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Regulation of Connexin43 Function by Activated Tyrosine Protein Kinases

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Gap junctions are specialized membrane structures that are involved in the normal functioning of numerous mammalian tissues and implicated in several human disease processes. This mini-review focuses on the regulation of gap junctions through phosphorylation of connexin43 induced by the v-Src or epidermal growth factor receptor tyrosine kinases. These tyrosine kinases markedly disrupt gap junctional communication in mammalian cells. Here, we describe work correlating the alteration of connexin43 function with the ability of the v-Src tyrosine kinase to phosphorylate connexin43 directly on two distinct tyrosine sites in mammalian cells (Y247 and Y265). We also present evidence that proline-rich regions and phosphotyrosine sites of connexin43 may mediate interactions with the SH3 and SH2 domains of v-Src. In contrast to v-Src, the activated epidermal growth factor receptor acts indirectly through activated MAP kinase which may stimulate phosphorylation of connexin43 exclusively on serine. This phosphorylation event is complex because MAP kinase phosphorylates three serine sites in connexin43 (S255, S279, and S282). These findings suggest novel interactions between connexin43, the v-Src tyrosine kinase, and activated MAP kinase that set the stage for future investigations into the regulation of gap junctions by protein phosphorylation.

KEY WORDS: Connexin43; gap junctional communication; v-Src tyrosine kinase; EGF receptor; signal transduction; MAP kinase; protein kinase C; phosphorylation; phosphotyrosine sites; phosphoserine sites.

INTRODUCTION

Gap junctions are plasma membrane channels that allow the exchange of ions, metabolites, and other small molecules below ~1 kDa between the cytoplasms of adjacent cells (Beyer, 1993; Wolburg and Rohlmann, 1995). They exist in virtually every tissue of multicellular animals and they are involved in numerous cellular functions, such as the conduction of ion currents in electrically excitable tissues (such as the myocardium, uterus, and nerves) and the passage of metabolites and nutrients in the lens and ovarian follicles. Gap junctions have also been implicated in the regulation of embryonic development, cellular differentiation, and growth control.

Connexins represent a gene family that currently comprises about a dozen distinct members (Beyer, 1993; Wolburg and Rohlmann, 1995). Connexins are transmembrane proteins that possess four hydrophobic, membrane spanning domains, two extracellular regions, and N-terminal, internal loop, and C-terminal regions that are located in the cytoplasm (see Fig. 1). Each connexon or hemichannel in the plasma membrane of a cell consists of a hexamer of connexin molecules. The assembly of individual connexons in one cell with the juxtaposed connexons of an adjacent cell forms the aqueous-filled, intercellular gap junction channels.

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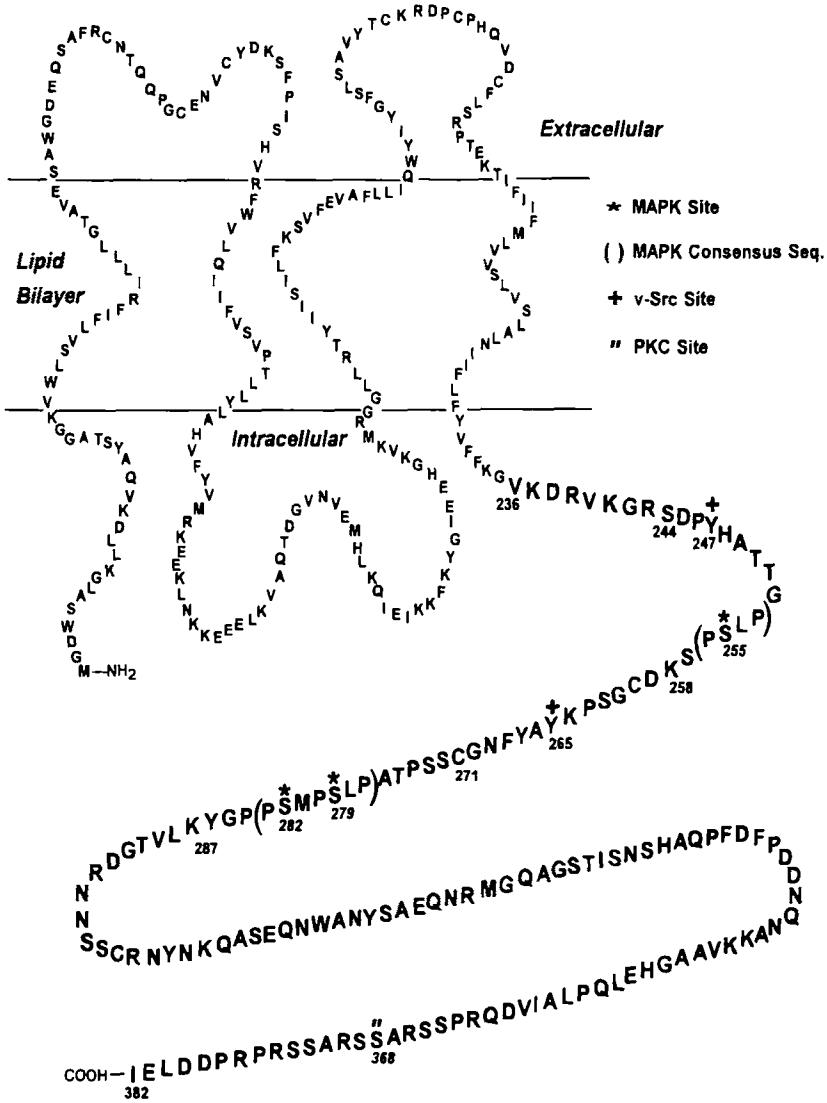

