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**pp60<sup>src</sup>-MEDIATED PHOSPHORYLATION OF  
CONNEXIN43, A GAP JUNCTION PROTEIN**

**A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION  
OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT  
OF THE REQUIREMENTS FOR THE DEGREE OF**

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## ABSTRACT

Several laboratories have demonstrated a decrease in gap junctional communication in cells transformed by the *src* oncogene of the Rous sarcoma virus. The decrease in gap junctional communication was associated with tyrosine phosphorylation of the gap junction protein, connexin43 (Cx43). This study was initiated to determine if the phosphorylation of Cx43 is the result of a direct kinase-substrate interaction between the highly active tyrosine kinase, pp60<sup>v-src</sup>, and Cx43. Confocal microscopy data indicates that the two proteins are within physical proximity allowing for a potential kinase-substrate interaction. Previous biochemical studies have been limited by the low levels of Cx43 protein in fibroblast cell lines. To obtain larger quantities of Cx43 we constructed a recombinant baculovirus expressing Cx43 in *Spodoptera frugiperda* (Sf-9) cells and subsequently purified the expressed Cx43 by immunoaffinity chromatography. We observed that this partially-purified Cx43 was phosphorylated on tyrosine *in vitro* in the presence of kinase-active pp60<sup>src</sup>. Phosphotryptic peptide mapping indicated that the *in vitro* phosphorylated Cx43 contained phosphopeptides which comigrated with a subset of tryptic peptides prepared from Cx43 phosphorylated *in vivo*. This phosphorylation event occurred in the COOH-tail region of the Cx43 molecule because pp60<sup>src</sup> phosphorylated a GST-fusion protein containing the COOH-tail region of Cx43 and the phosphotryptic peptides generated using

this construct comigrated with the subset mentioned above. Furthermore, coinfection of Sf-9 cells with recombinant baculoviruses encoding pp60<sup>v-src</sup> and Cx43 resulted in the accumulation of phosphotyrosine in Cx43. Taken together, the evidence presented in this dissertation demonstrates that kinase active pp60<sup>src</sup> is capable of phosphorylating Cx43 in a direct manner. Since the presence of phosphotyrosine on Cx43 is correlated with the down regulation of gap-junctional communication, these results suggest that pp60<sup>v-src</sup> regulates gap junctional gating activity via tyrosine phosphorylation of Cx43.

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# CHAPTER ONE

## INTRODUCTION

### Gap Junctions

Gap junctions, which are found in plasma membranes of almost all animal cells, mediate cell-to-cell communication and maintain normal organ function (Page and Manjunath, 1986; Garfield et al., 1988), embryonic development (Caveney, 1985; Fraser et al., 1987), and perhaps growth control (Mehta et al., 1986, 1991; Loewenstein, 1979, 1992; Yamasaki and Katoh, 1988; Zhu et al., 1991). Gap junctions are formed when a connexon (hemichannel) from one cell docks with a symmetrically opposed connexon from an adjacent cell forming a conduit which permits the passive diffusion of ions and small molecules between neighboring cells. The channel is approximately 1.2-2 nM in diameter, allowing molecules under 1000 daltons to pass through. Each connexon consists of an oligomer of six connexin proteins that form the central aqueous pore (Loewenstein, 1979; Makowski et al., 1977; Unwin and Zampighi, 1980) (Figure 1.1).

Connexins are a family of gap junction proteins that are composed of four transmembrane domains, two extracellular loops, an intracellular loop, and amino- and carboxy-terminal domains located in the cytoplasm (Hertzberg et al., 1988; Milks et al., 1988; Yancey et al., 1989). The transmembrane domains and extracellular loops are highly conserved between family members and are

believed to be responsible for channel structure, whereas the cytoplasmic domains are less conserved and may account for the differential regulation of gap junctions (Dermietzel et al., 1990; Spray and Burt, 1990) (Figure 1.1).

Connexins are expressed in a tissue-specific manner. For example, connexin43 (Cx43) is predominantly expressed in the heart (Beyer et al., 1987), whereas, connexin32 (Cx32) and connexin26 (Cx26) are expressed in the liver (Kumar and Gilula, 1986; Zhang and Nicholson, 1989). The function for the differential tissue distribution is at present poorly understood. In fact, new connexins continue to be identified with the development of powerful tools such as low stringency hybridization and DNA amplification with polymerase chain reaction (PCR), increasing our appreciation for the complexity of connexin expression in various tissues. The names of the respective connexin types are designated according to their predicted molecular mass.

Gap junction channels function to allow the passive diffusion of ions and small molecules such as metabolites and second messengers to be exchanged between neighboring cells. This may be one mechanism by which the cells maintain growth control and homeostasis. Gap junctional communication (gjc) has been implicated in the regulation of growth control based on experiments linking the permeability of the gap junction channel with cellular transformation (reviewed in Loewenstein and Rose, 1992). Several groups have demonstrated that the tumor promoter, 12-O-tetradecanoylphorbol 13-acetate (TPA), can inhibit gjc (Enomoto et al., 1981; Murray and

Fitzgerald, 1979; Yotti et al., 1979). Transformation of cells by viral oncogenes such as *v-ras* (Brissette et al., 1991; El-Fouly et al., 1989), *v-src* (Azarnia and Loewenstein 1984; Chang et al., 1985), *v-mos* (Atkinson and Sheridan 1988), and polyomavirus middle T antigen (Azarnia and Loewenstein 1987) has been correlated with an inhibition of gjc. Furthermore, Mehta et al. (1989) demonstrated that treatment of chemically transformed CH3 10T1/2 cells with retinoids simultaneously increased gjc and inhibited transformation. The most direct evidence supporting the role of gjc in transformation involved the introduction of Cx43 into communication-deficient, carcinogen-transformed cells. Re-expression of Cx43 resulted in restoration of gjc, an inhibition of growth, focus formation (Mehta et al., 1991) and tumorigenicity (Rose et al., 1993). Similar results were observed in communication-deficient C6 glioma cells transfected with Cx43 (Naus et al., 1992) and human tumor cells (SKHep) transfected with Cx32 (Eghbali et al., 1991).

The permeability of the gap junction channel is affected by various factors such as changes in free intracellular calcium levels (Rose and Loewenstein, 1975), intracellular pH (Spray et al., 1981), voltage (Bennett and Verselis, 1992; Spray et al., 1986), growth factors (Lau et al., 1992; Madhukar et al., 1989; Maldonado et al., 1988), and transforming oncogene activity (Atkinson and Sheridan, 1985; Azarnia et al., 1988). The mechanisms which mediate changes in gap junctional permeability are at present incompletely understood. However, the increased phosphorylation of connexin induced by various stimuli is associated with modulation of channel

gating (opening and closing) (Laird et al., 1991; Loewenstein, 1984; Stagg and Fletcher, 1990). For example, cyclic AMP treatment of liver hepatocytes increased gjc and Cx32 phosphorylation, which may be the result of activated cyclic AMP-dependent serine kinases (Saez et al., 1986, 1990). Expression of the *ras* oncogene or TPA treatment of mouse primary keratinocytes decreased gjc and increased serine phosphorylation on Cx43 (Brissette et al. 1991). Epidermal growth factor (EGF) treatment of T51B rat liver epithelial cells produced a rapid, transient decrease in gjc which was associated with increased serine phosphorylation on Cx43 (Kanemitsu and Lau, 1993; Lau et al. 1992).

