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Connexins as Substrates for Protein Kinases and Phosphoprotein Phosphatases

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1. Introduction

1.1 Mammalian connexins

Connexins are protein subunits expressed by cordates that form gap junction channels (GJCs) and hemichannels (HCs) (Goodenough, 1974; Makowski et al., 1977). A GJC is formed by the head-to-head docking of two HCs, each contributed by one of the two contacting cells (Meşe et al., 2007). Each HC is an oligomeric assembly of six identical (homomeric) or six different (heteromeric) Cx subunits (Sáez et al., 2005). GJCs and HCs subserve different functions; while GJCs communicate the cytoplasm of contacting cells, HCs provide a pathway for communication between the intracellular and extracellular compartments (Bruzzone and Dermietzel, 2006). Although both types of channels are permeable to ions and small molecules, GJCs and HCs composed of the same Cx subtype are likely to present differences in permeability and regulatory properties (Sáez et al., 2003; Meşe et al., 2007; Sáez et al., 2010).

The family of connexin genes has 20 members in the mouse genome and 21 members in the human genome (Eiberger et al., 2001; Willecke et al., 2002; Söhl and Willecke, 2003; 2004). Most Cx genes have a similar structure and contain the protein coding region as a single exon (Willecke et al., 2002; Söhl and Willecke, 2003; 2004; Pfenniger et al., 2011). Cxs were initially denoted according to the tissue of origin or the apparent size of a polypeptide as determined by SDS-PAGE. Shortly thereafter, it became clear that such designations were inappropriate, because many of these proteins are expressed in more than one tissue (Beyer et al., 1987) and their apparent molecular mass may vary with electrophoresis conditions (Green et al., 1988). Therefore, a standard nomenclature was developed to distinguish members of this family. The current nomenclature uses the abbreviated symbol "Cx" (for connexin) followed by a suffix that indicates the molecular mass of the Cx amino acid sequence (in kDa) predicted from its cDNA. In some cases, a prefix is added to indicate the species of origin. Hydropathicity plots of the Cx amino acid sequences have been used to

predict their membrane topology. These analyses predicted the presence of four hydrophobic domains, three hydrophilic cytoplasmic domains (the amino and carboxyl termini and an intracellular loop) and two extracellular loops (Heynkes et al., 1986; Paul, 1986; Beyer et al., 1987). This topology was supported by experiments that studied the binding of site-specific antibodies and protease sensitive sites (Zimmer et al., 1987; Hertzberg et al., 1988; Milks et al., 1988; Yancey et al., 1989; Zhang and Nicholson, 1994; Quist et al., 2000). The cytoplasmic loop and the carboxyl terminus vary extensively in length and amino acid composition and probably contain most of the regulatory sites of GJCs and HCs.

1.2 Mammalian protein kinases and phosphoprotein phosphatases

Most Cxs contain putative phosphorylation sites (Lampe and Lau, 2004). As with all phosphoproteins, their phosphorylation state will depend on the activities of protein kinases and phosphoprotein phosphatases. Mammalian cells express several different types of protein kinases and phosphoprotein phosphatases with more than 500 putative kinase genes in the human and mouse genome (Manning et al., 2002; Caenepeel et al., 2004). Protein kinases and phosphoprotein phosphatases have been subdivided according to their substrate specificities, activators, cofactors and/or amino acid sequence homology. It would be beyond the scope of this chapter to attempt to review them here and thus, we will briefly summarize the characteristics of the kinases and phosphatases that have most frequently been studied as possible effectors of the phosphorylation state of connexins.

1.3 Serine/threonine protein kinases

cAMP- and cGMP-dependent protein kinases (PKA and PKG, respectively) can be activated by increasing the concentration of the corresponding cyclic nucleotide (e.g., treatment with membrane permeable analogs of cAMP or cGMP such as 8-Bromo-cAMP and 8-Bromo-cGMP or forskolin, which activates adenylyl cyclase). The CAMKII isoenzymes are activated by binding of Ca^{2+}/calmodulin but other protein binding partners can also regulate their activity (Griffith, 2004). Casein kinase I (CK1) is a family of monomeric serine/threonine kinases that are constitutively active. This family shows a strong preference for pre-phosphorylated substrates. Several inhibitors for members of this family have been described including CKI-7 and IC261 (Perez et al., 2011). Protein kinase C (PKC) has several isoforms that have been subdivided in three subtypes: conventional, novel and atypical. They differ in their activation by Ca^{2+}, binding of diacylglycerol (DAG) and in their response to phorbol esters. Conventional PKCs bind Ca^{2+} and DAG. Novel PKCs lack amino acids involved in Ca^{2+} binding, but bind DAG. The catalytic activity of atypical PKCs is independent of Ca^{2+} and DAG; these PKC isoforms do not bind phorbol esters (Newton, 1995). The phorbol ester tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA) and 1-oleoyl-2-acetyl-sn-glycerol (OAG), an analog of diacylglycerol have been commonly used as activators of PKC. MAPKs are subdivided in three subfamilies: the extracellular signal-regulated kinases (ERKs), the c-Jun amino-terminal kinases (JNKs) and the p38 MAPKs. They are activated by protein kinase cascades [MKKK-MKK(or MEK for ERKs)-MAPK], although MKK-independent activation of p38α has been reported (Johnson and Lapadat, 2002). Finally, cyclin-dependent kinases (Cdks) constitute a family of serine/threonine

kinases that regulate proliferation, differentiation, senescence and apoptosis. In post-mitotic neurons, all Cdks, with the exception of Cdk5, are silenced.