Fig. 1. v-Src, MAP Kinase, and PKC Phosphorylation Sites in Connexin43. A schematic drawing of the primary structure of rat Cx43 as it is thought to be oriented in the plasma membrane. The folding of the C-terminal portion was drawn arbitrarily. The v-Src (Y247, Y265), MAP kinase (S255, S279, and S282), and the PKC (S368) sites of phosphorylation are indicated. The C-terminal, cytoplasmic portion of Cx43 that was expressed as a GST fusion protein extends from V236 to I382 and is printed in the figure in larger font size. The phosphotryptic peptide containing a v-Src and MAP kinase phosphorylation site extends from S244 to K258. The proline-enriched phosphotryptic peptide containing two MAP kinase sites and a v-Src tyrosine phosphorylation site extends from Y265 to K287. The MAP kinase consensus phosphorylation sequences are bracketed.

The regulation of gap junctional communication can occur potentially at multiple levels: transcription of the connexin gene, translation of connexin transcripts, assembly of connexins into connexons and transport to the plasma membrane, assembly of connexons into gap junction plaques, gating of the functional channels (mediated by Ca^{2+}, pH, lipophilic agents, transjunctional voltage), disassembly of the gap junctions, and degradation of the connexin proteins (reviewed in Saez *et al.* , 1993; Spray, 1994; Musil, 1994; Lampe, 1994).

Phosphorylation of connexin is another putative regulatory mechanism that may operate at several levels such as in the assembly of connexons into gap junctions in the plasma membrane and in the gating

of the formed gap junction. This mini-review focuses on the regulation of gap junctional communication by the phosphorylation of connexin43 (Cx43) induced by the activated $pp60^{v\text{-}src}$ (v-Src) tyrosine protein kinase and activated epidermal growth factor (EGF) receptor. It highlights the direct effects of v-Src on Cx43 and the indirect effects of the activated EGF receptor on Cx43 that may be mediated by the MAP kinase signal transduction pathway. The rapid nature of tyrosine kinase action on gap junctional communication suggests that the regulation of gap junctions by these tyrosine kinases may reside at the level of channel gating.

CONNEXIN43 PHOSPHORYLATION AND THE v-SRC TYROSINE PROTEIN KINASE

v-Src Tyrosine Protein Kinase

The v-Src tyrosine protein kinase is the prototypical member of the src family of cytoplasmic tyrosine kinases. It was one of the first retroviral oncogenes to be discovered and characterized as to its structure and function (reviewed in Parsons and Weber, 1989; Kefalas *et al.*, 1995). Several functional domains of v-Src are relevant to its interaction with Cx43 (see Fig. 2). The N-terminal 15 amino acids of v-Src contain signals for myristylation and palmitoylation that are essential for its localization to the inner surface of the plasma membrane. The N-terminal half of v-Src also contains a 60 amino acid SH3 (*Src homology* 3) domain that promotes protein–protein binding with proline-rich regions of interacting substrate proteins (Alexandropoulos *et al.*, 1995). Adjacent to the SH3 domain is the 100 amino acid SH2 domain which specifically binds phosphotyrosine (Marengere and Pawson, 1994). These SH2 and SH3 domains are critical regions involved in regulating the kinase activity of v-Src and mediating its downstream signal transduction events. The C-terminal portion of v-Src contains the catalytic kinase domain responsible for the phosphorylation of substrate proteins on tyrosine. Neoplastic transformation of mammalian and avian cells by v-Src is strictly dependent upon its tyrosine kinase activity and its localization to the plasma membrane where critical substrates are located (Kamps *et al.*, 1985; Parsons and Weber, 1989). The presence of phosphotyrosine in proteins from v-Src-transformed cells has served as a convenient marker for tyrosine kinase substrates that may be involved in neoplastic transformation.