### **pp60<sup>src</sup>**

In 1911 Peyton Rous provided the first clues to the viral etiology of cancer. He demonstrated that the sarcomas found in the breast muscles of chickens were associated with a viral agent, Rous sarcoma virus (RSV). Subsequent studies have identified the RSV gene responsible for the sarcomas, *v-src*. The cellular counterpart of this gene, *c-src*, was subsequently identified. It is expressed at low levels in all normal cells, whereas *v-src* is expressed at high levels and associated with cellular transformation. (Reviewed in Parsons and Weber, 1989)

The product of the *src* gene, pp60<sup>src</sup>, is a 60 KDa membrane associated tyrosine kinase. Membrane association is dependent on the addition of myristic acid, a 14-carbon saturated fatty acid, to a glycine at amino acid position 2. If the glycine is mutated to an

alanine, myristylation does not occur and pp60<sup>src</sup> does not associate with membrane structures and is unable to transform cells (Buss et al., 1986). The catalytic activity of the cellular form, pp60<sup>c-src</sup>, is tightly regulated. The mechanism of its regulation is dependent on a carboxy-terminal phosphotyrosine at amino acid position 527 (Cooper et al., 1986). This phosphotyrosine is thought to associate intramolecularly with its src homology 2 (SH2) domain, conformationally blocking its catalytic region (Liu et al., 1993). If the tyrosine at position 527 is mutated to a phenylalanine, there is no association with the SH2 domain and an increase in kinase activity results (Kmiecik and Shalloway, 1987; Piwnica-Worms et al., 1987). The viral form, pp60<sup>v-src</sup>, is highly active because it lacks 19 amino acids at the carboxy-terminus found in pp60<sup>c-src</sup> and is therefore missing the regulatory tyrosine 527. There are numerous mutations throughout the entire sequence of the viral gene, but this carboxy-terminal difference appears to have the most significant effect on pp60<sup>v-src</sup>'s catalytic activity.

Cells expressing the *v-src* oncogene demonstrate a loss of growth control and other characteristics of the neoplastically-transformed phenotype. *v-src*-transformed cells have a rounded, refractile appearance and grow in a disorganized, multi-layered fashion in contrast to normal cells which grow as organized monolayers. *v-src*-transformed cells are also characterized by their ability to grow without anchoring to a matrix. This is assayed by measuring the ability of the cells to grow in a semi-solid medium such as growth medium containing agar. Normal cells are not

capable of growth under these conditions. Moreover, *v-src*-transformed cell lines are capable of inducing tumors in nude mice. (Sefton and Hunter, 1986; Parsons and Weber, 1989).

Tyrosine phosphorylation in normal cells is a very rare occurrence. Analysis of total phosphoamino acids indicates that phosphotyrosine represents only 0.03% of the total phosphoamino acids in proteins of normal chicken embryo fibroblasts. Cells infected with the RSV may have ten times this amount (Hunter and Sefton, 1980). These results suggest that tyrosine phosphorylation of cellular proteins by pp60<sup>v-src</sup> has a direct role in cellular transformation. Several examples of pp60<sup>v-src</sup> substrates which have been identified are, cytoskeletal proteins (Reynolds et al., 1992; Turner et al., 1990), focal adhesion kinase (pp125<sup>FAK</sup>) (Schaller et al., 1992), and an RNA-binding protein (p68) (Fumagalli et al., 1994; Taylor and Shalloway, 1994). pp60<sup>v-src</sup> initiates a cascade of events by phosphorylating multiple substrates which ultimately results in a cellular population that possess different morphological and growth characteristics, thus the transformed state. pp60<sup>v-src</sup> substrates continue to be identified and characterized in an effort to understand the critical events which initiate and maintain pp60<sup>v-src</sup> mediated cellular transformation. We have focused our attention to the relationship between pp60<sup>src</sup> and a putative substrate, Cx43.

## **Tyrosine Phosphorylation on Cx43 and Decreased Gap Junctional Communication**

Tyrosine phosphorylation on Cx43 in *v-src* transformed cells correlates with a decrease in gjc (Crow et al., 1990, 1992; Filson et al., 1990). Cells infected with a temperature-sensitive mutant of RSV demonstrated a rapid reduction in gjc and a transformed morphology when shifted to the permissive temperature, where pp60<sup>v-src</sup> is active. At the nonpermissive temperature, where pp60<sup>v-src</sup> is inactive, the cells exhibited high levels of gjc and a normal morphology (Azarnia et al., 1984; 1988). Phosphorylated Cx43, purified from cells shifted to the permissive temperature, rapidly accumulated phosphotyrosine, concomitant with the disruption of gjc (Crow et al., 1992). In contrast, cells grown at the nonpermissive temperature, with normal levels of gjc contained Cx43 phosphorylated only on phosphoserine residues. These results suggested that pp60<sup>v-src</sup> may decrease gjc by inducing the phosphorylation of Cx43 on tyrosine.

Further evidence for this proposed mechanism came from experiments utilizing *Xenopus* oocytes expressing Cx43 and/or pp60<sup>v-src</sup>. When Cx43 was coexpressed with pp60<sup>v-src</sup>, junctional conductance was disrupted and Cx43 was phosphorylated on tyrosine (Swenson et al., 1990). Oocytes co-expressing pp60<sup>v-src</sup> and a mutated form of Cx43 (tyrosine 265 mutated to phenylalanine) did not demonstrate a decrease in gjc or tyrosine phosphorylation on Cx43. These results suggested that pp60<sup>v-src</sup> may directly regulate