1.4 Tyrosine kinases

The tyrosine kinases can be divided in two groups: receptor tyrosine kinases (RPTKs; e.g, growth factor receptors, ephrin receptors) and non-receptor (cytoplasmic) tyrosine kinases (NRPTKs; e.g., Src, FAK, JAK). RPTKs can be further subdivided into 20 subfamilies and NRPTKs into 10 subfamilies. In the case of RPTKs, ligand-induced oligomerization and conformational changes result in tyrosine autophosphorylation of the receptor subunits which activates the catalytic activity and mediate the specific binding of cytoplasmic signaling proteins containing Src homology-2 (SH2) and protein tyrosine-binding domains. The NRPTK, c-Src, contains an SH2 domain through which it can bind to specific tyrosine autophosphorylation sites in ligand-stimulated RPTKs and mediate mitogenic signaling. c-Src can also be activated by binding to proline-rich sequences in target proteins through its SH3 domain or by dephophorylation of Tyr527 (Blume-Jensen and Hunter, 2001). The viral form of Src, v-Src, is constitutively active and oncogenic. It contains a shorter sequence at the carboxyl terminus that lacks Tyr527, which is required for inactivation. v-Src has been extensively studied in relation to connexins for its effects on gap junction function.

1.5 Serine/threonine phosphoprotein phosphatases

The phosphoserine/phosphothreonine protein phosphatases have been classified in three subfamilies (PPM, FCP and PPP). Members of the PPP (PP1, PP2A and PP2B) and PPM (PP2C) subfamilies which use a metal ion-catalyzed reaction account for most of the serine/phosphothreonine phosphatase activity *in vivo* (Barford et al., 1998). Several phosphatase inhibitors with different specificities are available including calyculin A (which inhibits PP1 and PP2A), cyclosporine A (an inhibitor of PP2B), FK506 (an inhibitor of PP2B) and okadaic acid (which inhibits PP1).

1.6 Phosphotyrosine phosphatases

The phosphotyrosine phosphatases (PTPs) have been classified in class I-IV based on the amino acid sequence of their catalytic domains (class I-III are cysteine-based PTPs and class IV are aspartic-based PTPs). The cysteine-based family can be subdivided in classical PTPs, dual-specificity PTPs, cdc25 PTPs, and low-molecular weight PTPs. Classical PTPs can be further subdivided into transmembrane receptor-like enzymes and intracellular non-receptor PTPs. Eighty one of the 107 PTP genes in the human genome are active protein phosphatases (Alonso et al., 2004).

2. Methods used to demonstrate that connexins are phosphoproteins

The most frequently used experimental approaches to demonstrate that a particular Cx is a phosphoprotein include metabolic labeling of cultured cells with ^{32}P followed by immunoprecipitation and alkaline phosphatase treatment, phosphoamino acid analysis (Sáez et al. 1986; Takeda et al., 1989; Musil et al., 1990; Crow et al., 1990; Sáez et al., 1990; Lau et al.,

1992; Goldberg and Lau, 1993; Kurata and Lau, 1994; Doble et al., 1996; Warn-Cramer et al., 1996; Mikalsen et al., 1997; Cheng and Louis, 1999) or two-dimensional phosphopeptide mapping (Sáez et al., 1990; Kurata and Lau, 1994; Díez et al., 1995; Loo et al., 1995; Warn-Cramer et al., 1996; Berthoud et al., 1997; Díez et al., 1998; Kanemitsu et al., 1998) *in vitro* phosphorylation assays using fusion proteins or synthetic peptides containing the putative phosphorylation site(s) and purified protein kinases (Sáez et al., 1990; Loo et al., 1995; Warn-Cramer et al., 1996; Berthoud et al., 1997; Kanemitsu et al., 1998; Shah et al., 2002; O'Brien et al., 2004; Ouyang et al., 2005; Yogo et al., 2006; Alev et al., 2008; Morel et al., 2010); treatment of cultured cells with specific protein kinase or phosphoprotein phosphatase activators or inhibitors to alter ^{32}P incorporation or the immunoblot pattern of connexins (Lau et al., 1992; Husøy et al., 1993; Guan et al., 1996; Berthoud et al., 1997; Cruciani et al., 1999; Duthe et al., 2000; Li and Nagy, 2000; Sirnes et al., 2009; Morley et al., 2010); overexpression or knockdown of a specific protein kinase or phosphoprotein phosphatase (Kanemitsu et al., 1998; Lampe et al., 1998; Doble et al., 2000; Lin et al., 2001; Chu et al., 2002; Petrich et al., 2002; Doble et al., 2004; Peterson-Roth et al., 2009; Ai et al., 2011); mass spectrometry (MS) analyses of immunoprecipitated connexins or *in vitro* phosphorylated fusion proteins containing a Cx intracellular domain (Cooper et al., 2000; Yin et al., 2000; TenBroek et al., 2001; Cooper and Lampe, 2002; Cameron et al., 2003; Axelsen et al., 2006; Locke et al., 2006; Solan et al., 2007; Shearer et al., 2008; Locke et al., 2009; Wang and Schey, 2009; Huang et al., 2011) and more recently, luminescence resonance energy transfer (Bao et al., 2007). Mutagenesis of the identified phosphorylation sites has been used to determine the functional consequences of their phosphorylation/dephosphorylation in cultured cells as well as *in vivo* after transfection or knock-in of a phosphosite-directed mutant Cx (Lampe et al., 1998; Remo et al., 2011).

