Epidermal Growth Factor Stimulates the Disruption of Gap Junctional Communication and Connexin43 Phosphorylation Independent of 12-0-tetradecanoylphorbol 13-acetate-sensitive Protein Kinase C: The Possible Involvement of Mitogen-activated Protein Kinase

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We previously reported that epidermal growth factor (EGF) induced the disruption of gap junctional communication (gjc) and serine phosphorylation of connexin43 (Cx43) in T51B rat liver epithelial cells. However, the cascade of events linking EGF receptor activation to these particular responses have not been fully characterized. Furthermore, the serine kinase(s) acting directly on Cx43 remain unidentified. In the current study, we demonstrate that downmodulation of 12-0-tetradecanoylphorbol 13-acetate (TPA)-sensitive protein kinase C (PKC) activity does not affect EGF's ability to reduce junctional permeability or phosphorylate Cx43 in T51B cells. EGF in the presence or absence of chronic TPA treatment stimulated marked increases in Cx43 phosphorylation on numerous sites as determined by two-dimensional tryptic phosphopeptide mapping. Computer-assisted sequence analysis of Cx43 identified several protein kinase phosphorylation consensus sites including two sites for mitogen-activated protein (MAP) kinase. EGF stimulated activation of MAP kinase in a time- and dose-dependent manner where the kinetics of kinase activity corroborated its possible involvement in mediating EGF's effects. Moreover, purified MAP kinase directly phosphorylated Cx43 on serine residues *in vitro*. Two-dimensional tryptic and chymotryptic phosphopeptide mapping demonstrated that the *in vitro* phosphopeptides represented a specific subset of the *in vivo* phosphopeptides produced in response to EGF after chronic TPA treatment. Therefore, EGF-induced disruption of gjc and phosphorylation of Cx43 may be mediated in part by MAP kinase *in vivo*.

INTRODUCTION

One form of intercellular communication is mediated by gap junctions, aggregations of membranous channels that directly link the cytoplasms of adjacent cells (Flagg-Newton et al., 1979; Loewenstein, 1981). Gap junctions are composed of hexameric structures of individual proteins termed connexins. Connexins are now recognized as a growing family of proteins showing evolutionary conservation and diverse expression in various tissues suggesting that gap junctions play an important role in cellular function (Beyer et al., 1990; Dermietzel et al., 1990).

Gap junctions allow the exchange of regulatory ions and molecules involved in tissue homeostasis and differentiation and synchronization of tissue and organ systems (reviewed in Beyer et al., 1990). In addition, it has been suggested that regulatory molecules may pass through gap junctions and thus regulate cell growth and proliferation (Yamasaki and Enomoto, 1985; Mehta et al., 1986, 1991; Yamasaki, 1990; Loewenstein and Rose, 1992). Cells transformed by v-src or H-ras oncogenes exhibit reduced gap junctional communication (gjc) (Azarnia et al., 1988; Dotto et al., 1989; El-Fouly et al., 1989; Crow et al., 1990; Brissette et al., 1991). In addition, cells transformed by a temperature-sensitive

mutant of Rous sarcoma virus exhibited a rapid and reversible reduction in gjc when the cells were changed from the nonpermissive temperature to the permissive temperature required for cellular transformation (Atkinson et al., 1981; Azarnia and Loewenstein, 1984; Crow et al., 1992). Furthermore, numerous tumor promoters have been demonstrated to reduce junctional permeability in various cell lines (reviewed in Klaunig and Ruch, 1990). Finally, Mehta et al. (1991) demonstrated that incorporation of the Cx43 cDNA into the genome of communication-deficient transformed C3H 10T1/2 fibroblasts resulted in the production of functional Cx43 leading to the normalization of gjc and growth. Thus, the modulation of gjc is of particular interest because of its possible role in regulating various physiological functions.

The molecular basis by which gjc is regulated is largely unknown; however, posttranslational phosphorylation of the connexin protein has been associated with the modulation of gap junctional channels. Musil et al. (1990) examined Cx43 phosphorylation in communication-deficient and -competent cells and demonstrated that Cx43 phosphorylation may be required for the assembly and/or activation of gap junctions. Previous studies have demonstrated a direct correlation between increased levels of cyclic AMP (cAMP) and increased gjc (Flagg-Newton et al., 1981; Saez et al., 1986, 1990). These studies suggested that cAMP-dependent protein kinase may mediate the serine phosphorylation of Cx43 that was associated with the up-regulation of gjc. Conversely, reduced junctional permeability has been associated with the tyrosine and/or serine phosphorylation of Cx43 (Crow et al., 1990, 1992; Filson et al., 1990; Swenson et al., 1990; Berthoud et al., 1992; Moreno et al., 1992). We, and others, have demonstrated epidermal growth factor (EGF)-induced disruption of gjc (Maldonado et al., 1988; Madhukar et al., 1989; Lau et al., 1992). Unlike the tyrosine phosphorylation of Cx43 observed in Src-transformed cells (Crow et al., 1990, 1992; Filson et al., 1990), this effect was correlated with the serine phosphorylation of Cx43 suggesting that the EGF-receptor itself or other tyrosine kinases do not directly phosphorylate Cx43 but rather involve the activity of a serine kinase (Lau et al., 1992). As yet, the serine kinase(s) acting directly upstream of Cx43 is unidentified.