v-Src Induces Phosphorylation of Cx43 on Tyrosine

Early reports demonstrated that v-Src rapidly and profoundly disrupted gap junctional communication in fibroblasts (Atkinson *et al.*, 1981; Azarnia and Loewenstein, 1984; Chang *et al.*, 1985). Overexpression of activated forms of the cellular Src protein in NIH 3T3 cells also diminished gap junctional communication (Azarnia *et al.*, 1988). These alterations in gap junction function were accompanied by the rapid accumulation of phosphotyrosine on Cx43 without a major loss of antibody-reactive Cx43 gap junction plaques (Crow *et al.*, 1990, 1992; Filson *et al.*, 1990). Furthermore, the loss of gap junctional communication was not due to decreased expression of Cx43 because the levels of Cx43 mRNA and protein were actually

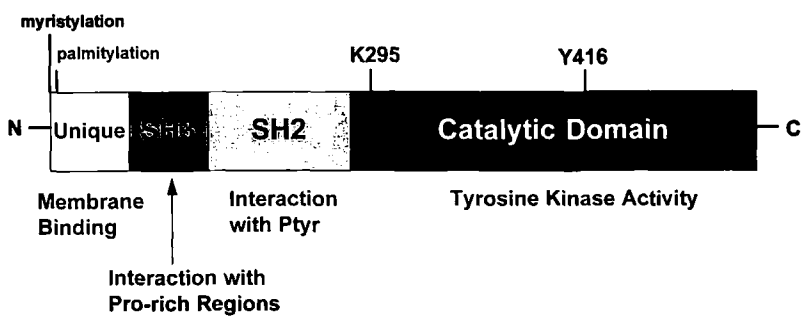

Fig. 2. Structure of the v-Src Oncogene Protein. The various domains of the v-Src protein are discussed in the text. The unique region differs between different members of the src family of tyrosine protein kinases. The ATP binding site of the catalytic domain is located at K295. Y416 is an autophosphorylation site and possibly modulates the kinase activity of v-Src.

elevated in v-Src-transformed cells (Stagg and Fletcher, 1990; Goldberg and Lau, 1993). The inhibition of gap junctional communication in *Xenopus* oocytes, co-injected with v-Src and Cx43 mRNA, was shown to be dependent upon the phosphorylation of Cx43 on tyrosine 265 (Swenson *et al.*, 1990). Another member of the Src family of tyrosine kinases, v-Fps, also effectively disrupted gap junctional communication and stimulated the phosphorylation of Cx43 on tyrosine (Kurata and Lau, 1994). These studies suggested that v-Src directly phosphorylated Cx43 on tyrosine and that the disruption of gap junctional communication may have resulted from effects on gating of the intercellular channels rather than on the diminished expression of Cx43, alterations in its localization to plasma membranes, or the disruption of gap junction plaques.

Cx43 Is Directly Phosphorylated by v-Src

Because v-Src can associate with and possibly activate tyrosine kinases, such as $p125^{Fak}$ in mammalian cells (Guan and Shalloway, 1992; Cobb *et al.*, 1994), it was conceivable that the tyrosine phosphorylation of Cx43 resulted not from v-Src itself, but instead from the action of an intermediate tyrosine kinase activated by v-Src. We were interested in determining if v-Src acted directly to phosphorylate Cx43 in fibroblasts and in elucidating the mechanism by which this action might occur. Full-length Cx43 and activated Src kinase were expressed in recombinant baculovirus-infected *S. frugiperda* (Sf-9) insect cells and isolated by immunoaffinity chromatography (Loo *et al.*, 1995). In addition, the C-terminal, cytoplasmically-localized portion of Cx43, corresponding to V236-I382 (see Fig. 1), was engineered as a glutathione-S-transferase fusion protein (GST-Cx43CT) for expression in bacteria. These purified proteins were used in phosphorylation reactions to examine the ability of activated Src kinase to phosphorylate Cx43 directly *in vitro*.