3. Metabolic labeling with ^{32}P

The first reports that demonstrated a particular Cx to be a phosphoprotein using metabolic labeling with ^{32}P showed phosphorylation of Cx32 in hepatocytes (treated with phorbol esters, OAG, forskolin or cAMP analogs)((Sáez et a., 1986; Takeda et al., 1989; Sáez et al., 1990) and phosphorylation of Cx43 in uninfected and Rous sarcoma virus (RSV)-transformed fibroblasts (Crow et al., 1990). Phosphoamino acid analysis indicated that hepatocyte Cx32 and Cx43 in uninfected fibroblasts were phosphorylated on seryl residues (Takeda et al., 1989; Crow et al., 1990; Sáez et al., 1990), but Cx43 was also phosphorylated in tyrosyl residues in RSV-transformed fibroblasts (Crow et al., 1990). Using metabolic labeling with ^{32}P, other studies described that EGF-induced phosphorylation of Cx43 on serine residues in T51B cells through activation of mitogen-activated protein kinase (MAPK) (Lau et al., 1992; Warn-Cramer et al., 1996), FGF-2 induced phosphorylation of Cx43 in cardiomyocytes (Doble et al., 1996), tyrosine phosphorylation of Cx43 in early passage hamster embryo fibroblast (Mikalsen et al., 1997), phosphorylation of Cx56 by PKC and Cx49 by casein kinase 1 (CK1) in lens fiber cells (Berthoud et al., 1997; Cheng and Louis, 1999). In some cases, the specific phosphorylation site has been identified in reconstituted connexons expressed in *Xenopus laevis* oocytes. Using this approach, it has been demonstrated that v-Src induces tyrosine phosphorylation of Cx43 but not Cx32 (Swenson et al., 1990), and that serine368 of Cx43 (but not serine372) is directly phosphorylated by PKC (Bao et al., 2004a; 2004b).

3.1 *In vitro* phosphorylation

Another widely used approach to identify putative phosphorylation sites is *in vitro* phosphorylation assays. In this case, a polypeptide, fusion protein or synthetic peptide (corresponding to a fragment of the connexin that includes the putative phosphorylation site(s)) is incubated with a purified protein kinase in the presence of [γ-^{32}P]ATP and its ability to be a substrate for that protein kinase is evaluated by the incorporation of ^{32}P. Sáez and collaborators (1990) also performed *in vitro* kinase assays using the catalytic subunits of PKA, PKC or CaMK II and purified gap junctions or synthetic peptides as substrates, and compared their two-dimensional pattern of phosphopeptides with those obtained from metabolically labeled cells. Using glutathione S-transferase (GST) fusion proteins of Cx56 containing the carboxyl terminus or the intracellular loop, *in vitro* phosphorylation of Cx56 by PKC and PKA have been demonstrated in serine118 (in the intracellular loop) and serine493 (in the carboxyl terminus)(Berthoud et al., 1997).

Phosphorylation of Cx43 is among the best characterized. Polypeptides, fusion proteins and several synthetic peptides containing putative phosphorylation sites within the carboxyl terminus of Cx43 have been used to carry out *in vitro* phosphorylation and identify phosphorylation sites. These experiments have demonstrated that Cx43 is a substrate of p34^{cdc2} kinase (cell division cycle 2 kinase also known as cyclin dependent kinase 1) which mediates phosphorylation of Cx43 on Ser255 and possibly Ser262 (Kanemitsu et al., 1998). Cx43 is also a substrate for PKC and PKA. Kinetic analyses of wild type and mutant (S364P and S365N) Cx43 peptides (containing amino acid residues 359-376) *in vitro* phosphorylated by PKA and PKC have suggested that phosphorylation of Ser364 may be required for subsequent phosphorylation by PKC (Shah et al., 2002). *In vitro* phosphorylation of Ser365, Ser368, Ser369, and Ser373 by PKA has been described using a His-tagged Cx43-CT (containing amino acid residues E227-I382)(Yogo et al., 2006).

Other studies have shown *in vitro* phosphorylation of perch Cx35 by PKA and mouse Cx36 by CaMKII using fusion proteins containing the carboxyl terminus or the intracellular loop (O'Brien et al., 2004; Ouyang et al., 2005; Alev et al., 2008). A polypeptide containing the polymorphic variants S319 and P319 of the carboxyl terminus of human Cx37 (amino acid residues 233-333) was *in vitro* phosphorylated by glycogen synthase kinase-3β (Morel et al., 2010). *In vitro* kinase assays have also been used to demonstrate that phosphorylation of Cx32 by PKC prevents its proteolysis by calpains (Elvira et al., 1993).