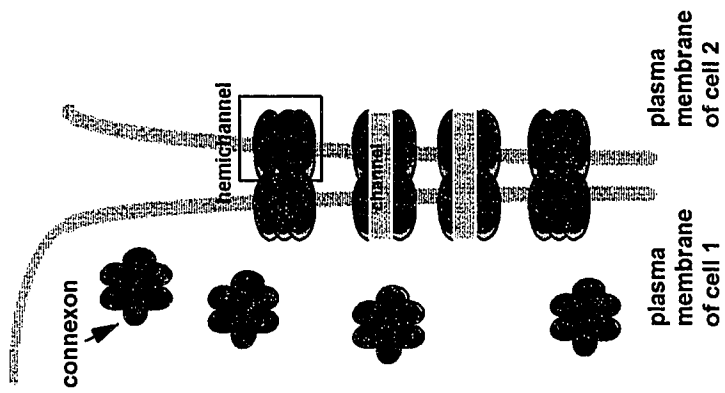
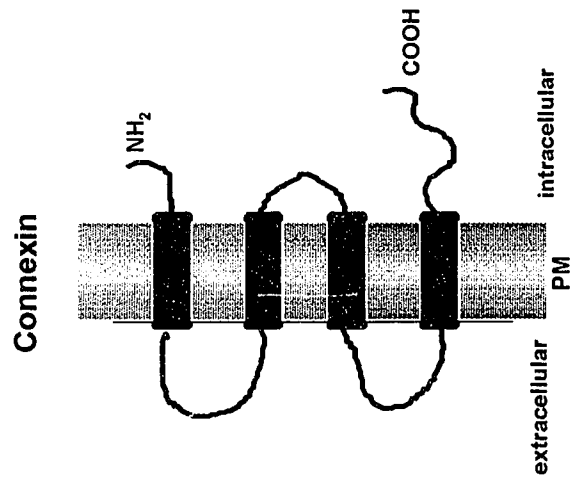
channel gating via tyrosine phosphorylation of Cx43 on residue 265, however, it was possible that an intermediate tyrosine kinase(s), activated by pp60<sup>v-src</sup>, may actually phosphorylate Cx43 in mammalian cells. One such tyrosine kinase could be the focal adhesion kinase, pp125<sup>FAK</sup> (Schaller et al., 1992), which is phosphorylated on tyrosine, activated in *v-src*-transformed cells (Guan and Shalloway, 1992) and associated with pp60<sup>src</sup> (Cobb et al., 1994).

The hypothesis that catalytically active pp60<sup>src</sup> regulates gjc directly, was further supported by results which demonstrated that membrane association is important for pp60<sup>src</sup>'s effects on gjc. Fibroblasts transfected with membrane associated, kinase active pp60<sup>c-src</sup> (pp60<sup>527F</sup>), exhibited a significant reduction in gjc (~12-fold), when compared to the normal parental cell. Whereas fibroblasts transfected with a mutant pp60<sup>c-src</sup>, which was kinase-active but non-myristylated (pp60<sup>2A527F</sup>) demonstrated a slight decrease in gjc (~twofold). Moreover, the reduction in gjc observed in cells transfected with pp60<sup>527F</sup> correlated with tyrosine phosphorylation on Cx43. Whereas, Cx43 in pp60<sup>2A527F</sup> did not exhibit any detectable phosphotyrosine (Crow et al., 1992).

Several groups have presented strong evidence supporting the hypothesis that pp60<sup>src</sup> phosphorylates Cx43 and that the observed tyrosine phosphorylation on Cx43 functions to downregulate gjc. Aside from the evidence presented by Swenson et al. (1990), most of the data demonstrating a direct kinase substrate relationship between pp60<sup>src</sup> and Cx43 have been correlative. In this

dissertation, we present *in vivo* and *in vitro* data which supports the hypothesis that Cx43 can serve as a direct substrate of pp60<sup>src</sup>. First, we demonstrate that Cx43 and pp60<sup>v-src</sup> are within physical proximity at the plasma membrane of *v-src*-transformed fibroblasts. Second, we demonstrate that Cx43 is phosphorylated by kinase-active pp60<sup>c-src</sup> *in vitro*. Third, we demonstrate that the *in vitro* phosphorylated Cx43 contained phosphopeptides which comigrated with a subset of tryptic peptides from Cx43 immunoprecipitated from *v-src*-transformed fibroblasts. Fourth, we demonstrate that pp60<sup>src</sup> phosphorylated Cx43 in the carboxy-terminal tail region. Last, we demonstrate that Cx43 is phosphorylated on tyrosine residue(s) in Sf-9 cells coinfecting with both Cx43 and pp60<sup>v-src</sup> recombinant baculoviruses. Overall these results represent a critical contribution to the ongoing effort to understand the parameters necessary for pp60<sup>v-src</sup>-mediated cellular transformation. The reagents generated in this study will be very useful for more detailed analyses of the sites of tyrosine phosphorylation and the effects of tyrosine phosphorylation on channel gating activities.

**Figure 1.1. Schematic diagram of a gap junction.** Connexons from one cell are docked to connexons from an adjacent cell forming the gap junction channels for the passive diffusion of ions and small molecules. Each connexon contains six connexin subunits. The connexin molecule consists of four transmembrane domains, two extracellular loops, one intracellular loop, and cytoplasmically located amino- and carboxy- terminal tails.



## CHAPTER 2

### MATERIALS AND METHODS

#### Cell Culture

Sf-9 insect cells (Invitrogen) were grown as monolayers in Grace's insect medium (GIBCO) supplemented with 3.3 g/liter yeastolate (GIBCO), 3.3 g/liter lactalbumin hydrolysate (GIBCO) and 10% fetal calf serum (HyClone) at 27°C. Sf-9 cells were infected with recombinant virus at a multiplicity of infection of 5-10 and harvested 48-72 hr post-infection. For coinfection experiments, cells were infected at a ratio of 1:2 for the recombinant pp60<sup>v-src</sup> and Cx43 baculoviruses, respectively. Rat-1 and *v-src*-transformed Rat-1 fibroblasts were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum (HyClone) at 37°C in a 5% CO<sub>2</sub>-95% air incubator.

#### Construction of Recombinant Transfer Vector and Baculovirus

pVL1393-Cx43 was constructed from the transfer vector pVL1393 (PharMingen; Summers and Smith, 1987) and the Cx43 cDNA (G2 clone) (Beyer et al., 1987) contained in Bluescript (Stratagene). The Bluescript-G2 construct was digested with *Ban* I (New England Biolabs) and the 1.8 kb *Ban* I fragment containing the Cx43 cDNA was blunt-ended with Klenow (Promega) then digested with *Eco*R1 (USB) resulting in a 1.2 Kb fragment containing the entire

Cx43 coding sequence with a 10-base-pair 5' untranslated region. This fragment was then inserted into a *Sma*I-*Eco*R1 site of pVL1393 transfer vector resulting in the recombinant transfer plasmid, pVL1393-Cx43. All DNA fragments were resolved by 1% agarose electrophoresis and isolated using Gene Clean (Bio 101 Inc.)

Recombinant baculovirus was generated by cotransfecting a monolayer of Sf-9 cells with 0.5 µg of the recombinant transfer plasmid, pVL1393-Cx43 and 2 µg of the BaculoGold baculovirus DNA (Pharmingen). Non-recombinant, BaculoGold baculovirus have a lethal deletion and do not survive in the Sf-9 cells. Four days after cotransfection, supernatant medium containing the recombinant baculovirus was collected and subjected to one round of plaque purification. A Western blot analysis was performed to confirm recombinant baculovirus-mediated expression of Cx43.

### **Western Blot Analysis**

Expression of Cx43 in Sf-9 cells was examined by harvesting cells at specified time points. Cells were rinsed once with phosphate buffered saline (PBS), lysed in 200 µl lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 2 mM EDTA, 1% Triton X-100), and clarified in a Beckman TL-100 ultracentrifuge at 400,000Xg for 20 min at 4°C.