These studies demonstrated that full-length Cx43 and the GST-Cx43CT fusion protein both served as excellent substrates of purified, activated Src kinase (Loo *et al.*, 1995). Most importantly, the phosphotryptic peptides resulting from the *in vitro* phosphorylations co-migrated with *in vivo* phosphopeptides from Cx43 immunoprecipitated from v-Src transformed cells. This suggested that v-Src phosphorylated Cx43 directly in intact cells. Further evidence of this direct action was provided by the demonstration that Sf-9 cells co-infected with Src and Cx43 recombinant baculoviruses contained Cx43 phosphorylated on tyrosine, in contrast to control Sf-9 cells infected only with the Cx43 baculovirus (Loo *et al.*, 1995).

Cx43 Interacts with SH2 and SH3 Domains of v-Src

These phosphorylation results were extended by examining whether Cx43 and v-Src were localized to similar regions in the plasma membrane by laser confocal microscopy. v-Src-transformed Rat-1 fibroblasts expressed high levels of v-Src which was located uniformly throughout the plasma membrane (L. Loo, M. Kanemitsu, and A. Lau, manuscript in preparation). As previously demonstrated, Cx43 was visualized in the plasma membrane as punctate reactions, probably corresponding to gap junction plaques (Crow *et al.*, 1990; Kurata and Lau, 1994). Horizontal, optical sections through the cell showed that a portion of v-Src was located with Cx43 in identical membrane regions (L. Loo, M. Kanemitsu, and A. Lau, manuscript in preparation). These results are in agreement with an earlier electron microscopic study demonstrating the co-localization of v-Src to regions of the plasma membrane enriched for gap junctions (Willingham *et al.*, 1979).

The ability of v-Src to phosphorylate Cx43 directly and the localization of both proteins to similar regions of the plasma membrane suggested that they associated with each other physically. This hypothesis was confirmed by the detection of Cx43 in co-immunoprecipitates prepared from v-Src-transformed mammalian cell lysates using Src antibodies (L. Loo, M. Kanemitsu, and A. Lau, manuscript in preparation). The reciprocal experiment using Cx43 antibody co-immunoprecipitates and immunoblotting with v-Src antibody was also successful (M. Kanemitsu, unpublished observations). The apparent association of Cx43 with v-Src in these co-immunoprecipitates was substantiated further by demonstrating the generation of phosphotyrosine-containing Cx43 when these immune complexes were used in *in vitro* kinase reactions (L. Loo, M. Kanemitsu, and A. Lau, manuscript in preparation).

What domains of Cx43 and v-Src are responsible for the apparent protein–protein interaction? We focused initially on the SH3 and SH2 domains of v-Src which interact with proline-rich regions and phosphotyrosine (see Fig. 2), respectively, because they are important in

regulating v-Src kinase activity and binding to other proteins (Superti-Furga and Courtneidge, 1995). A GST fusion protein containing the v-Src SH3 domain specifically associated with full-length Cx43 in lysates of recombinant baculovirus-infected insect cells or v-Src-transformed Rat-1 fibroblasts (M. Kanemitsu, L. Loo, A. Lau, and W. Eckhart, manuscript in preparation). The v-Src SH3 domain also specifically interacted with the C-terminal portion of Cx43 which further localized the relevant interacting region in Cx43. Binding of the v-Src SH3 domain to Cx43 was blocked by the addition of a synthetic Cx43 proline-enriched peptide, corresponding to C271-K287 of Cx43 (Fig. 1), suggesting that this region of Cx43 may be involved in the observed binding reaction. Importantly, this interaction may occur in intact cells because deletion of this proline-rich region disrupted the ability of Cx43 to co-immunoprecipitate with v-Src from cells co-transfected with the v-Src and mutant Cx43 genes (M. Kanemitsu, L. Loo, A. Lau, and W. Eckhart, manuscript in preparation). Similar experiments using the GST v-Src SH2 domain demonstrated that this Ptyr-binding domain could also bind specifically to Cx43 from v-Src-transformed cells, but not from non-transformed cells lacking v-Src (M. Kanemitsu, L. Loo, A. Lau, and W. Eckhart, manuscript in preparation). Binding of the v-Src SH2 domain occurred only with Cx43 that was phosphorylated on tyrosine.

