptic peptides contain a potential serine or threonine phosphorylation site at position 5 (see Fig. 1). GST was not significantly phosphorylated by MAPK (0.13% and 0.35% of wt GST-Cx43-CT in the absence of other MAPK substrates); thus, its level of phosphorylation is too low to account for the radioactivity in cycle 5. Furthermore, predicted tryptic peptides of GST do not contain phosphorylatable residues at position 5 or recognized MAPK phosphorylation consensus sequences. A more plausible explanation for the ^{32}P -radioactivity obtained in cycle 5 is partial hydrolysis of the Tyr²⁶⁵-Lys²⁸⁷ peptide (peptide b) that occurred during the Edman degradation procedure. Hydrolysis of the Thr²⁷⁴-Pro²⁷⁵ bond would produce a shortened peptide and yield phosphorylation peaks at cycles 5 and 8. This possibility is consistent with the data (Fig. 5C) and with the lack of evidence for other phosphopeptide contaminants in the sample analyzed (Fig. 5B). A small increase in radioactivity in cycle 8 (reflecting the same difference in radioactivity compared with cycle 5 as seen between cycles 18 and 15) is consistent with this hypothesis. Although the radioactivity at cycles 5 and 15 was determined to be equivalent, when corrected for repetitive losses at each cycle, the radioactivity at cycle 15 was actually 4 times that in cycle 5, suggesting that ~20% of the peptide may have been hydrolyzed.

There are three sites in the Tyr²⁶⁵-Lys²⁸⁷ peptide that are potential candidates for alternate MAPK phosphorylation sites: Ser²⁷², Ser²⁷³, and Thr²⁷⁶. Phosphoserine was the only radiolabeled phosphoamino acid detected in MAPK-phosphorylated wt GST-Cx43-CT and in Cx43 phosphorylated *in vivo* in EGF-treated cells (14), consistent with phosphorylation at the consensus MAPK serine sites. Phosphoserine was also the only radiolabeled phosphoamino acid detected in the MAPK-phosphorylated GST-Cx43-CT mutants with altered consensus MAPK sites in the Tyr²⁶⁵-Lys²⁸⁷ peptide (double mutant S279A,S282A and triple mutant S255A,S279A,S282A; data not shown). Since threonine was not phosphorylated in these mutants, the alternate sites of MAPK phosphorylation on peptide b must be Ser²⁷² and Ser²⁷³. All of the data presented are consistent with the migration of peptide b as the doubly phosphorylated Tyr²⁶⁵-Lys²⁸⁷ peptide (phosphorylated at Ser²⁷⁹

and Ser²⁸² in wt GST-Cx43-CT) and peptide c migrating as the singly phosphorylated form of the Tyr²⁶⁵-Lys²⁸⁷ peptide.

DISCUSSION

Earlier studies from this laboratory demonstrated an increase in serine phosphorylation on Cx43 in response to signals transduced by the activated EGF receptor (13, 14). GJC was transiently disrupted in T51B rat liver epithelial cells following EGF treatment and coincided with increased phosphorylation on Cx43. Treating stimulated cells with okadaic acid, a serine/threonine phosphatase inhibitor, prevented both the dephosphorylation of Cx43 and the restoration of GJC. These studies suggested that phosphorylation on Cx43 at specific serine sites was directly related to the disruption of GJC. The MAPK signaling cascade is activated by EGF and leads to the activation of downstream protein kinases, such as MEK and MAPK (20–22). MAPK was activated in EGF-treated T51B cells, and putative consensus MAPK phosphorylation sequences are present in the cytoplasmic, carboxyl-terminal tail of Cx43. Furthermore, purified MAPK phosphorylated recombinant Cx43 *in vitro* on serine residues in tryptic peptides that comigrated with EGF-responsive peptides obtained from Cx43 phosphorylated *in vivo* in the EGF-stimulated cells (14).