Analyses of two dimensional maps of mixes of tryptic phosphopeptides from a connexin immunoprecipitated after metabolic labeling and from a (poly)peptide after *in vitro* phosphorylation together with phosphopeptide sequencing have been used often to identify the phosphorylated sites of the immunoprecipitated connexin and changes in their phosphorylation state under different experimental conditions.

4. Pharmacological modulation of phosphoprotein phosphatases

Changes in the phosphorylation state of Cxs can be induced by activating or inhibiting a specific intracellular phosphoprotein phosphatase. This type of approach allows identification of the protein phosphatases involved in the effects observed.

Using this approach, it has been demonstrated that treatment of V79 fibroblasts with several phosphoprotein phosphatase inhibitors (i.e., calyculin A, cyclosporin A or FK506) does not change the immunoblot pattern of Cx43 (Husøy et al., 1993; Cruciani et al., 1999). However, the dephosphorylation of immunoprecipitated Cx43 from TPA-exposed V79 cells is more efficiently reduced by PP2A than by PP1, PP2B or PP2C inhibitors (Cruciani et al., 1999). In WB-F344 cells, a rat liver epithelial cell line, calyculin A prevents the dephosphorylation of Cx43 induced by 18β-glycyrrhetinic acid (Guan et al., 1996). However, in primary cultures of astrocytes, calyculin A had little effect on hypoxia-induced Cx43 dephosphorylation; in this cell type, inhibition of PP2B with cyclosporin A or FK506 reduced Cx43 dephosphorylation after hypoxia (Li and Nagy, 2000). Calyculin A significantly retarded the loss of channel activity seen in ventricular myocytes in ATP-deprived conditions; conversely, stimulation of endogenous PP1 activity by treatment with p-nitrophenyl phosphate or 2,3-butanedione monoxime (a dephosphorylating chemical agent) induced a reversible interruption of cell-to-cell communication (Duthe et al., 2000; 2001).

The effect of okadaic acid on Cx43 also varies depending on cell type. It inhibits dephosphorylation of Cx43 in untreated and EGF-treated T5lB rat liver epithelial cells and prevents the dephosphorylation of Cx43 induced by 18β-glycyrrhetinic acid in WB-F344 rat liver epithelial cells (Lau et al., 1992; Guan et al., 1996). Okadaic acid also significantly retards the loss of gap junction channel activity seen in ventricular myocytes in ATP-deprived conditions (Duthe et al., 2000; 2001). In other cell types, it has little or no effect on the immunoblot pattern of Cx43 (Berthoud et al., 1992; Husøy et al., 1993; Cruciani et al., 1999), and has little effect on hypoxia-induced Cx43 dephosphorylation in primary cultures of astrocytes (Li and Nagy, 2000). Altogether these results suggest the involvement of different protein phosphatases in the phosphorylation state of Cx43 in different cell types under various experimental conditions.

5. Genetic activation or inhibition of a protein kinase or phosphatase

In some studies, changes in the phosphorylation state of Cxs have been induced by genetic manipulation through chemical-induced mutagenesis of genomic DNA or transfection with mammalian expression vectors and/or infection with virus containing cDNAs coding for a protein of interest. These methods can be used to modify the kinase activity using cDNAs encoding active or dominant negative mutant forms of a specific kinase. Lampe et al. used the FT210 cell line which contains a temperature-sensitive mutant of $p34^{cdc2}$/cyclin B kinase to demonstrate that the formation of the phosphoform of Cx43 present in mitotic cells was dependent on the activity of this kinase. However, the two-dimensional tryptic phosphopeptide map of immunoprecipitated Cx43 from mitotic cells had many major and minor tryptic phosphopeptides that could not be attributed to direct $p34^{cdc2}$/cyclin B kinase phosphorylation of the Cx43CT (Lampe et al., 1998). Doble et al. (2000) used transient tranfection and adenoviral infection of truncated or dominant-negative forms of PKCε to demonstrate that this kinase is required for Cx43 phosphorylation in cardiomyocytes (Doble et al., 2000).

The mechanism by which v-Src affects Cx43 phosphorylation and function has been extensively explored. Several studies have shown that expression of v-Src in mammalian fibroblasts leads to phosphorylation of Cx43 in tyrosyl residues (Crow et al., 1990). Mutants of Cx43 and v-Src SH2 and SH3 domains have been used to demonstrate that the SH2 and

SH3 domains of v-Src interact with Cx43; the SH3 domain binds to a proline-rich motif and the SH2 domain binds to a phosphorylated tyrosyl residue in the carboxyl terminus of Cx43 (Kanemitsu et al., 1997). Two specific phosphorylation sites for v-Src have been identified in Cx43, Tyr247 and Tyr265, by stably re-expressing wild type or mutant Cx43 with v-Src in Cx43 knockout cells (Lin et al., 2001). Moreover, using a triple serine-to-alanine mutant at the MAPK sites (S255/279/282A) it has been shown that phosphorylation of Cx43 by MAPK is not required for v-Src-induced disruption of gap junctional intercellular communication (Lin et al., 2006).