Results from these *in vitro* experiments suggest that the SH3 and SH2 domains of v-Src may be important in binding to proline-containing regions and phosphotyrosine of Cx43 *in vivo*. Our current model for this potential association postulates that the SH3 domain of v-Src initially interacts with the proline-containing region of Cx43 which is followed by the phosphorylation of Cx43 on tyrosine by v-Src. Subsequently, this protein–protein interaction may be strengthened by the interaction between the SH2 domain of v-Src and the newly created phosphotyrosine site(s) on Cx43. The association of these proteins and Cx43 tyrosine phosphorylation may then effect the observed changes in channel function. Experiments utilizing domain mutants of v-Src and Cx43 are currently underway to test the validity of this model in intact cells.

Cx43 Phosphotyrosine Sites

Availability of sufficient amounts of purified Cx43 for use in *in vitro* phosphorylation reactions with enzymatically-active v-Src has permitted the study of the tyrosine sites in Cx43 that are phosphorylated by v-Src in neoplastically-transformed fibroblast cells. Swenson *et al.* (1990) previously demonstrated the importance of Y265 phosphorylation for the ability of v-Src to disrupt Cx43 function in *Xenopus* oocytes. However, we have detected more than one phosphotyrosine-containing tryptic peptide in Cx43 isolated from v-Src-transformed fibroblasts (Kurata and Lau, 1994). Our results indicated that the minor of two phosphotyrosine-containing Cx43 peptides may contain Y265 (Fig. 1). Phosphorylation of a Cx43 deletion mutant indicated that the major, more highly phosphorylated site mapped to a phosphotryptic peptide (S244-K258 in Fig. 1) containing Y247 (W. Kurata and A. Lau, unpublished observations). Site-directed mutagenesis is underway to examine the relative contributions of these phosphotyrosine sites to the ability of v-Src to disrupt Cx43-mediated gap junctional communication.

Phosphorylation of Cx43 on Serine

The results presented above demonstrated that v-Src and Cx43 associate with each other and that Cx43 is phosphorylated directly by v-Src on tyrosine. However, they do not exclude the possibility that Cx43 in v-Src-transformed cells may also be phosphorylated on serine. We, and others, have observed reproducible increases in the phosphoserine content of specific phosphopeptides of Cx43 from v-Src cells, suggesting that Cx43 may be a target of serine kinases activated by v-Src as downstream components of a signal transduction pathway (Kurata and Lau, 1994; Filson *et al.*, 1990).

The serine kinase(s) that may be responsible for these phosphorylation events is currently unknown; however, there are several potential candidates. First, protein kinase C α has been reported recently to be activated specifically in v-Src-transformed cells (Zang *et al.*, 1995). In addition, numerous reports have indicated that activation of PKC by phorbol ester tumor promoters (TPA) generally results in a marked reduction in gap junctional communication accompanied by phosphorylation of Cx43 on serine (Saez *et al.*, 1993). Our recent identification of S368 as a major Cx43 serine site that is phosphorylated by PKC *in vitro* and *in vivo* in cells stimulated with TPA will allow us to test this hypothesis (P. Lampe, W. Kurata, M. Bazzi, R. Johnson, and A. Lau, manuscript in preparation; see Fig. 1).

Second, the proline-directed MAP kinase may also phosphorylate Cx43 in v-Src-transformed cells. MAP kinase has been reported to be constitutively-

activated in v-Src-transformed Rat-1a fibroblasts (Gupta *et al.*, 1992). This is probably mediated by binding of the SH2-containing Shc adapter protein to v-Src (Rozakis-Adcock *et al.*, 1992) and the subsequent activation of the MAP kinase signal transduction pathway (Fig. 3). As described below, activation of MAP kinase by EGF is correlated with a rapid and marked disruption of gap junctional communication. In support of this possibility, it has long been recognized that v-Src requires the downstream action of the $p21^{ras}$ protein in order to transform cells (Smith *et al.*, 1986). The Ras oncoprotein also disrupts gap junctional communication and stimulates the serine phosphorylation of Cx43 (Bignami *et al.*, 1988; Brissette *et al.*, 1991). Because Ras is not a serine kinase, these effects may be the consequence of the participation of Ras in the signal transduction pathway leading to activation of MAP kinase. Knowledge of the respective phosphorylated tyrosine and serine sites in Cx43 and the availability of site-directed mutants will permit the elucidation of the role these phosphorylated sites may play in the ability of v-Src to disrupt gap junctional communication and induce neoplastic cellular transformation.