Previous studies have characterized 12-*O*-tetradecanoylphorbol 13-acetate (TPA)- and 1-oleoyl-2-acetyl-glycerol (OAG)-induced disruption of gjc (Enomoto and Yamasaki, 1985; Gainer and Murray, 1985; Dotto et al., 1989; Somogyi et al., 1989; Katoh et al., 1990). In addition, others have demonstrated TPA-induced phosphorylation of MP26 lens gap junction protein, Cx26 or Cx43 in vivo (Lampe and Johnson, 1989; Brissette et al., 1991; Berthoud et al., 1992) or direct phosphorylation of Cx32 by purified protein kinase C (PKC) in vitro (Takeda et al., 1987; Saez et al., 1990). Moreover,

EGF has been demonstrated to stimulate the phospholipase C/phosphatidylinositol pathway producing the second messengers inositol 1,4,5-trisphosphate and diacylglycerol (DAG) that mediate the release of intracellular Ca^{2+} and PKC activation, respectively (Berridge, 1987; Nishizuka, 1988). These studies suggest that PKC may mediate EGF's ability to disrupt gjc and phosphorylate Cx43 in T51B cells.

In addition, computer-assisted sequence analysis of Cx43 has identified several protein kinase phosphorylation consensus sites, including two mitogen-activated protein (MAP) kinase sites (Alvarez et al., 1991). Ligand-mediated activation of the EGF receptor has been demonstrated to result in the stimulation of MAP kinase (Ahn et al., 1990a,b, 1991). In addition, sites for PKC, p34^{cdc2} , casein kinase I, and glycogen synthase kinase-3 were also identified (Kennelly and Krebs, 1991). It is possible that Cx43 is a target of multiple signaling pathways. In the current study, we investigate further the possible EGF-stimulated signaling pathways and attempt to identify the serine kinase(s) responsible for Cx43 phosphorylation in T51B rat liver epithelial cells.

MATERIALS AND METHODS

Cell Culture

T51B rat liver epithelial cells (originally provided by Alton Boynton, Pacific Northwest Research Foundation) were cultured in basal medium, Eagle's (BME) (GIBCO, Grand Island, NY) supplemented with 10% bovine calf serum (BCS) (Colorado Serum, Denver, CO) and maintained at 37°C in a humidified 5% CO_2 incubator. Confluent and quiescent T51B cell cultures were used for all experiments unless stated otherwise. For experiments requiring chronic TPA treatment, T51B cells were incubated with TPA (100 ng/ml) in BME-1% BCS for 24 h at 37°C.

Assay of GJC

Gjc was measured by the microinjection-dye transfer method (Crow et al., 1990; Lau et al., 1992). Briefly, T51B cells preincubated with phosphate-buffered saline (PBS) or TPA (100 ng/ml) (Sigma, St. Louis, MO) for 24 h were rinsed and equilibrated in BME-1% BCS at 37°C for 30 min. After the equilibration period, the cells were treated with PBS (control), EGF (25 ng/ml) (Collaborative Research, Bedford, MA), or TPA (10 ng/ml) for the indicated times. Individual cells were microinjected pneumatically with a glass micropipette (Flaming-Brown P80/PC micropipette puller, Sutter Instrument, Novato, CA) containing 10% (wt/vol) Lucifer yellow dissolved in 0.33 M lithium chloride using a Zeiss micromanipulator (Thornwood, NY) and Eppendorf pneumatic injector (Eppendorf, Madison, WI) and visualized on a Nikon diphot phase-contrast inverted microscope (Garden City, NY) with epifluorescence. Gjc was quantitated by counting the number of cells that contained the Lucifer yellow dye 1 min after microinjection of a single cell.

Metabolic Labeling, Immunoprecipitation, and Quantitation of Cx43

T51B cells, either untreated or chronically exposed to TPA, were metabolically labeled as previously described (Crow et al., 1990) with [^{32}P] (NEN, NEX-053) at 0.5–3.0 mCi/ml in BME-1% BCS phosphate-deficient medium (GIBCO) for 3 h at 37°C. During the labeling period, cells were treated with PBS (control), EGF (25 ng/ml), or TPA (10 ng/ml) for the indicated times. After the labeling period, the cells

were rinsed with PBS, lysed in RIPA (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100 [Sigma, St. Louis, MO], 0.1% sodium dodecyl sulfate, 10 mM Tris-HCl [pH 7.2]) buffer, clarified, and immunoprecipitated with either normal rabbit serum or rabbit antiserum directed against a peptide encoding amino acids 368–382 of rat heart connexin43 (CT368) as previously described (Crow et al., 1990). The immunoprecipitates were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 7.5–15% polyacrylamide gels (Laemmli et al., 1970). The gels were stained with Coomassie blue (Sigma), destained in 30% methanol/10% acetic acid (vol/vol), dried, and exposed to X-OMAT XAR-5 film (Eastman Kodak, Rochester, NY) for 1–3 d at -70°C . Radioactive quantitation of the Cx43 proteins was accomplished by excising and rehydrating gels bands followed by scintillation counting in a Beckman LS3801 spectrometer (Fullerton, CA).

Phosphoamino Acid Analysis

Phosphoamino acid analysis of Cx43 phosphorylated by purified MAP kinase *in vitro* was conducted as described by Kamps and Sefton (1989). After the *in vitro* kinase reaction, Cx43 was immunoprecipitated with the Cx43 CT368 peptide antiserum. The immunoprecipitates were resolved by SDS-PAGE and electrotransferred to an Immobilon P membrane (Millipore, Bedford, MA) as described by Matsudaira (1987). The samples were acid hydrolyzed, lyophilized, and mixed with a solution containing unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards. The samples were electrophoresed in pH 1.9 buffer in the first dimension then in pH 3.5 buffer in the second dimension on thin layer cellulose sheets (Kamps and Sefton, 1989). Standards were visualized by ninhydrin staining and the sheets were exposed to Kodak X-OMAT XAR-5 film with an intensifying screen for 2–4 d at -70°C .