Several studies have been carried out on cardiac cells. Phosphorylation of Cx43 in Ser262 regulates DNA synthesis in cardiomyocytes forming cell-cell contact (Doble et al., 2004). Expression of an activated mutant of mitogen-activated protein kinase kinase 7 (a JNK-specific upstream activator) in cultured cardiomyocytes and in the heart *in vivo* demonstrated that Cx43 expression is regulated by JNK, although this effect may not be mediated by direct phosphorylation of Cx43 (Petrich et al., 2002). Transgenic mice with cardiac-specific overexpression of a constitutively active form of calcineurin (a calcium-dependent serine/threonine phosphatase) showed differences in the distribution of Cx43 in the ventricles, and Cx43 was mainly present in the nonphosphorylated form (Chu et al., 2002). Overexpression of p21-activated kinase 1 (PAK1, an activator of PP2A) increased PP2A activity and induced dephosphorylation of Cx43 in rabbit myocytes and Cx43-overexpressing HEK293 cells (Ai et al., 2011).

6. Genetic modification of a phosphosite-specific mutant connexin

A more recent approach is the generation of connexin knock-in mice in which the coding region of the wild type protein is replaced by DNA encoding a phosphosite-specific mutant. The only available report to date using this approach showed that mice in which Cx43 was replaced by a Cx43 mutant at the CK1 sites in which serines 325/328/330 were replaced with phosphomimetic glutamic acids (S3E) were resistant to gap junction remodeling and less susceptible to the induction of arrhythmias. In contrast, mice in which a Cx43 mutant with serines 325/328/330 mutated to non-phosphorylatable alanines (S3A) was knocked-in in place of Cx43 had severe alterations in gap junction formation and function, and had a proarrhythmic phenotype (Remo et al., 2011). This report shows a mechanistic link between the phosphorylation state of Cx43 and arrhythmic susceptibility (Remo et al., 2011).

7. Phosphospecific antibodies

Antibodies that recognize a specific phosphorylated (or dephosphorylated) site in a connexin have been developed. These have been extensively used to identify the state of phosphorylation of the phosphosite they recognize and to determine associated changes in connexin distribution in cells under different physiological and pathological conditions. Using this approach, it has been described that ischemic preconditioning prevents the changes in the phosphorylation state of Cx43 observed in a model of ischemia/reperfusion in pig hearts (Schulz et al., 2003). It has also been reported that PKC phosphorylates Cx43 in Ser368 (Solan et al., 2003), and that scratch wounding of primary human keratinocytes causes a PKC-dependent increase in phosphorylation at this site in cells adjacent to the scratch (Richards et al., 2004). Leykauf et al. used a specific antibody against PSer279-

PSer282 of Cx43 to demonstrate that different phosphorylated forms of Cx43 coexist at the plasma membrane (Leykauf et al., 2003). Two antibodies recognizing the same phosphosites were used to show that EGF and activation of its receptor with quinones induce phosphorylation of Cx43 in these serine residues (Abdelmohsen et al., 2003; Leykauf et al., 2003). Using an antibody that specifically recognizes Cx43 phosphorylated at serines 325, 328 and/or 330 (PS325/328/330), Lampe and colleagues showed that while Cx43 relocalizes to the lateral edges in ischemic hearts, Cx43 phosphorylated at these residues remained mostly at the intercalated disk (Lampe et al., 2006). An antibody that recognizes dephosphorylated Ser364/Ser365 and binds preferentially to Golgi-localized Cx43 in cultured cells has been used to demonstrate conformational changes in Cx43 (Sosinsky et al., 2007). Other studies have described that phosphorylation of connexin 43 at Ser262 is associated with a cardiac injury-resistant state (Srisakuldee et al., 2009).

Phosphospecific antibodies have been used in combination with PKC or MEK inhibitors to determine the protein kinase pathway involved in the effects observed. Sirnes et al. reported that TPA induces phosphorylation of Ser255 and Ser262 of Cx43 in a MAPK-dependent manner (Sirnes et al., 2009). A MAPK-dependent phosphorylation of serines 255, 262 and 279/282 of Cx43 has also been demonstrated using phosphospecific antibodies and a MEK inhibitor in follicles exposed to luteinizing hormone (Norris et al., 2008). In MC3T3-E1 osteoblasts, treatment with fibroblast growth factor 2 induces a PKCδ-dependent increase in phosphorylation at Ser368 of Cx43 (Niger et al., 2010). Solan and Lampe used several anti-Cx43 phosphospecific antibodies that recognize Src, MAPK or PKC sites and LA-25 cells (which express a temperature-sensitive v-Src) grown at the permissive and non-permissive temperatures to show that distinct tyrosine and serine residues are phosphorylated in response to v-Src activity (Solan and Lampe, 2008). Li et al. used antibodies that specifically recognize PSer110 and PSer276 in Cx35 to demonstrate that the level of phosphorylation of these serines depends on PKA activity and regulates photoreceptor coupling in zebrafish retina (Li et al., 2009).

8. Mass spectrometry analyses

Another technique that has been used to identify putative phosphorylation sites is mass spectrometry (MS) analysis of connexins isolated from tissue or cultured cells or *in vitro* phosphorylated (poly)peptides. For this purpose, the immunoprecipitated/isolated connexin or *in vitro* phosphorylated polypeptide is digested with a protease or a mix of proteases, the sample is enriched in phosphopeptides and subjected to MS. This technique is highly sensitive and it does not require the use of radioactivity.