CONNEXIN43 PHOSPHORYLATION AND THE ACTIVATED EGF RECEPTOR

EGF Receptor and the MAP Kinase Signal Transduction Pathway

A remarkable signal transduction pathway that is responsible for the propagation of an extracellular signal throughout the interior of the cell has been the focus of recent, intense research efforts. This MAP kinase signal transduction pathway contrasts with other pathways that generate a second messenger, such as cyclic AMP. It is initiated by ligand activation of a growth factor receptor and proceeds through a defined sequence of protein–protein interactions that are coupled to a protein kinase cascade (reviewed recently in Seger and Krebs, 1995; Malarkey *et al.*, 1995). Because this pathway is critically involved in the regulation of mammalian cell growth and differentiation, its alteration by oncogene proteins results in the modified regulation of cellular proliferation and neoplastic cellular transformation. This signaling pathway is directly relevant to the gap junction field because it may be involved in the regulation of Cx43 function by growth factors and by numerous oncogene products, such as src, fps, ras, and neu (Jou *et al.*, 1995).

This novel signal transduction pathway and how it might relate to EGF-induced phosphorylation of Cx43 (Seger and Krebs, 1995; Malarkey *et al.*, 1995) is illustrated in Fig. 3. Briefly, the binding of EGF to its receptor results in receptor dimerization, followed by activation of the receptor's tyrosine kinase activity, and the intermolecular phosphorylation of specific tyrosine sites in the cytoplasmic domain of the EGF receptor where they create binding sites for various adapter proteins and enzymes. Grb2, an adapter protein, binds to a phosphotyrosine site of the activated EGF receptor via its SH2 domain. Grb2 also complexes with the mSos guanine nucleotide exchange factor through

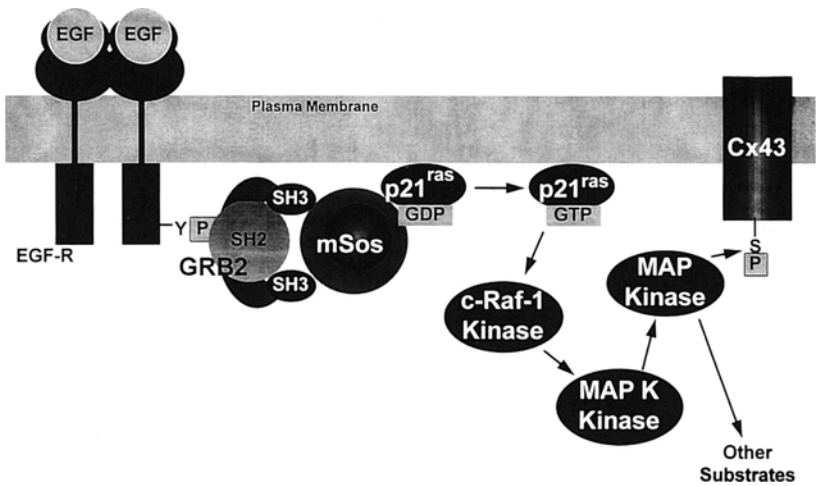

Fig. 3. Interaction of the MAP Kinase Signal Transduction Pathway with Connexin43. The MAP kinase signal transduction pathway as it is thought to be activated by the EGF receptor leading to a series of protein–protein interactions, protein activations, and protein kinase cascades that lead to Cx43 phosphorylation (see text for discussion).

interaction of the SH3 domains of Grb2 with the proline-rich regions of the mSos protein. This interaction facilitates the localization of mSos to the plasma membrane where it can stimulate the exchange of GDP for GTP on $p21^{ras}$ resulting in activation of Ras. Activated Ras-GTP binds to and recruits the Raf serine protein kinase to the plasma membrane, leading to Raf activation. Activated Raf phosphorylates and activates the dual-specificity MAP kinase kinase (also known as MEK), which in turn activates MAP kinase by phosphorylation on tyrosine and threonine residues. Numerous substrate proteins located in the nucleus, cytosol, cytoskeleton, and plasma membrane serve as targets of activated MAP kinase (Seger and Krebs, 1995). As we describe in the following sections, activated MAP kinase may also target Cx43 located in the plasma membrane.