In this study, we have utilized a GST fusion protein of the carboxyl-terminal tail of Cx43 as a substrate and demonstrated that the *in vitro* MAPK phosphorylation sites in Cx43 are located in the carboxyl-terminal tail. Tryptic peptides of MAPK-phosphorylated wt GST-Cx43-CT (Fig. 3B) comigrated (Fig. 3D) with a subset of the tryptic peptides obtained from Cx43 phosphorylated *in vivo* in EGF-treated cells (Fig. 3C) and produced the same phosphorylation pattern as that obtained for full-length Cx43 phosphorylated by MAPK *in vitro* (Fig. 3A). These data provided additional support for the potential of MAPK to mediate phosphorylation on Cx43 in EGF-treated cells. Using a combination of tryptic peptide analysis of MAPK-phosphorylated GST-Cx43-CT mutants, phosphoamino acid analysis, and sequence analysis of isolated peptides, we have identified three primary MAPK phosphorylation sites in Cx43 at Ser²⁵⁵, Ser²⁷⁹, and Ser²⁸². Tryptic analysis of MAPK-phosphorylated deletion mutants indicated that the major phosphopeptides contained the consensus MAPK sequences. Phosphopeptides d and e were absent in tryptic maps of the MAPK-phosphorylated $\Delta 253$ –256 deletion mutant and the S255A serine site mutant (Fig. 4B). Thus, Ser²⁵⁵ is the MAPK target site in the Ser²⁴⁴-Lys²⁵⁸ peptide in wt GST-Cx43-CT. Peptides d and e are likely to represent different tryptic digestion products containing the Ser²⁴⁴-Lys²⁵⁸ peptide, due to inefficient cleavage at Lys-Asp bonds (Lys²⁵⁸-Asp²⁵⁹). Two phosphopeptides migrated as peptide d, and two or three phosphopeptides migrated as peptide e (see Fig. 3B or 4A). However, with more complete digestion, using sequencing grade TPCK-treated trypsin in the absence of the RNase A carrier protein, the peptides migrating at positions d and e resolved to single phosphopeptides (see Fig. 5A). Peptide e probably represents the Ser²⁴⁴-Lys²⁵⁸ peptide and peptide d the larger, less hydrophobic Ser²⁴⁴-Lys²⁶⁴ peptide. It is important to note that phosphopeptides d and e are present in tryptic maps of Cx43 obtained from EGF-treated cells (Fig. 3C) and were shown to be EGF-responsive peptides in the earlier study (14), suggesting that Ser²⁵⁵ may be an *in vivo* target for phosphorylation on Cx43 in cells stimulated with EGF.

Two other primary MAPK phosphorylation sites were identified in the tryptic peptide corresponding to Tyr²⁶⁵-Lys²⁸⁷. The identity of this peptide was determined by amino acid sequence analysis of peptide b isolated from tryptic digests of MAPK-phosphorylated wt GST-Cx43-CT. Edman degradation confirmed that the preferred MAPK phosphorylation sites on

this peptide are the consensus MAPK sites, Ser²⁷⁹ and Ser²⁸² (see Fig. 5C), and that peptide b is the doubly phosphorylated form of the Tyr²⁶⁵–Lys²⁸⁷ peptide. When one of the consensus sites in the Tyr²⁶⁵–Lys²⁸⁷ peptide is altered by the conservative substitution of alanine for serine, MAPK phosphorylates this peptide at an alternate site and doubly phosphorylated peptide b is still present in the tryptic maps of the single site mutants, S279A and S282A. The phosphotryptic map of the S279A,S282A double mutant confirmed that phosphorylation can occur at two alternate sites on the Tyr²⁶⁵–Lys²⁸⁷ peptide in the absence of both consensus MAPK sites (Fig. 4C). However, the extent of phosphorylation on peptides b and c appears to be reduced relative to phosphorylation of these peptides in wt GST-Cx43-CT (see Fig. 4A) and the consensus Ser²⁵⁵ phosphorylation site appears to be favored relative to phosphorylation at the alternate sites in the Tyr²⁶⁵–Lys²⁸⁷ peptide (compare phosphorylation of the S279A,S282A double mutant in Fig. 4C with phosphorylation of wt GST-Cx43-CT in Fig. 4A).