2-Dimensional Tryptic or Chymotryptic Phosphopeptide Mapping

T51B cells metabolically labeled with [^{32}P] or *in vitro* kinase reactions were solubilized in RIPA buffer and immunoprecipitated with Cx43 CT368 peptide antiserum; the proteins were separated by SDS-PAGE as previously described. The labeled Cx43 proteins were excised and eluted from unfixed, unstained wet gels and digested with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone or alpha-chymotrypsin (Worthington, Freehold, NJ) as previously described (Boyle et al., 1991). The resulting phosphopeptides were separated on cellulose thin-layer chromatography (TLC) plates (Curtin Matheson Scientific, Houston, TX) by electrophoresis using pH 1.9 buffer [2.5% (vol/vol) formic acid (88%), 7.8% (vol/vol) glacial acetic acid] for 1 h at 1 kV in the first dimension and by ascending chromatography using isobutyric acid buffer [62.5% (vol/vol) isobutyric acid, 1.9% (vol/vol) *n*-butanol, 4.8% (vol/vol) pyridine, 2.9% (vol/vol) glacial acetic acid] in the second dimension as described by Boyle et al. (1991). The TLC plates were exposed to Kodak X-OMAT XAR-5 film with an intensifying screen (Dupont, Wilmington, DE) for 12–14 d at -70°C .

In Vitro PKC and MAP Kinase Assays

PKC activity was assayed as described by Meier et al. (1991). T51B cells were unstimulated or EGF-treated (25 ng/ml) for 15 min after preincubation with PBS or TPA (100 ng/ml) for 24 h at 37°C . Cells were scraped from culture dishes in extraction buffer (20 mM *N*-2-hydroxyethylpiperazine [HEPES], pH 7.5, 10 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 2 mM EDTA, 2 mM dithiothreitol [DTT]) and Dounce homogenized with 30 strokes. The crude soluble fraction was obtained after centrifugation in a TLA 100.2 rotor at $100\,000 \times g$ for 20 min at 4°C in a Beckman TL-100 ultracentrifuge. The resulting pellet was resuspended by passage through a 22G 1 1/2 in needle in extraction buffer containing 1% Triton X-100 (vol/vol) and incubated on ice for 30 min. The crude particulate fraction was obtained by centrifugation in a TLA 100.2 rotor at $400\,000 \times g$ for 20 min at 4°C in a Beckman TL-100 ultracentrifuge. Cytosolic and membrane-bound PKC was partially purified

by diethylaminoethyl (DEAE)-Sephacel chromatography as described by Meier et al. (1990). All chromatography steps were conducted at 4°C . PKC activity in DEAE-Sephacel eluates of cytosolic and particulate fractions were assayed as described by Meier et al. (1991) immediately after column chromatography. Samples were resolved by SDS-PAGE on a 12% polyacrylamide gel as previously described. Quantitation of the phosphorylated histone protein was accomplished by excising and rehydrating the gel band, followed by scintillation counting (Beckman LS 5000TD).

Myelin basic protein (MBP) kinase activity was assayed as described by Boulton et al. (1991). T51B cells were treated with PBS (control) or EGF for the indicated times and concentrations. For experiments requiring chronic TPA treatment, T51B cells were preincubated with TPA (100 ng/ml) for 24 h at 37°C before the addition of EGF as described above. After the treatment period, the cells were scraped from the culture dish in extraction buffer (50 mM beta-glycerophosphate, pH 7.4, 1 mM EGTA, 1 mM DTT, 2 mM phenylmethylsulfonyl fluoride [PMSF] 0.1 mM sodium orthovanadate) and then passed through a 26G 3/8 in needle five times. Samples were centrifuged at 14 000 rpm in a microcentrifuge at 4°C . The resulting cytosolic fraction was assayed immediately as previously described (Boulton, 1991). The samples were resolved by SDS-PAGE on a 12% polyacrylamide gel, prepared for autoradiography, and quantitated as described above.

Cytosolic fractions prepared as described above were also analyzed for MAP kinase activity in MBP-containing polyacrylamide gels as previously described (Kameshita and Fujisawa, 1989; Tobe et al., 1991; Wang and Erikson, 1992). A control experiment measuring autophosphorylation was performed with a gel in the absence of MBP. The gel was autoradiographed using Kodak X-OMAT XAR-5 film for 1–3 d at -70°C . Quantitation of MBP phosphorylated by MAP kinase was measured by scintillation counting as described previously.