EGF Disrupts Gap Junctional Communication and Induces Phosphorylation of Cx43 on Serine

Initial studies in this area reported that the growth factors, PDGF and EGF, disrupted gap junctional communication. However, the mechanism(s) underlying this effect was not identified (Maldonado *et al.*, 1988; Madhukar *et al.*, 1989). Because the EGF receptor is a tyrosine protein kinase, we became interested in the possibility that it effected the disruption of gap junctional communication in a manner similar to the v-Src tyrosine kinase, through the direct phosphorylation of Cx43 on tyrosine. In agreement with previous reports, we also observed that EGF induced a very rapid, but transient decrease in gap junctional communication in T51B rat liver epithelial cells (Lau *et al.*, 1992). The loss of gap junctional communication correlated well kinetically with a marked increase in phosphorylation of Cx43 and the emergence of more slowly migrating, phosphorylated forms of Cx43. Restoration of gap junctional communication occurred concomitantly with dephosphorylation of Cx43 and re-appearance of the pattern of phosphorylated Cx43 proteins observed in the unstimulated cells. A cause and effect relationship was suggested further by our observation that the phosphatase inhibitor, okadaic acid, not only blocked the dephosphorylation of Cx43, but also blocked the restoration of gap junctional communication (Lau *et al.*, 1992). To our surprise, phosphoamino acid analysis clearly demonstrated that EGF did not stimulate tyrosine phosphorylation on Cx43, but induced only the accumulation of phosphoserine on Cx43 (Lau *et al.*, 1992).

Examination of the levels and distribution of gap junction plaques in the liver epithelial cells by immunofluorescence microscopy demonstrated discrete punctate antibody reactions in the plasma membranes both before and after EGF stimulation (Lau *et al.*, 1992). These observations indicated that the striking loss of gap junctional communication was unlikely to be the result of gross alterations in gap junction plaque organization.

Thus, these data clearly demonstrated that the EGF receptor tyrosine kinase did not act directly on Cx43 like v-Src, but in contrast, it activated a downstream serine kinase(s) that phosphorylated Cx43. They also suggested that the phosphorylation and dephosphorylation of Cx43 on serine were responsible for the loss and restoration of gap junction function (perhaps by modulating gating), respectively. These EGF-stimulated phosphorylation events occurred over the basal level of serine phosphorylation which correlates with the assembly of functional gap junction plaques (Musil and Goodenough, 1991; Musil *et al.*, 1990). It is evident that phosphorylation of Cx43 and its potential for regulating Cx43 function are complex. In addition, these data highlighted the likely significance of phosphoserine phosphatases for the dephosphorylation of Cx43 and regulation of gap junctional communication. The nature of this putative regulatory phosphatase(s) is presently unclear.

EGF-Induced Cx43 Phosphorylation Is Mediated by MAP Kinase and Not TPA-Sensitive PKC

To clarify the mechanism by which EGF induced the phosphorylation of Cx43, we initiated an investigation of the serine protein kinases that are activated by the EGF receptor as components of a downstream signal transduction cascade. We initially examined the involvement of protein kinase C (PKC) as a mediator of EGF's actions because the PKC agonist, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), produces a profound disruption of gap junctional communication and stimulation of Cx43 phosphorylation in a wide variety of cell types (Oh *et al.*, 1991; Berthoud *et al.*, 1992). In addition, EGF is known to stimulate signal transduction pathways that lead to the activation of PKC (Berridge, 1987; Nishizuka, 1988). We found that TPA treatment of our T51B epithelial cells

resulted in the marked depression of gap junctional communication and the stimulation of Cx43 phosphorylation on serine (Kanemitsu and Lau, 1993). EGF treatment of these cells also activated PKC as measured by a 6-fold increase in membrane-bound PKC activity relative to the nontreated cell controls and it occurred in a time course that was compatible with the induced phosphorylation of Cx43 (Kanemitsu and Lau, 1993). Thus, PKC appeared to be a likely candidate to mediate EGF's effects on Cx43 phosphorylation and function.

We tested the involvement of PKC in Cx43 phosphorylation by treating the T51B epithelial cells with TPA overnight (24 h), which down-regulates TPA-sensitive PKC activity. This experimental manipulation resulted in the desired biochemical effects: down-modulation of PKC activity in the presence or absence of EGF, and the complete block of Cx43 phosphorylation and disruption of gap junctional communication in response to acute TPA treatment (Kanemitsu and Lau, 1993). However, downregulation of PKC was completely ineffective at preventing EGF-induced disruption of GJC or Cx43 phosphorylation. These results clearly indicated that the TPA-sensitive forms of PKC were not involved in mediating EGF's action on Cx43 and that another serine kinase was responsible for this activity.

As described above, EGF stimulates a remarkable series of protein kinase phosphorylations that result in successive enzyme activations (Seger and Krebs, 1995; Malarkey *et al.*, 1995). Any one of these kinases might be capable of phosphorylating Cx43 and inducing the observed changes in its activity. We focused initially on MAP kinase, one of the last enzymes in this cascade, as the most likely candidate because it is central to the action of activated growth factor receptors. In addition, we identified putative MAP kinase consensus phosphorylation sequences (P-X_{1-2}-S/T-P) in the cytoplasmic, carboxy-terminal tail of Cx43 (Kanemitsu and Lau, 1993; bracketed in Fig. 1).