Samples for the biochemical subcellular localization experiments were obtained by lysing recombinant baculovirus-infected cells with cytosol extraction buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM EDTA pH 7.5, 1 mM EGTA, 25 µg/ml leupeptin and 25 µg/ml aprotinin) with 30 strokes in a Dounce homogenizer. The

samples were centrifuged at 131,000Xg for 20 min at 4°C in a Beckman TL-100 ultracentrifuge. The supernatant was collected and stored as the cytosolic fraction. The pellet was resuspended by passage through a 22 gauge, 3/8 inch needle 5 times in cytosol extraction buffer supplemented with 1% Triton X-100 followed by centrifugation at 400,000Xg for 20 min at 4°C in a Beckman TL-100 ultracentrifuge. The supernatant was collected and stored as the particulate fraction.

Protein concentrations were measured with the Bio-Rad protein assay kit. Whole and fractionated cell samples were boiled in an equal volume of 2X sample buffer (to achieve a final concentration of 2% SDS, 5% β-mercaptoethanol, 5% glycerol, 0.004% bromophenol blue, and 0.03 M Tris pH 6.8), separated on 12% SDS-polyacrylamide gels (Laemmli, 1970), and electrotransferred to Immobilon-P membranes (Millipore Corp.). The membranes were blocked overnight with blocking buffer (25 mM Tris buffered saline pH 7.4, 1% bovine serum albumin), washed 30 min with wash buffer (25 mM Tris buffered saline pH 7.4, 0.05% Tween 20, 1 mM EDTA), and incubated with rabbit antiserum directed against peptide 368-382 of Cx43 (anti-CT 368; 1:500 dilution) for two hr at room temperature. The membranes were then washed for 45 min in wash buffer, incubated with 1 μCi of [<sup>125</sup>I]-goat anti-rabbit IgG (ICN Pharmaceuticals, Inc.) for one hr at room temperature, and washed for 30 min to remove unbound secondary antibody. Membranes were then autoradiographed using Kodak X-OMAT XAR-5 film with the aid of an intensifying screen at -70°C. Immunoreactive bands

were excised and quantitated with a Micromedics 4-channel Gamma Counter.

### **Immunofluorescence Microscopy**

Sf-9 cells were infected as described above and harvested 48 hr post-infection. Cells were fixed in 3% paraformaldehyde/PBS pH 7.4 for 20 minutes at 4°C, washed 2 times in PBS, permeablized in 0.2% Triton X-100/PBS for 2 min at 4°C and washed 2 times in PBS. Cells were incubated with Cx43 rabbit antiserum (anti-CT 368; 1:500 dilution) overnight at 4°C, washed 2 times, and blocked with normal goat serum (1:50 dilution) for 30 min at 4°C. After washing twice with PBS, the cells were incubated with fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:80 dilution) (Sigma) for 1 hr at 4°C and washed 3 times in PBS. Cells were wet-mounted in 50% PBS/50% glycerol containing 1 mg/ml *p*-phenylenediamine (Sigma), visualized and photographed with a Zeiss Axioplane Universal microscope equipped with epifluorescence.

Rat-1 *v-src*-transformed cells were grown on coverslips under conditions described above then fixed in 3% paraformaldehyde/PBS pH 7.4 for 20 min at room temperature and washed with PBS. Cells were then permeablized in 0.2% Triton X-100/PBS pH 7.4 for 2 min at room temperature and washed twice in PBS and twice with 1% bovine serum albumin (BSA)/PBS. Cells were incubated 2 hr with the appropriate primary antibody at the following dilutions in 1% BSA/PBS: anti-CT 368, 1:1000 dilution; anti-*v-src* EB7 (a generous gift from Sarah Parsons, Univ. of Virginia), (1:700 dilution). After

washing 3 times with 1% BSA/PBS the cells were blocked with normal goat serum for 30 min and washed twice with 1% BSA/PBS. Cells were then incubated with either fluorescein isothiocyanate-conjugated goat anti-rabbit secondary antibody (1:80 dilution) (Sigma) or tetramethylrhodamine isothiocyanate-conjugated goat anti-mouse secondary antibody (1:64 dilution) (Sigma) followed by 3 washes in PBS. The cells were then mounted in 50% glycerol/ 50% PBS/ 1 mg/ml *p*-phenylenediamine (Sigma) and visualized with both the Zeiss Axioplane Universal microscope equipped with epifluorescence.

### **Confocal Microscopy**

Samples for confocal microscopy were prepared identically to those for epifluorescence. Images were acquired on a Bio Rad MRC-600 laser scanning system adapted to a Zeiss Axiovert 10 microscope with a 63X objective. Optical sections were approximately 1.5-2.0  $\mu\text{m}$  in height. Both fluorochromes were excited with a single argon laser source at 514 nm. The signals were split with filter blocks A1 (rhodamine) and A2 (fluorescein) and collected with the Bio Rad confocal microscopy software program, COMOS. The images were merged and processed in Adobe Photoshop 3.0.

### **Radiolabeling, Immunoprecipitation, and Quantitation of Cx43**

Sf-9 cells were infected with recombinant baculovirus encoding Cx43 as described above. At 24 hr post-infection, the cells were

radiolabeled with either [ $^{35}\text{S}$ ]-EXPRESS protein labeling mix (NEG-072; New England Nuclear) at 100  $\mu\text{Ci/ml}$  in Grace's methionine-free medium (GIBCO) or [ $^{32}\text{P}_i$ ] (NEX-053; New England Nuclear) at 0.5 mCi/ml in Grace's phosphate-deficient medium (Cell Culture Facility, University of California San Francisco) supplemented with 0.4% Fetal Calf Serum, 5% complete TNM-FH media, 20  $\mu\text{M}$  2-[N-morpholino]ethanesulfonic acid pH 6.1 (Sigma). Cells were labeled overnight (16 hr) at 27°C, harvested and washed once with PBS. Fibroblasts were grown to confluency and labeled with [ $^{35}\text{S}$ ]-EXPRESS protein labeling mix at 100  $\mu\text{Ci/ml}$  or [ $^{32}\text{P}_i$ ] at 0.5 mCi/ml in phosphate-deficient medium for 3 hr at 37°C. The cells were lysed in RIPA buffer (150 mM NaCl, 1% Na deoxycholate, 1% Triton X-100, 0.1% Na dodecylsulfate, 10 mM Tris pH 7.2, 50 mM NaF, 160  $\mu\text{M}$   $\text{Na}_2\text{VO}_4$ , 1 mM phenylmethyl sulfonyl fluoride [PMSF]), then clarified by centrifugation at 400,000Xg for 20 min at 4°C. Lysates were incubated with either non-immune rabbit serum or Cx43 rabbit antiserum. Immune complexes were precipitated with activated protein A-*Staphylococcus aureus*. Immunoprecipitated proteins were resolved on a 7.5-15% SDS-polyacrylamide gradient gel (Laemmli, 1970). Gels were dried and autoradiographed using Kodak X-OMAT XAR-5 film at -70°C. Gels containing [ $^{35}\text{S}$ ]-labeled proteins were fluorographed prior to drying. Gel pieces containing [ $^{35}\text{S}$ ]- or [ $^{32}\text{P}$ ]-labeled Cx43 proteins were excised, rehydrated, and counted in 7.5% Scintigest (New England Nuclear)-Scintiverse fluid (Fisher Scientific) using a Beckman LS5000 liquid scintillation counter.