In Vitro Phosphorylation of Cx43 by MAP Kinase

In vitro phosphorylation of Cx43 by MAP kinase was performed using the kinase buffer and assay conditions as described by Thomas et al. (1992). Cx43 was partially purified from Cx43 recombinant baculovirus-infected Sf9 cells (L. Loo and A. Lau, unpublished data). The cells were Dounce homogenized in homogenization buffer (50 mM tris(hydroxymethyl)aminomethane, pH 7.5, 0.15 M NaCl, 2 mM EDTA, 1 mM EGTA, 25 $\mu\text{g}/\text{ml}$ leupeptin, 25 $\mu\text{g}/\text{ml}$ aprotinin) with 30 strokes. The crude particulate fraction was obtained after centrifugation in a TLA 100.2 rotor at $100\,000 \times g$ for 20 min at 4°C in a Beckman TL-100 ultracentrifuge. The pellet was resuspended in homogenization buffer by passage through a 22 G 1 1/2 in needle and treated at 65°C for 5 min to minimize endogenous kinase activity. Heat treatment did not degrade or affect the mobility of Cx43 as determined by Western blot analysis. Seventy micrograms of the particulate fraction was incubated in kinase buffer (30 mM HEPES, pH 7.4, 10 mM MgCl_2 , 1 mM DTT, 20 μM ATP, 20 μCi [γ - ^{32}P] ATP [≈ 6000 Ci/mmol], 5 mM benzamide) with 18 μg purified MAP kinase (Ray and Sturgill, 1988a) from EL4 cells (a gift of Mike Weber, University of Virginia, Charlottesville, VA) for 30 min at 30°C . Kinase reactions were terminated by the addition of RIPA buffer and were followed by immunoprecipitation with Cx43 CT368 peptide antiserum. The immunoprecipitated proteins were resolved by SDS-PAGE as described above.

RESULTS

Down-Modulation of TPA-sensitive PKC Does Not Affect EGF-induced Disruption of gjc

EGF (25 ng/ml) or TPA (10 ng/ml) treatment of T51B cells resulted in rapid reductions in gjc (Figure 1A). A 60% reduction in gjc occurred 10 min after EGF addition that reached maximum levels of disruption (76% reduction) by 30 min (Figure 1A, $\square$). Gradual re-establishment

Figure 1. Effect of chronic TPA treatment on EGF-induced disruption of gjc and Cx43 phosphorylation in T51B rat liver epithelial cells. (A) T51B cells were preincubated with PBS or TPA (100 ng/ml) for 24 h before acute treatment with EGF (25 ng/ml) or TPA (10 ng/ml) and assayed for gjc at the indicated times as described in MATERIALS AND METHODS. Changes in gjc after EGF and TPA treatment were determined relative to the average control values of 101 and 72 cells communicating for untreated and 24-h TPA-treated T51B cells, respectively. These data represent the averages ($\pm$ SEM) of 8–17 determinations. (B) T51B cells treated as described above were metabolically labeled with 32 P, and Cx43 was immunoprecipitated from cell lysates with Cx43 CT368 peptide antiserum as described in MATERIALS AND METHODS. The proteins were resolved by SDS-PAGE and autoradiographed using Kodak X-OMAT XAR-5 film for 1–3 d at -20°C . The Cx43 proteins were excised from the gel, rehydrated, and quantitated by scintillation counting. Increases in Cx43 phosphorylation were determined relative to the average control value of 197–248 cpm. These data represent the averages ($\pm$ SEM) of 2–3 individual experiments.

lishment of gjc occurred at the 1-h time point with restoration nearly to levels observed in control cells occurring by 3 h. These results are essentially similar to those reported previously (Lau et al., 1992). Gjcs were reduced by 47% and 79% after TPA treatment for 10 and 30 min, respectively (Figure 1A, ○). The lowest levels of gjc (99% reduction) were observed at the 1-h time point and unlike the restoration in gjc observed in EGF treated cells, disruption of gjc persisted at 3 h after TPA addition. Restoration of gjc to levels observed in control cells occurred at 12 h after preincubation of T51B cells with TPA (100 ng/ml) and was maintained for 24 h. This pretreatment desensitized cells to subsequent TPA treatments where only nominal decreases in gjc were observed 10 and 30 min after acute TPA treatment (11% and 5% reduction, respectively) (Figure 1A, ●). Gjcs appeared slightly elevated above control levels at

the 1- and 3-h time points. In contrast, chronic TPA exposure of T51B cells did not affect EGF's ability to alter gjc where the time course, levels of disruption, and subsequent restoration of gjc closely paralleled that observed in EGF-treated naive cells (Figure 1A, compare ■ and □).

Down-Modulation of TPA-sensitive PKC Does Not Affect EGF-induced Cx43 Phosphorylation

To determine if the observed changes in gjc caused by EGF and TPA correlated with phosphorylation of Cx43, [32 P]-labeled cell lysates were immunoprecipitated with Cx43 CT368 peptide antiserum and the proteins were resolved by SDS-PAGE. A 2.1-fold increase in Cx43 phosphorylation occurred as early as 10 min after EGF addition with maximum levels of phosphorylation (2.9-fold increase) occurring at the 30-min time point (Figure 1B, □). These changes in Cx43 phosphorylation inversely correlated with those observed in gjc. The levels of Cx43 phosphorylation decreased at the 1-h time point, approaching levels observed in control cells by 3 h after EGF addition. TPA treatment also resulted in 2.0- and 2.7-fold increases in Cx43 phosphorylation at 10 and 30 min, respectively (Figure 1B, ○). In contrast to decreasing levels of Cx43 phosphorylation observed 1 h and 3 h after EGF treatment, maximum levels (3.2-fold increase) of Cx43 phosphorylation occurred 1 h after TPA treatment. Moreover, Cx43 phosphorylation levels remained elevated at the 3-h time point that correlated with the prolonged disruption of gjc (Figure 1A). Acute TPA treatment of T51B cells after chronic TPA exposure resulted in little change in Cx43 phosphorylation like that observed in control cells (Figure 1B, ●). However, chronic TPA exposure did not affect EGF's ability to induce Cx43 phosphorylation, in that the time course and levels of Cx43 phosphorylation and dephosphorylation closely paralleled those observed for EGF treatment of naive cells (Figure 1B, ■). These latter results were most significant because they suggested that EGF's action on Cx43 phosphorylation was independent of TPA-sensitive PKC activity.