Other explanations for the presence of phosphopeptides b and c in the S279A,S282A double mutant, such as comigration of a contaminating peptide or phosphorylation by a contaminating kinase, are unlikely. GST was not a substrate for MAPK, and no candidate serine MAPK sites are present in the GST-Cx43-CT fusion protein except for the described alternate sites, Ser²⁷² and Ser²⁷³, located adjacent to a COOH-terminal proline. Peptides b and c migrated as dimers on some two-dimensional maps in the chromatographic dimension (see Figs. 4A and 5A). This was dependent on the extent of migration relative to the solvent front with increasing migration resulting in a loss of the dimeric form. Such differences in migration may be due to oxidation differences or to hydrophobicity differences due to phosphorylation at different sites on the peptide (33). No evidence of a contaminating phosphopeptide was found in the amino acid sequence analysis of peptide b isolated from wt GST-Cx43-CT. Also, contaminating kinases were not present in wt GST-Cx43-CT (see Fig. 2, lane 5), and two very different MAPK preparations yielded the same phosphorylation patterns for the site-directed mutants, even with reduced amounts of enzyme (3–10-fold), arguing against the presence of a common contaminating serine kinase in the MAPK preparations.

The singly phosphorylated Tyr²⁶⁵–Lys²⁸⁷ peptide appears to be a better substrate for MAPK than unphosphorylated peptide, because the doubly phosphorylated form is apparent in tryptic maps of all of the site-directed mutants (see Fig. 4). Peptide b was observed on tryptic maps when either preparation of MAPK was used and with decreased amounts of enzyme, suggesting a preference for double phosphorylation, at tandem sites, on the Tyr²⁶⁵–Lys²⁸⁷ peptide. Peptide b was also present in tryptic maps of Cx43 phosphorylated *in vivo* in EGF-treated cells (Fig. 3C), consistent with double phosphorylation on the Tyr²⁶⁵–Lys²⁸⁷ peptide *in vivo*.

Since phosphoserine was the only radiolabeled phosphoamino acid detected in the MAPK-phosphorylated S279A,S282A double mutant, the alternate phosphorylation sites in the Tyr²⁶⁵–Lys²⁸⁷ peptide are Ser²⁷² and Ser²⁷³. These sites are present in the Cx43 Δ 274–284 deletion mutant, but were not phosphorylated (peptides b and c were missing rather than shifted in position). Presumably, MAPK did not phosphorylate these serine sites, since the adjacent proline residue (Pro²⁷⁴) was deleted. A proline residue COOH-terminal to serine/threonine was determined to be critical for recognition by MAPK and a NH₂-terminal proline, 1–2 amino acids away, constituted an optimal phosphorylation sequence in studies with peptide substrates (23, 25). Although alternate MAPK phosphorylation sites are present in the Tyr²⁶⁵–Lys²⁸⁷ peptide, phosphorylation appears to be tightly controlled with only two

sites phosphorylated in wt GST-Cx43-CT and in Cx43 phosphorylated *in vivo*. Peptides b and c migrated diagonally in relation to each other on all tryptic maps, consistent with phosphoisomers, whereas peptide a migrated diagonally below peptide b on some tryptic maps (see Figs. 3A and 5A) but not on others (see Figs. 3B and 4D). The identity of peptide a is not known; however, it does not appear to be a phosphoisomer of peptide b or to be a major phosphopeptide of Cx43 phosphorylated *in vivo* (Fig. 3C).

The Ser²⁷³ alternate phosphorylation site in Cx43 has an adjacent COOH-terminal proline as a minimal MAPK recognition signal. The Ser²⁷² site (COOH-terminal proline 2 amino acids away) does not appear to conform to a recognized MAPK phosphorylation site. MAPK phosphorylated a synthetic peptide with an alanine residue inserted between the phosphorylation site and the COOH-terminal proline 85-fold less effectively than a peptide with the COOH-terminal proline residue adjacent to the phosphorylation site (23). Amino acids flanking Ser²⁷² in Cx43 may permit a more favorable geometry for MAPK phosphorylation at a site 1 amino acid away from the COOH-terminal proline or the presence of a phosphate group on Ser²⁷³ may favor subsequent phosphorylation at the adjacent Ser²⁷² site. It is important to remember that the phosphorylation at Ser²⁷² and Ser²⁷³ observed in these studies is induced by the elimination of the primary consensus MAPK phosphorylation sites and probably does not reflect events occurring *in vivo*. However, the potential for phosphorylation to occur at these secondary sites must be considered in DNA transfection experiments designed to examine the effects of these mutations on connexin phosphorylation and function.