## **Phosphoamino Acid Analysis**

[<sup>32</sup>P]-labeled Cx43 was immunoprecipitated and gel-purified as described. The proteins were electrotransferred to an Immobilon-P membrane (Millipore) and autoradiographed to determine the position of Cx43. The region of the membrane containing Cx43 was excised and rewetted. Cx43 was then directly acid-hydrolyzed from the membrane by incubating in 5.7 N HCl at 110°C for 60 min. The supernatant was lyophilized to a pellet and resuspended in 5-10 µl phosphoamino acid standards mix containing equal portions of P-ser: P-thr: P-tyr (Kamps and Sefton, 1989). The samples were then resolved on thin-layer cellulose plates (MN Polygram) in two dimensions in pH 1.9 buffer [2.5% (v/v) formic acid, 8.7% (v/v) acetic acid] at 1000V for 32 min and in pH 3.5 buffer [0.5% (v/v) pyridine, 5% (v/v) acetic acid] at 1000V for 21 min, as described in Kamps and Sefton (1989). The positions of the phosphoamino standards were revealed with 0.2% ninhydrin in ethanol. Autoradiography was performed with Kodak X-OMAT XAR-5 film with the aid of an intensifying screen at -70°C.

## **Preparation of Cx43 Monoclonal Antibodies** (completed by John Berestecky, Monoclonal Antibody Facility, University of Hawaii)

Monoclonal antibodies were made according to established techniques (Goding, 1983) against a synthetic peptide corresponding to amino acids 241-254 of the C-terminal cytoplasmic domain of Cx43. The peptide was conjugated to rabbit serum albumin via an

amino-terminal cysteine (Lerner et al., 1981) and BALB/C mice were immunized with the conjugate in Freund's adjuvant.  $5 \times 10^7$  immune spleen cells were fused with  $5 \times 10^7$  X63-Ag8.653 myeloma cells. Hybridoma clones were selected in HAT medium and supernatants were screened against the Cx43 peptide by ELISA. Two clones F-3 (IgG<sub>1</sub>) and D-7 (IgG<sub>1</sub>) out of the 208 hybridomas screened were found to be reactive with the Cx43 peptide and with Cx43 expressed both in fibroblasts and infected Sf-9 cells.

### **Preparation of Cx43 Immunoaffinity Matrix**

The immunoaffinity matrix was prepared with the D-7 monoclonal antibodies covalently bound to protein-G Sepharose 4B Fast Flow beads (Sigma). Briefly, mouse ascites fluid was subjected to a 50% ammonium sulfate precipitation (Harlow and Lane, 1988). The ammonium sulfate precipitate was resuspended in PBS and dialyzed overnight against PBS. The antibodies were then incubated with the protein G Sepharose beads overnight at 4°C with gentle end-over-end mixing. The beads were washed with 50 bed volumes of 200 mM triethanolamine (TEA) in PBS (pH 8.0), and incubated for 2 hr at room temperature with end-over-end mixing in 40 mM dimethylpalmilidate (DMP), 200 mM TEA, PBS (pH 8.0), followed by a 200 mM TEA (pH 8.0) wash and overnight incubation at room temperature in 40 mM DMP, 200 mM TEA (pH 8.0) with end-over-end mixing. The beads were finally washed with 200 mM TEA (pH 8.0), incubated with 40 mM DMP, 200 mM TEA (pH 8.0) for 2 hr and incubated in 40 mM at room temperature for 2 hr with end-over-

end mixing. The beads were thoroughly washed with PBS and stored at 4°C in PBS containing merthiolate to prevent bacterial contamination (Harlow and Lane, 1988; Schnider et al., 1982).

### **Immunoaffinity Purification of Cx43**

Cx43 was purified from recombinant baculovirus-infected Sf-9 cells. At 72 hr post-infection  $4 \times 10^8$  cells were harvested from a suspension culture and washed in PBS at 4°C. The following steps were completed at 4°C. Cells were disrupted with 75 strokes (pestle A) in a Dounce homogenizer in 5 ml homogenization buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 2 mM EDTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM PMSF). This homogenate was clarified in a Beckman TL100 ultracentrifuge at 100,000Xg for 30 min at 4°C. The supernatant was discarded and the pellet resuspended in 5 ml cell extraction buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.1% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, 25 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM PMSF) by gentle passage through a 22 gauge, 1-1/2 inch needle. The resuspended sample was Dounce homogenized with 20 strokes (pestle A) and incubated on ice for 15 min. The sample was clarified in a Beckman TL100 ultracentrifuge at 400,000Xg for 20 min at 4°C. The supernatant was incubated with 1 ml of Cx43 monoclonal antibody-protein G Sepharose for 3 hr with end-over-end mixing at 4°C. The slurry was then packed into a column and washed with 20 bed volumes of cell extraction buffer, 5 bed volumes of high salt wash buffer (10 mM Tris-HCl, pH 7.2, 1 M NaCl, 1% Triton X-100), 5 bed volumes of high

salt glycine wash buffer (100 mM glycine, pH 4.0, 1 M NaCl, 1 % Triton X-100), and 5 bed volumes of glycine wash buffer (100 mM glycine, pH 5.5, 1% Triton X-100). Cx43 was eluted from the immunoaffinity matrix with a low pH buffer (100 mM glycine, pH 2.5, 1% Triton X-100) and the eluate was neutralized immediately with 1 M Tris-HCl pH 8.0.