Chronic TPA Treatment of T51B Cells Down-modulates Protein Kinase C Activity

To confirm that 24-h TPA treatment of T51B cells down-regulated PKC activity, DEAE-Sephacel eluates of cytosolic and particulate fractions of T51B cells were assayed for PKC activity using histone type IIIS as a substrate, in the absence or presence of Ca^{2+} , phosphatidylserine (PS), and DAG. The PKC activity values shown in Figure 2 represent the relative increases in histone phosphorylation over the nominal activity observed in the absence of Ca^{2+} , PS, and DAG. PKC activity was predominantly localized in the cytosolic fraction (6.3-fold increase) of unstimulated T51B cells with the membrane-bound PKC showing limited activity

Figure 2. Effect of chronic TPA treatment on EGF-induced PKC activation in cytosolic and particulate fractions of T51B cells. T51B cells were preincubated with PBS or TPA (100 ng/ml) for 24 h before treatment with PBS or EGF (25 ng/ml) for 15 min. Crude cytosolic and particulate fractions were fractionated on DEAE-Sephacel as described in MATERIALS AND METHODS. Cytosolic and membrane-bound PKC was batch-eluted with 0.1 M and 0.15 M NaCl, respectively. DEAE-Sephacel eluates (2 μ g) of cytosolic and particulate fractions were assayed for PKC activity in the presence or absence of Ca^{2+} , phosphatidylserine, and diacylglycerol. The samples were resolved by SDS-PAGE and autoradiographed using Kodak X-OMAT XAR-5 film for 1 d at -20°C . The phosphorylated histone proteins were excised from the gel, rehydrated, and quantitated by scintillation counting. The nominal histone phosphorylation observed in the absence of Ca^{2+} , phosphatidylserine, and diacylglycerol was regarded as nonspecific kinase activity, and the values represented in Figure 2 are expressed relative to this nonspecific kinase activity. Each point represents the average ($\pm$ SEM) of two independent experiments.

(2.1-fold increase) (Figure 2). However, upon EGF treatment for 15 min, membrane-bound PKC activity markedly increased 6.3-fold above the nominal kinase activity (Figure 2). Increased PKC activity in the particulate fraction of T51B cells in response to EGF was presumably because of PKC translocation to the membrane after activation (reviewed in Niedel and Blackshear, 1986). PKC activity in the cytosolic fraction of EGF-treated cells was slightly reduced from that observed in unstimulated cells (Figure 2). Most significantly, chronic TPA treatment greatly diminished PKC activity in both cytosolic and particulate fractions of T51B cells (Figure 2). In addition, EGF no longer stimulated PKC activity in chronically TPA-treated cells (Figure 2). These findings strongly suggested that 24-h TPA treatment of T51B cells results in the down-modulation of TPA-sensitive PKC.

EGF Stimulates the Phosphorylation of Cx43 at Numerous Sites In Vivo

The phosphorylation sites of ^{32}P -labeled Cx43 from T51B cells were examined by two-dimensional tryptic phosphopeptide mapping as previously described (Boyle

et al., 1991). Tryptic digestion of Cx43 from untreated T51B cells generated numerous phosphopeptides that were nominally phosphorylated (Figure 3A). However, EGF treatment for 30 min resulted in marked increases in phosphorylation of the phosphopeptides (Figure 3B). The tryptic phosphopeptide map of Cx43 from cells chronically treated with TPA appeared similar to that observed for untreated cells, although several phosphopeptides showed slightly higher levels of phosphorylation (compare Figure 3, A and C, $\diamond$). EGF treatment after chronic TPA treatment resulted in a tryptic phosphopeptide map similar to that of EGF alone with the exception of several missing phosphopeptides, possibly containing the TPA-sensitive PKC phosphorylation sites (compare Figure 3, B and D, $\blacklozenge$). These results indicated that Cx43 becomes highly phosphorylated on numerous sites in response to EGF. Furthermore, chronic TPA treatment does not affect EGF's ability to stimulate the phosphorylation of these sites with exception of three phosphopeptides that may contain TPA-sensitive PKC phosphorylation sites.

EGF Stimulates Activation of the p42 and p44 Isoforms of MAP Kinase

To measure EGF activation of MAP kinase directly, cytosolic fractions of EGF-stimulated T51B cells were assayed for MBP kinase activity. We found that phosphorylation of MBP by EGF-activated MBP kinase occurred in a rapid and transient manner in vitro. A 2.9-fold increase in MBP kinase activity was observed 2 min after EGF stimulation that reached maximum levels within 5 min (4.2-fold increase) and progressively declined nearly to the control cell levels by 1 h (Figure 4A, $\square$). Preincubation of T51B cells with TPA (100 ng/ml) for 24 h did not interfere with EGF's ability to stimulate MBP kinase activity, because the time course of its activation closely paralleled that of EGF alone (Figure 4A, $\blacksquare$).

EGF-induced MBP kinase activation was also dose-dependent. Treatment of T51B cells with increasing concentrations (0.5–25 ng/ml) of EGF for 5 min resulted in a marked increase in MBP kinase activity that appeared to plateau at 10 ng/ml (Figure 4B, $\square$). A concentration of 0.2 ng/ml was required to reach half maximal stimulation of MBP kinase activity (Figure 4B). A very similar dose-response curve was observed for cells chronically treated with TPA before stimulation by EGF (Figure 4B, $\blacksquare$).