The first studies using this technique to identify phosphorylation sites in Cxs were reported several years ago (Cooper et al., 2000; Yin et al., 2000). Cooper et al. showed that *in vitro* phosphorylation of the carboxyl terminus of Cx43 with $p34^{cdc2}$/cyclin B kinase resulted in phosphorylation of Ser255 using liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Cooper et al., 2000). Yin et al. demonstrated that lens Cx45.6 is phosphorylated in the chicken lens *in vivo* at Ser363 using nanoelectrospray and tandem mass spectrometry (Yin et al., 2000).

Several studies using mass spectrometry analysis have been performed on Cx43. Ser364 was identified as a phosphorylation site in Cx43 using matrix-assisted laser

desorption/ionization-time of flight (MALDI-TOF MS) and LC-MS/MS (TenBroek et al., 2001). MALDI-TOF MS in combination with metabolic labeling of normal rat kidney (NRK) epithelial cells (in the presence and absence of a casein kinase 1 inhibitor) and *in vitro* phosphorylation of Cx43CT fusion proteins with casein kinase 1δ (CK1δ) have been used to determine that serines 325, 328 or 330 are potential sites of CK1 phosphorylation in these cells (Cooper and Lampe, 2002). Cameron et al. (2003) used MALDI-TOF MS to identify Ser255 of Cx43 as the preferred site for big MAPK 1 (BMK1)/ERK5 phosphorylation. This finding was further supported by the lack of phosphorylation of GST fusion proteins containing mutant carboxyl termini of Cx43 in which Ser255 had been mutated to alanine (S255A and S255A/S279A/ S282A). Axelsen et al. (2006) reported the time course of changes in phosphorylation of Cx43 immunopurified from perfused rat hearts under non-ischemic and ischemic conditions. These authors identified thirteen phosphorylation sites using MALDI MS and LC-MS/MS in non-ischemic conditions and detected site-specific changes