### **Purification of Activated pp60<sup>c-src</sup>**

The immunoaffinity column was generated by covalently coupling the monoclonal antibody (MAb 327) directed against pp60<sup>src</sup> (Lipsich et al., 1983, generously provided by Joan Brugge) to 1 ml protein-A agarose beads (Bio Rad) under the same conditions described above. Activated pp60<sup>c-src</sup> was immunoaffinity purified from Sf-9 cells infected with the recombinant baculovirus containing the c-src gene (Morgan et al., 1989, kindly provided by David Morgan). The purification steps were performed as previously described in Morgan et al. (1989). Briefly, two 100 mm dishes of Sf-9 cells infected with recombinant baculovirus expressing pp60<sup>c-src</sup> were harvested 48 hr post infection and lysed in 5 ml lysis buffer [50 mM HEPES pH 7.4, 1% (w/v) Triton X-100, 5 mM EDTA, 1 mM PMSF, 1 mg/ ml leupeptin]. The lysate was clarified on a Beckman TL100 ultracentrifuge at 20,000g for 30 min at 4°C. Glycerol was added to the clarified lysate to a final concentration of 10%. The lysate was then incubated with the immunoaffinity matrix as a slurry with gentle end-over-end mixing for 3 hr at 4°C. The matrix was packed onto the column and washed with 15 ml lysis buffer, 15

ml salt wash [50 mM HEPES pH 7.4, 1% (w/v) sodium cholate, 500 mM NaCl, 10% (v/v) glycerol], and 15 ml KSCN wash [1 M potassium thiocyanate (KSCN) pH 8.0, 1% (w/v) sodium cholate, 10% (v/v) glycerol]. The column was eluted with a high pH elution buffer [10 mM diethylamine pH 11.0, 1% (w/v) sodium cholate, 150 mM NaCl, 10% (v/v) glycerol, 1 mM dithiothreitol] in 500  $\mu$ l fractions which were immediately neutralized with 0.5 M Tricine pH 8.5. An *in vitro* autophosphorylation assay was completed with an aliquot of each elution fraction to detect the presence of pp60<sup>c-src</sup>.

### ***In vitro* Protein Kinase Assay**

*In vitro* protein kinase assays were performed using immunoaffinity purified, kinase-active pp60<sup>c-src</sup> and/or partially purified Cx43 in a reaction volume of 20  $\mu$ l containing 0.10  $\mu$ g of Cx43, 0.10  $\mu$ g of pp60<sup>c-src</sup>, kinase buffer (20 mM HEPES pH 7.4, 12 mM MnCl<sub>2</sub>, 20 mM MgCl<sub>2</sub>, 1 mM DTT, 100  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>) and 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP (NEN, NEG-0027, 6000 Ci/mmol) for 15 min at room temperature. The reactions were stopped by the addition of 2X sample buffer and boiling.

For depletion experiments, the kinase reaction was performed in two incubations. The first incubation included partially-purified Cx43, pp60<sup>c-src</sup>, kinase buffer, and 5 nM unlabeled ATP. Following incubation for 15 min at room temperature, pp60<sup>src</sup> was immunodepleted from the reaction mixture by the addition of pp60<sup>src</sup> monoclonal antibodies (MAb327) chemically coupled to Protein A agarose. The pp60<sup>src</sup> depleted sample was then divided into two

aliquots. To one portion, 10  $\mu$ Ci of  $\gamma$ -[ $^{32}$ P]ATP alone was added, and to the other portion, 10  $\mu$ Ci  $\gamma$ -[ $^{32}$ P]ATP plus purified pp60<sup>c-src</sup> were added. These reactions were then incubated an additional 15 min at room temperature to permit radiolabeling to occur. The reactions were stopped with the addition of 2X sample buffer and boiling. The proteins were resolved on a 12% SDS-polyacrylamide gel. The gels were dried and autoradiographed using Kodak X-OMAT XAR-5 film.

### **Generation of GST-Cx43CT and GST-Cx43 Fusion Proteins**

Nucleotide sequences between 907-1356 of the Cx43 cDNA corresponding to amino acids 236-382 (GST-Cx43CT) were amplified by polymerase chain reaction (PCR) (Perkin Elmer 480). The primer sequences for PCR amplification were 5'GTATGGATCCGTTAAGGATCGCGTCAAGGG3' and 5'CTATGAATTCGCCGGTTTAAATCTCCAGGT3' PCR amplification reactions were performed using the "hot start technique". In this technique nonspecific amplifications are kept to a minimum by keeping the primer and template separate until the temperature exceeds 80°C so that amplification begins at an elevated temperature. Ampli-Wax PCR Gems (Perkin Elmer) was added to 20  $\mu$ l lower PCR mix containing 10 mM Tris HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 2 mM MgCl<sub>2</sub>, 1 mM dNTP's and 600 pmol of each primer. The tubes were then placed into the thermal cycler and heated to 80°C for 5 min. At this time, the Ampli-Wax is melted forming a layer on top of the reaction mixture. When the tubes are

cooled to 25°C the Ampli-Wax resolidifies forming a barrier for the upper and lower mixtures. The upper PCR mix (30 µl) was then added to the tubes. This consists of 10 mM Tris HCl pH 8.3, 50 mM KCl, 0.01% gelatin, 1 unit Ampli-Taq (Perkin Elmer), and 50 ng template. The samples were then subjected to the following cycling conditions: one cycle of 94°C for 5 min, followed by 25 cycles of: 94°C for 1 min, 58°C for 1 min, 72°C for 1.5 min. The PCR amplified DNA products were then run on a 1% agarose gel and isolated using Gene Clean (Bio 101 Inc.). The PCR products were digested with *Bam*HI and *Eco*RI and directionally cloned into *Bam*HI and *Eco*RI sites of the pGEX-KG expression vector (Hakes and Dixon, 1992; generous gift from Jack Dixon). GST fusion proteins were expressed in *E. coli* upon induction with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hr at 37°C. The cells were lysed by brief sonication in PBS then incubated in 1% Triton X-100 for 15 min on ice. Insoluble material was removed by a 10 min spin on a microcentrifuge at 4°C. The lysate was then incubated with glutathione-Sepharose 4B beads (Pharmacia) for 30 min followed by extensive washes with PBS.

The cDNA for the whole Cx43 molecule was similarly PCR amplified with the appropriate primers (5'GTATGGATCCATGGGTGACTGCAGTGCCTT3' and 5'CTATGAATTCGCCGGTTTAAATCTCCAGGT3') and cloned into the pGEX-KG expression vector.

## ***In vitro* Phosphorylation of GST-Cx43CT Fusion Protein**

For *in vitro* kinase assays, 15  $\mu$ l of GST or GST-Cx43CT bound to glutathione-Sepharose 4B beads (washed once in kinase buffer) were incubated with purified kinase-active pp60<sup>c-src</sup>, kinase buffer (20 mM HEPES, pH 7.4, 12 mM MnCl<sub>2</sub>, 1 mM DTT, 100  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>) and 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]ATP (NEN, NEG-0027, 6000Ci/mmol) in a total volume of 40  $\mu$ l for 15 min at room temperature. Reactions were stopped with the addition of an equal volume of 2X sample buffer, boiled and resolved on a 12% SDS-polyacrylamide gel (Laemmli, 1970).