These time- and dose-dependent changes in MBP kinase activity were accompanied by the appearance of slower migrating species of p42 and p44 isoforms of MAP kinase that were also phosphorylated on tyrosine (M. Kanemitsu and A. Lau, unpublished data).

The cytosolic fraction of T51B cells was assayed in situ to confirm that the MBP kinase activity was actually because of MAP kinase. Cytosolic fractions were re-

Figure 3. Tryptic phosphopeptide maps of ^{32}P -labeled Cx43. Cx43 from ^{32}P -labeled T51B cells either untreated (A), EGF-treated for 30 min (B), TPA-treated for 24 h (C), and EGF-treated for 30 min after chronic TPA treatment (D) were immunoprecipitated with Cx43 CT368 peptide antiserum, resolved by SDS-PAGE, and autoradiographed. Cx43 was eluted from unfixed, unstained wet gels, digested with TPCK-trypsin, and analyzed by electrophoresis in the first dimension (pH 1.9 buffer) and by ascending chromatography (isobutyric acid buffer) in the second dimension as described in MATERIALS AND METHODS. The closed arrows indicate the positions of three phosphopeptides possibly containing TPA-sensitive PKC phosphorylation sites. The origin is indicated by an arrow.

solved by SDS-PAGE in MBP-containing polyacrylamide gels, and the kinase reaction was conducted directly in the gel containing the renatured proteins. We found that EGF transiently stimulated 44-kDa, and to a lesser extent, 42-kDa MBP kinase activities that reached maximum levels within 5 min of EGF treatment (Figure 4C). This activity progressively decreased nearly to levels observed in control cells by 60 min (Figure 4C). No other protein kinases that phosphorylated MBP were detectable throughout the gel. Control experiments conducted using a gel polymerized in the absence of MBP substrate, indicated that the observed MBP kinase activities were not because of autophosphorylation. These combined results strongly suggested that the cytosolic fractions of EGF-treated T51B cells contain p42 and p44 MBP kinases that electrophoretically and kinetically behave as activated MAP kinases. Furthermore, EGF-activation of MAP kinase persisted in T51B cells chronically treated with TPA and correlated with the phosphorylation of Cx43 and disruption of gjc.

MAP Kinase Phosphorylates Cx43 In Vitro

To determine if Cx43 is a direct substrate of MAP kinase, partially purified Cx43 in particulate fractions of Cx43 recombinant baculovirus-infected Sf9 cells was subjected to phosphorylation by purified MAP kinase in

vitro (a gift of Dr. M. Weber, University of Virginia, Charlottesville). We discovered that phosphorylation of Cx43 in the presence of MAP kinase was 5.1-fold (± 1.1 SEM) above that observed in the absence of MAP kinase (Figure 5). In vitro phosphorylated Cx43 contained only phosphoserine as determined by phosphoamino acid analysis (Figure 5, inset). These results are similar to the serine phosphorylation of Cx43 observed in T51B cells in response to EGF treatment in vivo (Lau *et al.*, 1992). The slight phosphorylation of Cx43 observed in the absence of MAP kinase was possibly because of endogenous insect cell kinases that survive the heat inactivation process designed to reduce endogenous kinase activity present in Sf9 cell membrane fractions. Nonetheless, in the presence of MAP kinase, Cx43 was markedly phosphorylated on serine in vitro suggesting that Cx43 may be an in vivo substrate of MAP kinase.

Common Phosphopeptides Exist in Cx43 Phosphorylated In Vivo and by MAP Kinase In Vitro

Two-dimensional tryptic phosphopeptide mapping of Cx43 phosphorylated by MAP kinase in vitro revealed that several unique sites were phosphorylated directly by MAP kinase (Figure 6A, phosphopeptides a–d). One phosphotryptic peptide appeared to represent the major

Figure 4. Time- and dose-dependence of EGF-induced MBP kinase activation. T51B cells were treated with EGF for the indicated times (A) and various concentrations for 5 min (B) after preincubation with PBS (□) or TPA (100 ng/ml, ■) for 24 h. Cytosolic fractions (10 μ g) were assayed for MBP kinase activity in vitro as described in MATERIALS AND METHODS. The samples were resolved by SDS-PAGE and autoradiographed. Phosphorylated MBP was excised from the gel, rehydrated, and quantitated by scintillation counting. These data represent the averages ($\pm$ SEM) of 2 individual experiments. (C) Cytosolic fractions (20 μ g) of T51B cells were resolved by SDS-PAGE in MBP-containing gels followed by denaturation with 6 M guanidine HCl and renaturation, and the kinase assay was performed in situ as described in MATERIALS AND METHODS. The arrows indicate p42 and p44 isoforms of MAP kinase.

site of in vitro phosphorylation (Figure 6A, phosphopeptide b). These in vitro-produced phosphopeptides appeared to migrate very similarly to phosphopeptides produced from Cx43 labeled in vivo after EGF-treatment (compare Figure 6A with 6B). Comigration experiments confirmed that the in vitro-produced phosphotryptic peptides did indeed represent a unique subset of the in vivo-labeled ones (compare Figure 6C with 6A and 6B).