Ser²⁶² (in the Ser²⁴⁴–Lys²⁶⁴ peptide) also has an adjacent COOH-terminal proline, but is not readily phosphorylated by MAPK *in vitro*. However, this site may be minimally phosphorylated in the absence of consensus MAPK phosphorylation sites, since the phosphopeptide marked as d in the MAPK-phosphorylated S255A,S279A,S282A triple mutant (Fig. 4D) comigrated with peptide d of wt GST-Cx43-CT (data not shown). Ser²⁶² is flanked by proline and glycine residues and may not be readily accessible to MAPK in GST-Cx43-CT.

Although there are numerous reports of posttranslational modifications on connexin molecules associated with alterations in GJC, little is known about the specific molecular sites in the molecule essential for the regulation of GJC. One study in *Xenopus* oocytes, transfected with Cx43 mRNA, linked phosphorylation at Tyr²⁶⁵ in Cx43 with altered GJC (12). Oocytes coexpressing pp60^{v-src} lost the ability to communicate, whereas GJC was not disrupted in oocytes that coexpressed pp60^{v-src} with a mutant form of Cx43 that could not be phosphorylated at position 265 (Y265F). This study concluded that phosphorylation at the Tyr²⁶⁵ site on Cx43 was sufficient for directly disrupting GJC. However, these results should be interpreted cautiously, since events demonstrated in an oocyte system may not reflect completely the signaling events occurring in mammalian cells, where more than one tyrosine residue in Cx43 may be phosphorylated, directly or indirectly by pp60^{v-src} (15, 17). Moreover, pp60^{v-src} associates with the Shc adaptor protein, resulting in the activation of the Ras/Raf signal transduction pathway and leading to the activation of MAPK (36).

A study by Britz-Cunningham *et al.* (37) demonstrated that a serine to proline mutation at position 364 of Cx43 (S364P) was associated with congenital heart defects in children. Cx43 is the main connexin expressed in heart tissue (38), and right ventricular cardiac malformation was the primary cause of neonatal death in mice lacking the Cx43 gene (39). The cytoplasmic, carboxyl-terminal tail of Cx43 contains consensus phosphorylation sequences for several protein kinases (40) that

may regulate GJC, such as PKC, MAPK, and glycogen synthase kinase 3. L929 cells transfected with the S364P mutant of Cx43 did not exhibit the enhanced GJC observed in L929 cells transfected with wt Cx43 and differed in their responses to microinjected cAMP-dependent protein kinase and PKC (37). Additional mutations in serine or threonine residues in the carboxyl-terminal tail of Cx43 were noted in children with viscerotaxial heterotaxia syndromes; however, the functional significance of these mutations has not been characterized (37). Nevertheless, it is clear that alterations in specific phosphorylated residues in the carboxyl-terminal tail of Cx43 can affect the regulation of GJC and the normal development of the heart.

The data presented in this study provide strong support for MAPK's role in mediating EGF-induced phosphorylation on Cx43 and identify the specific phosphorylated serine sites in the cytoplasmic, carboxyl-terminal tail of the protein that may be functionally related to the disruption of GJC. Importantly, the tryptic peptides containing the phosphorylated Ser²⁵⁵ and Ser²⁷⁹/Ser²⁸² sites comigrated with major phosphopeptides of Cx43 phosphorylated *in vivo*, suggesting that these peptides are phosphorylated in response to activation of the EGF receptor in intact cells. Although alternate signaling pathways, leading to activation of other kinases such as JNK (41, 42) or involving the Rac and/or Rho GTPases (43–45), have not been excluded from acting on Cx43 by these studies, we have demonstrated that Cx43 is a substrate for MAPK. Thus, Cx43 joins a growing number of MAPK substrates that include ribosomal S6 kinase (46), c-Myc (24), c-Jun (24, 47), myelin basic protein (48), and the transcription factor p62^{TCF} (49). Cx43 is also a substrate for the pp60^{src} tyrosine kinase (17), the p130^{gag-fps} tyrosine kinase (either directly or indirectly; Ref. 16), and the PKC serine kinase.³ Identification of the protein kinases responsible for phosphorylating Cx43 at specific sites is important to a better understanding of how GJC may be regulated in normal development and differentiation, synchronous contraction, and cell growth.

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