## **Two-Dimensional Tryptic Phosphopeptide Mapping**

Tryptic phosphopeptide maps of phosphorylated Cx43 were prepared as described in Boyle et al. (1991). Briefly, *v-src*-transformed Rat-1 fibroblasts were metabolically-labeled with 3 mCi of [<sup>32</sup>P]<sub>i</sub> for 3 hr at 37°C then solubilized in RIPA buffer. Cx43 was then immunoprecipitated from clarified lysates with CT368 peptide antiserum. For *in vitro* labeling experiments, partially-purified Cx43 from infected Sf-9 cells was phosphorylated by activated pp60<sup>c-src</sup> in *in vitro* kinase reactions as described above. The radiolabeled proteins were resolved on 12% SDS-polyacrylamide gels and autoradiographed. Gel pieces containing phosphorylated Cx43 were carefully sliced out of the wet, unfixed gel and ground into very fine pieces. Cx43 was then eluted from the gel pieces in 50 mM NH<sub>4</sub>HCO<sub>3</sub>

pH 7.3-7.6, 0.1% SDS, and 0.5%  $\beta$ -mercaptoethanol for 2-3 hr at room temperature. Following this step, the gel pieces were cleared from the mixture by spinning in a microcentrifuge for 10 min. Cx43 was then precipitated with 20% TCA using RNase A as a carrier protein. The precipitate was washed once with ethanol then resuspended in performic acid (nine parts 98% formic acid and one part hydrogen peroxide) and incubated on ice for 60 min. The performic acid was removed with a lyophilization step and the pellet was resuspended in 50  $\mu$ l 50 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.0 and 25  $\mu$ l (stock concentration, 1mg/ml) TPCK-trypsin (Worthington) then incubated overnight at 37°C. Another 10 mg TPCK-trypsin was added to the digest and incubated for 2 hr at 37°C the following day. After the digestion steps were completed, the ammonium bicarbonate was removed with repeated lyophilization. The sample was then resuspended in pH 1.9 electrophoresis buffer (2.5% (vol/vol) formic acid (88%), 7.8% (vol/vol) glacial acetic acid) and lyophilized to a sample volume of approximately 10  $\mu$ l. The sample was then spotted onto cellulose thin-layer chromatography (TLC) plates (Curtin Matheson Scientific) and electrophoresed in the first dimension using pH 1.9 buffer for 1.25 hr at 1000 V followed by ascending chromatography in the second dimension 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]. The positions of the phosphopeptides were determined by autoradiography using Kodak X-OMAT XAR-5 film at -70°C with the aid of an intensifying screen.

**CHAPTER THREE**  
**RESULTS-**  
**REGIONS OF COLOCALIZATION OF CONNEXIN43**  
**AND pp60<sup>v-src</sup> IN**  
**V-SRC-TRANSFORMED FIBROBLASTS**

Gap junctions consists of a few to thousands of hemiconnexons from one cell docked to hemiconnexons from another cell densely clustered into large plaques. In immunohistochemical studies these gap junction plaques appear as punctate reactions in the plasma membrane (Hertzberg et al., 1988).

Willingham et al. (1979) demonstrated that pp60<sup>v-src</sup> was located in the inner surface of the plasma membrane of *v-src*-transformed NRK cells and that it was particularly concentrated near the junctions between neighboring cells. The morphological appearance of the junctions were similar to gap junctions, but immunological identification was not performed to confirm their identification.

Since our efforts were focused on determining if Cx43 is a substrate of pp60<sup>v-src</sup>, we felt it important to determine if Cx43 and pp60<sup>v-src</sup> were in physical proximity of each other. Cx43 and pp60<sup>v-src</sup> may appear to be within physical proximity of each other when observed by epifluorescence microscopy. In reality, due to the limitations of standard microscopy they may be on different horizontal planes of the cell, appearing to be superimposed upon

each other. The confocal microscope may overcome this visual deception because of its ability to examine a thinner horizontal optical plane. Several groups have successfully demonstrated that the proteins of their studies were colocalized with the confocal microscope (Coghlan et al., 1995; Dickson et al., 1992). We felt it would also be a useful tool to determine if Cx43 and pp60<sup>v-src</sup> were colocalized in regions such as gap junction plaques of *v-src*-transformed fibroblasts.

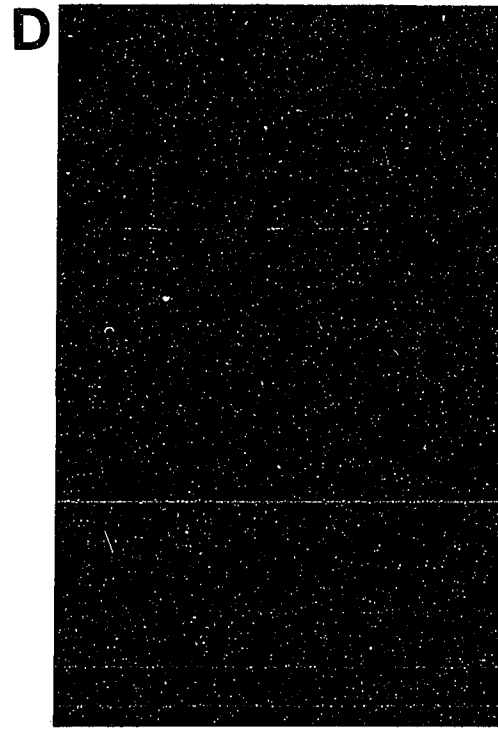
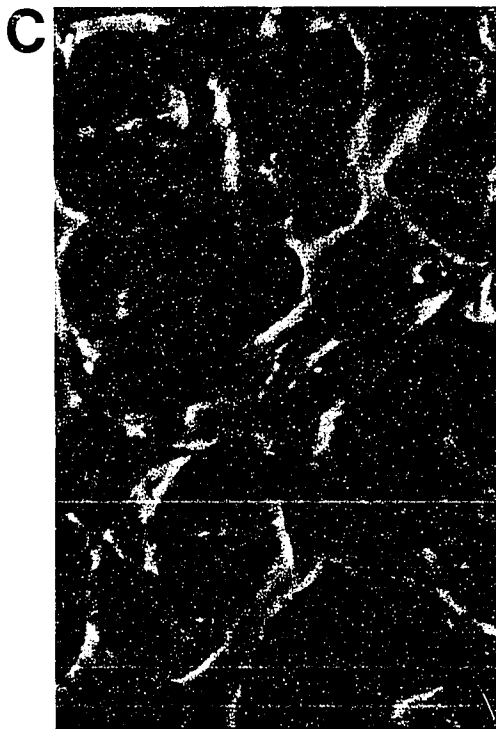
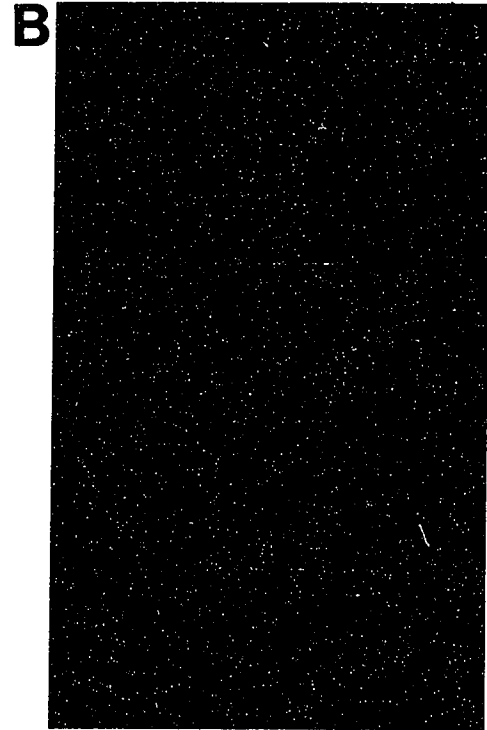
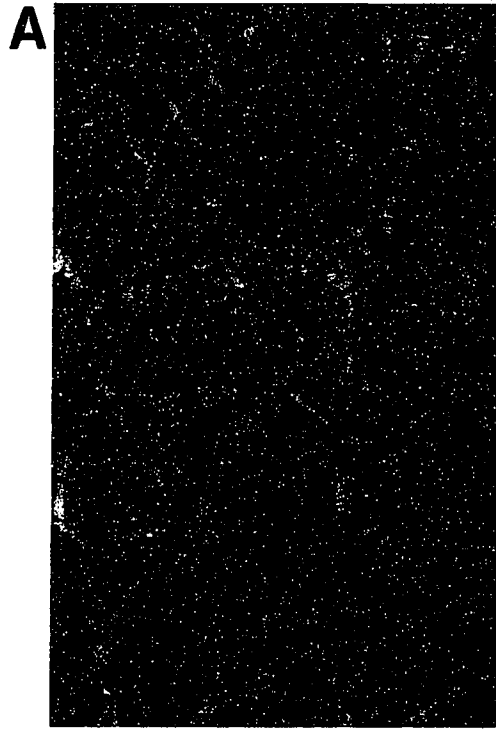
The confocal microscopes allows the investigator to optically section the sample into horizontal planes ranging from 1.0-2.0  $\mu\text{m}$  in height. It does so by collecting light that is reflected only from the plane of focus, filtering out light above and below the plane (Lichtman 1994).