EGF treatment of T51B liver epithelial cells activated MAP kinase with the kinetics and dose response behavior that were consistent with MAP kinase directly phosphorylating Cx43 (Kanemitsu and Lau, 1993). Moreover, the ability of EGF to activate MAP kinase was not blocked by the pretreatment of cells with TPA that down-regulated PKC activity. Exploiting our *in vitro* phosphorylation systems, we demonstrated that purified MAP kinase efficiently phosphorylated Cx43 isolated from recombinant baculovirus-infected Sf-9 insect cells. Phosphorylation occurred exclusively on serine. Most significantly, MAP kinase phosphorylated Cx43 *in vitro* on phosphotryptic peptides that co-migrated with a subset of phosphotryptic peptides obtained from Cx43 phosphorylated *in vivo* in EGF-stimulated cells (Kanemitsu and Lau, 1993). These combined results indicated the likelihood that Cx43 was phosphorylated directly *in vivo* by MAP kinase activated as a consequence of the signal transduction process initiated by exposure of cells to EGF.

MAP Kinase Phosphorylation Sites in Cx43

To gain more insight into the importance of MAP kinase phosphorylation on Cx43 function, it was essential to identify the precise sites targeted by the enzyme. Earlier attempts to identify phosphorylation sites utilizing [^{32}P]-labeled Cx43 isolated from metabolically-labeled cells were unsuccessful due to the difficulty of preparing sufficient amounts of sample required for the various analytical procedures. For the MAP kinase site(s) identification we took a different, more expedient approach and utilized the cytoplasmic, C-terminal tail of Cx43 (V236-I382) fused to GST as a substrate for *in vitro* MAP kinase phosphorylation reactions. However, to ensure the biological relevance of our results we focused our efforts on the major *in vitro* Cx43 phosphotryptic peptides that co-migrated with phosphotryptic peptides isolated from *in vivo* phosphorylated Cx43. Our analyses were further facilitated by the use of deletion and site-directed mutants of Cx43 and Edman degradation to directly determine the position of phosphorylation in the peptides of interest (Warn-Cramer *et al.*, 1996).

By this approach we successfully identified three MAP kinase serine phosphorylation sites localized in two tryptic peptides of Cx43 (Warn-Cramer *et al.*, 1996). Direct protein sequencing results indicated that one of the phosphotryptic peptides corresponded to Y265-K287 of Cx43 which contained two overlapping MAP kinase consensus phosphorylation sequences (Fig. 1). S279 and S282 within the consensus sequences were determined to be phosphorylated by MAP kinase. In addition, a secondary phosphotryptic peptide corresponding to S244-K258 of Cx43 contained the third phosphorylated site at S255 which also represented a MAP kinase consensus phosphorylation sequence (Fig. 1). Our data did not permit us to ascertain if the three Cx43 serine sites were phosphorylated *in vitro* or *in vivo* in a hierarchical manner; however,

the availability of various serine site mutants will allow us to answer this question. The presence of three potential MAP kinase phosphorylation sites in Cx43 indicated that the regulation of Cx43 by this enzyme is likely to be complex. These novel data create the opportunity for a detailed examination of the role these phosphoserine sites may play in the regulation of Cx43 function.

FUTURE DIRECTIONS

The substantial progress that has been accomplished by many investigators in this field represents an exciting beginning of the investigation to establish phosphorylation as a biologically-relevant mechanism regulating the activity of Cx43. However, these studies also raise many important questions that await future answers. Are the phosphorylated tyrosine and serine sites both necessary and sufficient for these tyrosine kinases to regulate Cx43 function? Does phosphorylation of Cx43 at these sites affect channel gating? And if so, which channel parameters are altered: unitary conductance and/or the probability of the open state? Which protein kinases are responsible for the basal level of Cx43 phosphorylation that is correlated with gap junction channel assembly? Another area of equal importance, but which up to now has only been touched upon lightly and indirectly is: Which protein phosphatases are involved in dephosphorylating Cx43 and possibly regulating its function? Does Cx43 associate with other cellular proteins that also contain SH3 domains? And finally, in a mechanistic sense, how is the oncogene-induced disruption of gap junctional communication related to the neoplastic transformation of mammalian cells? These are only some of the exciting questions that will fascinate gap junction researchers in the coming years.

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