	Connexin	Cell Type	Kinase or Phosphatase	Identification site	References
Metabolic labeling	Cx32	Rat hepatocytes and purified liver gap junction	cAMP-PK, PKC, Ca2+/CaM-PK-II	Ser233	Sáez et al., 1990. Eur J Biochem 192:263-73.
	Cx43	Rat liver epithelial cells (T51B)	-	Serine residues	Lau et al., 1992. Mol Biol Cell 3:865-74.
	Cx43	Rat neonatal cardiomyocytes	-	Serine residues	Doble et al., 1996. Circ Res 79:647-658
	Cx43	in vitro	MAPK	Ser255, Ser279, Ser282	Warn-Cramer et al., 1996. J Biol Chem 271:3779-86
	Cx43	Hamster fibroblast	-	Tyrosine residues	Mikalsen et al., 1997. FEBS Lett 401:271-5.
	Cx49	Sheep lens fiber cell	casein kinase 1	Ser/Thr	Cheng and Louis, 1999. Eur J Biochem 263:276-86.
	Cx43	Expression in Xenopus	PKC	Ser368	Bao et al., 2004. J Biol Chem 279:20058-66.
	Cx43	Expression in Xenopus	PKC	Ser368	Bao et al., 2004 Am J Physiol Cell Physiol 286: C647-C654
	Cx43	Expression in Xenopus	pp60v-src	Tyr265	Swenson et al., 1990. Cell Regul 1:989-1002
Kinase assays	Cx56	chicken lens primary cultures	PKC	Ser118	Berthoud et al., 1997. Eur J Biochem 244:89-97.
	Cx43	Rat-1 fibroblast	p34cdc2/cyclin B	amino acids 241-264	Kanemitsu et al., 1998. Cell Growth Differ 9:13-21.
	Cx43	Mouse fibroblast (L929)	PKA-PKC	Ser364	Shah et al., 2002. Mol Cell Biochem 238:57-68
	Cx43	Rat granulosa cells	PKA	Ser365, Ser368, Ser369, and Ser373	Yogo et al., 2006. J Reprod Dev 52:321-8
	Cx35	HeLa Cells	PKA	cytoplasmic domain	O'Brien et al., 2004. J Neurosci 24:5632-5642
	Cx35	HeLa Cells	PKA	intracel loop & cytoplasmic domain	Ouyang et al., 2005. Brain Res Mol Brain Res 135:1-11.
	Cx36	GST-Cx36 fusion protein	CaMKII	cytoplasmic domains	Alev et al., 2008. Proc Natl Acad Sci U S A 105:20964-9.
	Cx37	HeLa and SK-HEP-1 cells	GSK-3ß	Ser319 and Pro319	Morel et al., 2010.Carcinogenesis 31:1922-1931.
Pharmacological Modulation	Cx43	Hamster embryo cells and lung fibroblasts	PKC	-	Husoy et al., 1993 Carcinogenesis. 1993 14(11):2257-65.
	Cx43	Rat liver epithelial cells	PP1 and PP2A	-	Guan et al., 1996 Mol Carcinog 16, 157-164
	Cx43	Hamster fibroblast	PP1, PP2A, PP2B and PP2C	-	Cruciani et al., 1999 Exp Cell Res 252: 449-463
	Cx43	Astrocytes	PP1, PP2A and PP2B	-	Li and Nagy, 2000 Eur J Neurosci 12, 2644-2650.
	Cx43	neonatal rat cardiomyocytes	serine/threonine protein kinases	-	Duthe et al., 2000 Gen Physiol Biophys 19: 441-449.
	Cx43	neonatal rat cardiomyocytes	PP1 and PP2A	-	Duthe et al., 2001 Am J Physiol Cell Physiol. 2001 281:C1648-56.
	Cx43	Madin Darby canine kidney (MDCK) cells	PKC, cAMP- or cGMP-dependent PK	-	Berthoud et al., 1992 Eur J Cell Biol 57: 40-50
Genetic Modulation	Cx43	Rat1 fibroblasts	p34cdc2 kinase	Ser255	Lampe et al., 1998. J Cell Sci 111:833-41.
	Cx43	neonatal rat cardiomyocytes	PKC epsilon	-	Doble et al., 2000 Circ Res 86:293-301
	Cx43	Cx43 knockout mouse cell line.	v-Src	Y247, Y265	Lin et al., 2001 J Cell Biol 154:815-27.
	Cx43	neonatal rat cardiomyocytes	c-jun	-	Petrich et al., 2002 Circ Res 91:640-7.
	Cx43	Transgenic Mouse hearts	PP3	-	Chu et al., 2002 Cardiovasc Res 54: 105-116.
	Cx43	embryonic fibroblasts	v-Src	Tyrosine residues	Peterson-Roth et al. 2009 Cancer Res 69:3619-3624
	Cx43	left ventricular myocytes	p21-activated kinase 1 and PP2A	-	Ai et al., 2011 Cardiovasc Res. 92(1):106-14.
	Cx43	Cx43 germline knock-in mice	-	Ser325, Ser 328, Ser330	Remo et al., 2011 Circ Res. 108(12):1459-66.
	Cx43	in vitro	v-Src	Y265	Kanemitsu et al., 1997 J Biol Chem 272:22824-31.
	Cx43	neonatal rat cardiomyocytes	PKC	Ser262	Doble et al., 2004 Journal of Cell Science 117:507-514
	Cx43	Cx43 knockout mouse fibroblasts	V-Src	-	Lin et al., 2006 Cell Commun Adhes 13:199–216.
Phosphospecific antibodies	Cx43	rabbit lens ephitelial cells	PKC-gamma	-	Lin et al., 2003
	Cx43	rat kidney epithelial cells	PKC	Ser368	Solan et al., 2003
	Cx43	liver epithelial cells	ERK1, ERK2	Ser279, Ser282	Abdelmohsen et al., 2003
	Cx43	Pig hearts	PKCα, p38MAPKα, and p38MAPKβ	-	Schulz et al., 2003 FASEB J 17:1355-7
	Cx43	Rat liver epithelial cells	MAPK	-	Leykauf et al., 2003 Cell Tissue Res 311:23-30
	Cx43	Humna keratinocyte	PKC	Ser368	Richards et al., 2004 J Cell Biol. 167:555-62.
	Cx43	Cx43 knockout mouse fibroblasts and heart	casein kinase 1	Ser325, Ser328, Ser330	Lampe et al., 2006 J Cell Sci 119:3435–3442
	Cx43	Rat kidney epithelial cells	vSrc	Y247, Y265, Ser262, Ser279/282, Ser368	Solan and Lampe, 2008 Cell Commun Adhes 15:75-84.
	Cx43	Mouse ovarian follicles	MAPK	Ser255, Ser262, Ser279/282	Norris et al., 2008 Development 135:3229-3238
	Cx35	Zebrafish retina	PKA	Ser110, Ser276	Li et al., 2009 J Neurosci 29:15178-15186.
	Cx43	Rat liver epithelial cell	PKC and MAPK	Ser368, Ser255, Ser262	Simes et al., 2009 Biochem Biophys Res Commun 382:41-45.
	Cx43	Rat hearts	PKC	Ser262	Srisakuldee et al., 2009 Cardiovasc res 83:672-81.
	Cx43	MC3T3 osteoblasts	PKC delta	Ser368	Niger et al., 2010 BMC Biochemistry 11:14
Mass spectrometry analyses	Cx45.6	Avian lens primary cell cultures	casein kinase II	Ser363	Yin et al., 2000 J Biol Chem 275:6850-6.
	Cx43	mouse fibroblasts	PKA	Ser364	TenBroek et al., 2001 J Cell Biol 155:1307-18
	Cx43	rat kidney cells	casein kinase 1 (gamma)	Ser325, Ser328, Ser330	Cooper and Lampe, 2002 J Biol Chem 277:44962-8.
	Cx43	HEK-293	BMK1/ERK5	Ser255	Cameron et al., 2003 J Biol Chem 278:18682-8.
	Cx43	isolated perfused rat hearts	PKA, PKCα, PKCε, PKG, AMP-dependent,	30 residues	Axelsen et al. 2006 J Mol Cell Cardiol 40:790-8.
	Cx26, Cx32	HeLa Cells	-	-	Locke et al. 2006 FASEB J 20:1221-3.
	Cx43	NRK-E51, MDCK	PKC	Ser365	Solan et al. 2007 J Cell Biol 179:1301-9.
	Cx44, Cx49	Bovine Lens Fiber	-	Ser and Thr residues in the C-tail of Cx44 and Ser residues in Cx49	Shearer et al. 2008 Invest Ophthalmol Vis Sci 49:1553-1562.
	Cx46, Cx50	Bovine lens	-	9 residues in Cx46 and 18 residues in Cx50	Wang and Schey 2009 Exp Eye Res 89:898-904
	Cx26	in vitro	-	Thr123, Thr177, Ser183, Thr186, Tyr233, Tyr235, Tyr240	Locke et al 2009 Biochem. J 424:385–398
	Cx43	in vitro	CaMKII	Ser296, Ser365, Ser369, Ser373, Ser244, Ser306	Huang et al. 2011 J Proteome Res 10:1098-109
LRET	Cx43	purified WT Cx43	PKC	-	Bao et al., 2007. Proc Natl Acad Sci U S A 104:4919-24.