Furthermore, phosphopeptide mapping of Cx43 digested with chymotrypsin verified that purified MAP kinase phosphorylated Cx43 in vitro at sites that also appeared to be phosphorylated in vivo in response to EGF stimulation (Figure 6, D-F, phosphopeptides e-h). These combined peptide mapping results suggested that MAP kinase may phosphorylate Cx43 directly in vivo.

DISCUSSION

In previous work, we demonstrated that EGF induced a rapid and transient disruption of gjc in T51B rat liver epithelial cells (Lau *et al.*, 1992). This effect was associated with the serine phosphorylation of Cx43. Although the cascade of events linking growth factor receptor activation to the disruption of gjc and Cx43 phosphorylation is still largely unknown, several of the previously reported growth factor-induced signaling cascades may mediate EGF's effects in T51B cells.

Previous studies have characterized TPA- and OAG-induced disruption of gjc (Enomoto and Yamasaki, 1985; Gainer and Murray, 1985; Dotto *et al.*, 1989; Somogyi *et al.*, 1989; Katoh *et al.*, 1990). In addition, others have demonstrated TPA-induced phosphorylation of Cx26,

Figure 5. Phosphorylation of Cx43 by MAP kinase in vitro. Partially purified Cx43 from particulate fractions (70 μ g) of Cx43 recombinant baculovirus-infected Sf9 cells were incubated in the absence (–) or presence (+) of purified MAP kinase (18 μ g) as described in MATERIALS AND METHODS. Cx43 was immunoprecipitated with the Cx43 CT368 peptide antiserum, resolved by SDS-PAGE, and autoradiographed using Kodak X-OMAT XAR-5 film for 1–2 d at -20°C . Phosphorylated Cx43 was excised from the gel, rehydrated, and quantitated by scintillation counting. Increases in Cx43 phosphorylation in the presence of MAP kinase were determined relative to the average control value (absence of MAP kinase) of 627–749 cpm. These data represent the averages ($\pm$ SEM) of two individual experiments. Phosphoamino acid analysis (inset) of Cx43 phosphorylated by purified MAP kinase in vitro was conducted as described in MATERIALS AND METHODS. The positions of the unlabeled phosphoserine (P-S), phosphothreonine (P-T), and phosphotyrosine (P-Y) standards are outlined.

Figure 6. Tryptic and chymotryptic phosphopeptide maps of Cx43 metabolically labeled in vivo and phosphorylated by purified MAP kinase in vitro. Cx43 from particulate fractions of Cx43 recombinant baculovirus-infected Sf9 cells and phosphorylated by purified MAP kinase in vitro (A and D) and ^{32}P -labeled T51B cells treated with EGF for 30 min after chronic TPA treatment (B and E) were immunoprecipitated with Cx43 CT368 peptide antiserum and resolved by SDS-PAGE as described in MATERIALS AND METHODS. Two-dimensional tryptic and chymotryptic phosphopeptide analysis of Cx43 was performed as described in Figure 3 and MATERIALS AND METHODS. C and F represent the results from mixing the in vitro and in vivo radiolabeled phosphopeptides. Comigrating phosphopeptides representing possible MAP kinase phosphorylation sites are designated a–d (trypsin) and e–h (chymotrypsin). The origin is indicated by an arrow.

Cx43 and MP26, a lens gap junction protein in vivo (Lampe and Johnson, 1989; Brissette *et al.*, 1991; Oh *et al.*, 1991; Berthoud *et al.*, 1992), or direct phosphorylation of Cx32 by purified PKC in vitro (Takeda *et al.*, 1987; Saez *et al.*, 1990). Furthermore, EGF has been demonstrated to stimulate the phospholipase C/phosphatidylinositol pathway ultimately leading to the activation of PKC (Berridge, 1987; Nishizuka, 1988). Although these earlier studies suggest that EGF may stimulate PKC activity in T51B cells, the current study demonstrates that EGF stimulates the disruption of gjc and serine phosphorylation of Cx43 in a manner that is independent of TPA-sensitive PKC. Although there are PKC isoforms insensitive to down-regulation, such as PKC-zeta (Olivier and Parker, 1992; Ways *et al.*, 1992), chronic TPA treatment of T51B cells clearly diminished TPA-sensitive PKC activity (Figures 1 and 2). However, it did not affect EGF's ability to phosphorylate Cx43 or disrupt gjc (Figure 1). Whether EGF treatment of T51B cells stimulates the activation of TPA-insensitive PKC isoforms that mediate EGF's effects is not yet known.

Most notably, two-dimensional tryptic phosphopeptide mapping demonstrated that the marked phosphorylation of Cx43 on numerous sites in response to EGF was (with three exceptions) largely unaffected by chronic TPA treatment (Figure 3). The three missing Cx43 phosphopeptides may possibly contain TPA-sensitive PKC phosphorylation sites that do not appear to be necessary for EGF's effects on Cx43. As mentioned earlier, computer-assisted sequence analysis of Cx43 identified several protein kinase phosphorylation consensus sites including two MAP kinase sites (Pro-Leu-Ser-Pro) located at residues 252–255 and 276–279 (Alvarez *et al.*, 1991). In addition, sites for PKC, p34^{cdc2} , casein kinase I, and glycogen synthase kinase-3 were also observed (Kennelly and Krebs, 1991). Our data suggest that one or more of these other serine kinases may mediate EGF's effects on gjc and Cx43 phosphorylation.