Table 1. Techniques used for identification of connexins as substrates for protein kinases and phosphoprotein phosphatases.

in Cx43 phosphorylation during the course of ischemia. Phosphorylation of Ser365 has also been demonstrated in Cx43 immunoprecipitated from NRK cells using liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/ESI MS/MS) (Solan et al., 2007). Fifteen putative phosphorylation sites on Cx43 have also been identified after *in vitro* phosphorylation of a GST fusion protein containing the Cx43CT with CaMK II by high-resolution mass spectrometry (Huang et al., 2011).

Post-translational modification by phosphate has also been identified by mass spectrometry in Cx26 and Cx32; Cx26 is phosphorylated in the intracellular loop and the second extracellular loop, and Cx32 is phosphorylated in the amino and carboxyl termini (Locke et al., 2006). Two studies have used mass spectrometry to identify phosphorylation sites in the bovine lens fiber connexins, Cx44 and Cx49. While phosphorylation sites were identified only on the carboxyl terminus of Cx44, phosphosites were identified in both the intracellular loop and carboxyl terminus of Cx49 (Shearer et al., 2008; Wang and Schey, 2009).

9. Luminescence resonance energy transfer

Another recent approach used to evaluate the functional effect(s) of phosphorylation of Cxs is the generation of hemichannels of known composition, stoichiometry that can be assessed by luminescence resonance energy transfer (LRET)(Bao et al., 2007). This method uses terbium ions (Tb^{3+}), which have a long lifetime emission as donor and fluorescein as acceptor. The technique is based on the detection of LRET between Cx43 subunits labeled with Tb^{3+} and those labeled with fluorescein. The composition of the HCs can be determined based on the number of acceptor-labeled monomers per HC. Using HC of known composition, Bao and colleagues have determined that in a Cx43 HC all six subunits have to be phosphorylated by PKC at Ser368 to abolish sucrose permeability, although the HC pore still has a sizable diameter and allows permeation of smaller molecules (Bao et al., 2007).

10. Conclusions and future directions

In summary, connexins are substrates for various protein kinases and phosphoprotein phosphatases. Several of the phosphorylation sites have been identified, and the effect of phosphorylation at many of these sites on connexin channel activity has been studied. In some cases, pathophysiological conditions that alter their phosphorylation state have been reported. Although significant progress has been made in the area of connexin phosphorylation, there are many associated aspects that require further investigation.

A question that remains unanswered is whether all connexins are phosphoproteins. Does phosphorylation affect connexin channel function in all members? Does phosphorylation at a specific site induce consistent functional changes in gap junction channels and hemichannels? Or, can phosphorylation at a specific site induce changes in one channel type, and not in the other?. Because phosphorylation has been implicated in several steps of the connexin's life cycle, it is also important to determine which phosphorylation events are associated with proper trafficking to the plasma membrane, formation of gap junctional plaques or internalization and degradation. Are connexins sorted/targeted to different compartments depending on their cohort of phosphorylated sites? Where do these phosphorylation events take place? Since some hierarchy in the phosphorylation events has been shown for Cx43, it is interesting to know whether changes in phosphorylation are also associated with other post-

translational modifications. Do these have a hierarchical sequence? Because connexins and changes in the activity of protein kinases/phosphoprotein phosphatases have been associated with disease, it would be important to know how the phosphorylation state of connexins is affected in disease. What are the intracellular signals and mechanisms of regulation of phosphorylation/dephosphorylation of connexins? What are the endogenous activators of the protein kinases/phosphoprotein phosphatases involved? Although the answers to some of these questions are known for some of the phosphorylation sites identified, especially in the case of Cx43, these questions have not been addressed for most connexins.

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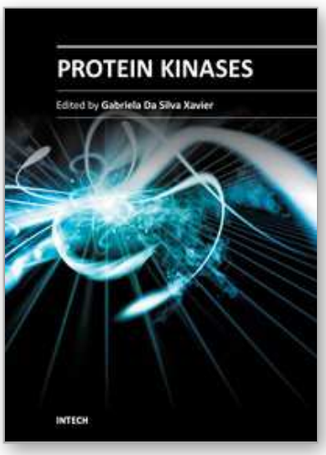

Protein Kinases

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Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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