Ahn *et al.* (1990a,b, 1991) recently identified a protein kinase cascade stimulated by EGF leading to the activation of MAP kinase. In addition, Northwood and Davis (1990) demonstrated EGF's ability to activate

MAP kinase independent of PKC. These data, taken together with the presence of two MAP kinase sites in Cx43, suggested that EGF-induced disruption of gjc and Cx43 phosphorylation may be mediated by MAP kinase.

Accordingly, we found that T51B cells contained p42 and p44 isoforms of MAP kinase as determined by immunoblot analysis using rabbit antiserum against MAP kinase (M. Kanemitsu and A. Lau, unpublished data). Moreover, EGF treatment of T51B cells resulted in the tyrosine phosphorylation (M. Kanemitsu and A. Lau, unpublished data) and activation of p42 and p44 isoforms of MAP kinase in a time- and dose-dependent manner (Figure 4). These results are consistent with previous reports describing MAP kinase as a mitogen-activated serine/threonine kinase requiring tyrosine and threonine phosphorylation for activity (Ray and Sturgill, 1988a,b; Rossomando *et al.*, 1989; Anderson *et al.*, 1990; Ferrel and Martin, 1990; Boulton *et al.*, 1991; Payne *et al.*, 1991). In addition, the kinetics of EGF-induced activation of MAP kinase in T51B cells was similar to previous studies demonstrating EGF-induced MAP kinase activation in human foreskin fibroblasts, human epithelial carcinoma A431 cells, and rat embryonic fibroblastic 3Y1 cells (Gotoh *et al.*, 1990; Chao *et al.*, 1992). EGF-induced stimulation of MAP kinase activity occurred before the disruption of gjc and Cx43 phosphorylation that suggested that MAP kinase may mediate those responses.

This hypothesis was further substantiated when we demonstrated the ability of purified MAP kinase to phosphorylate Cx43 on serine residues in vitro (Figure 5). Moreover, tryptic and chymotryptic phosphopeptide mapping demonstrated that Cx43 was phosphorylated by MAP kinase in vitro at several sites that represented a subset of the sites phosphorylated in vivo in response to EGF (Figure 6). Trypsin and chymotrypsin cleavage sites exist between the two MAP kinase consensus sites located at residues 252–255 and 276–279. It is possible that the major phosphopeptides may contain these distinct phosphorylation sites. However, the possibilities that they may be the result of partial digestion or that the minor phosphopeptides were produced by the endogenous Sf9 insect cell kinase activity described earlier have not yet been ruled out. Nevertheless, these results strongly suggest that Cx43 may be an in vivo substrate of MAP kinase.

Although previous studies have associated EGF receptor activation to the activation of MAP kinase (Ahn *et al.*, 1990a,b, 1991), the possible intermediate events involved have only recently become clearer. It has become evident that EGF-stimulated signaling involves, in part, multiple protein-protein interactions and kinase cascades downstream of the activated receptor tyrosine kinase (Hernandez-Sotomayor and Carpenter, 1992; Margolis, 1992). Upon ligand-induced receptor activation, SH2-containing Shc proteins associate with the receptor and become tyrosine phosphorylated (Pelicci

et al., 1992). Grb-2, a 23-kDa SH2-containing protein also forms a complex with Shc and the receptor (Rozakis-Adcock *et al.*, 1992). These protein interactions have been implicated in the activation of Ras in *Caenorhabditis elegans*, *Drosophila*, and mammalian HER 14 cells (Clark *et al.*, 1992; Lowenstein *et al.*, 1992; Olivier *et al.*, 1993). Ras activation has been demonstrated to ultimately lead to MAP kinase activation (de Vries-Smits *et al.*, 1992; Hattori *et al.*, 1992; Pomerance *et al.*, 1992; Wood *et al.*, 1992) mediated by a complex protein kinase cascade involving Raf-1 (Dent *et al.*, 1992; Howe *et al.*, 1992; Kyriakis *et al.*, 1992; Troppmair *et al.*, 1992; Wood *et al.*, 1992), possibly a MAP kinase kinase (Gomez *et al.*, 1992), and MAP kinase kinase (Adams and Parker, 1992; Haystead *et al.*, 1992; Matsuda *et al.*, 1992; Nakiely *et al.*, 1992; Wu *et al.*, 1992). Thus, this signaling pathway may not only mediate the reduced junctional permeability observed in EGF-treated cells, but also cells transformed by the *ras* oncogene (Dotto *et al.*, 1989; El-Fouly *et al.*, 1989; Brissette *et al.*, 1991).

A number of proteins have been identified as substrates of MAP kinase including MBP (Erickson *et al.*, 1990), MAP-2 (Ray and Sturgill, 1987), ribosomal S6 kinase (Sturgill *et al.*, 1988), c-jun (Alvarez *et al.*, 1991; Pulverer *et al.*, 1991), transcription factor p62^{TCF} (Gille *et al.*, 1992), c-myc (Alvarez *et al.*, 1991) and the EGF receptor (Countaway *et al.*, 1989; Northwood *et al.*, 1991; Takishima *et al.*, 1991). This study suggests that Cx43 may also be a substrate of MAP kinase.

Although these data demonstrate that MAP kinase may mediate EGF's ability to stimulate the disruption of gjc and Cx43 phosphorylation, further characterization of the phosphorylation sites will be required. Examination of phosphorylation mutants and other genetic manipulations will enable us to establish the functional significance of these EGF-induced events. Moreover, EGF's ability to modulate gjc and Cx43 phosphorylation in T51B cells provides an excellent system for facilitating further characterization of EGF-stimulated protein kinase signaling pathways involved in regulating critical cellular functions.

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