The initial subcellular location of the two proteins were identified with primary rabbit and mouse antibodies directed against Cx43 and pp60<sup>v-src</sup>, respectively. The primary antibodies are then reacted to FITC conjugated goat anti-rabbit and TRITC conjugated goat anti-mouse antibodies and viewed with standard epifluorescence (Figure 3.1 and 3.2). Our results were consistent with previous studies demonstrating Cx43 as punctate reactions in the plasma membrane (Figure 3.1, panel D) (Crow et al., 1990; Hertzberg et al., 1988) and pp60<sup>v-src</sup> localized throughout the plasma membrane and associated with cytoplasmic structures believed to be perinuclear regions (Figure 3.2, panel D) (Shriver and Rohrschneider 1981; Willingham et al., 1979). Specificity of primary antibody immunostaining was confirmed by staining with secondary

antibody alone, which gave a faint, diffuse staining pattern for both FITC conjugated secondary antibody (Figure 3.1, panel B) and tetramethyl-rhodamine conjugated secondary antibody (Figure 3.2, panel B).

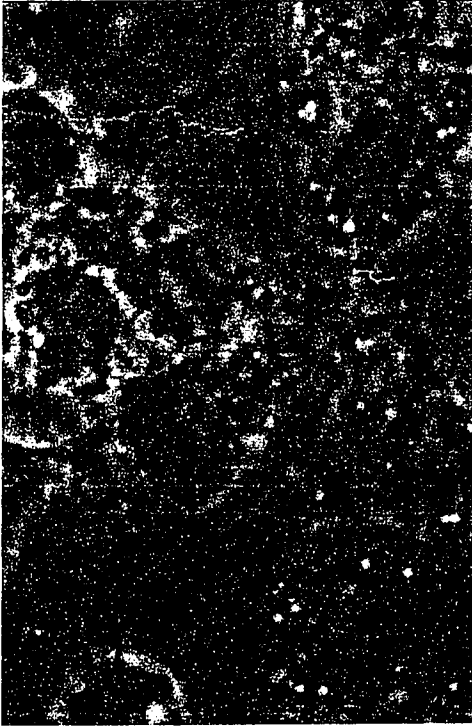
Individual confocal images of pp60<sup>v-src</sup> (Figure 3.3) and Cx43 (Figure 3.3) were obtained from the same optical plane then superimposed, demonstrating the presence of both proteins in overlapping regions in the plasma membrane (Figure 3.4). An optical section above and below the initial plane of focus was obtained to ensure that the region of colocalization was not smaller than the height of the optical section. If this region is smaller, we would not be capable of determining their location relative to each other within the optical plane. The gap junction plaques and pp60<sup>v-src</sup> were observed in all three optical sections. These results indicated that Cx43 and pp60<sup>v-src</sup> can be found in overlapping regions, suggesting that they are in physical proximity. Images of single labeled samples (Cx43 antibodies or pp60<sup>v-src</sup> antibodies) were obtained to confirm that the subcellular localizations of Cx43 and pp60<sup>v-src</sup> in the double labeled experiments were consistent with those of the single labeled samples. Similar staining patterns were observed for both Cx43 and pp60<sup>v-src</sup>, suggesting that binding of the primary antibodies did not interfere with each other in the double labeled samples.

**Figure 3.1. Subcellular localization of pp60<sup>v-src</sup> in v-src-transformed fibroblasts.** v-src-transformed fibroblasts were fixed, permeabilized, then examined by indirect immunofluorescent staining with pp60<sup>src</sup>-directed monoclonal antibody and TRITC-conjugated goat anti-mouse antibody as described in Materials and Methods. Phase (panel A and C) and fluorescent (panel B and D) images were photographed with a Zeiss Axioplane Universal microscope with epifluorescence.

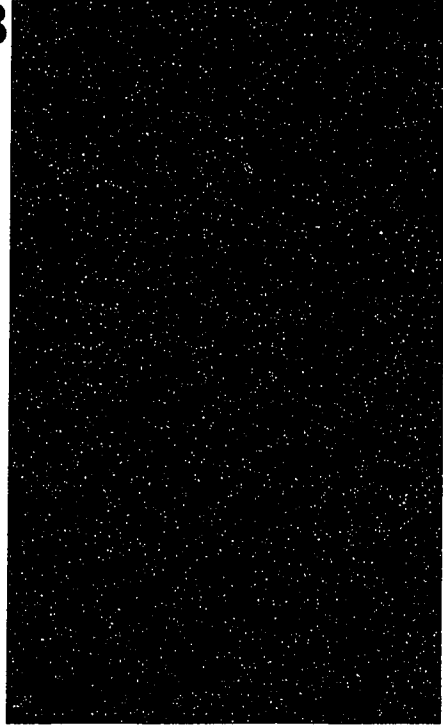


**Figure 3.2. Subcellular localization of Cx43 in *v-src*-transformed fibroblasts.** *v-src*-transformed fibroblasts were fixed, permeabilized, then examined by indirect immunofluorescent staining with Cx43-directed polyclonal antibody and FITC-conjugated goat anti-mouse antibody as described in Materials and Methods. Phase (panel A and C) and fluorescent (panel B and D) images were photographed with a Zeiss Axioplane Universal microscope.

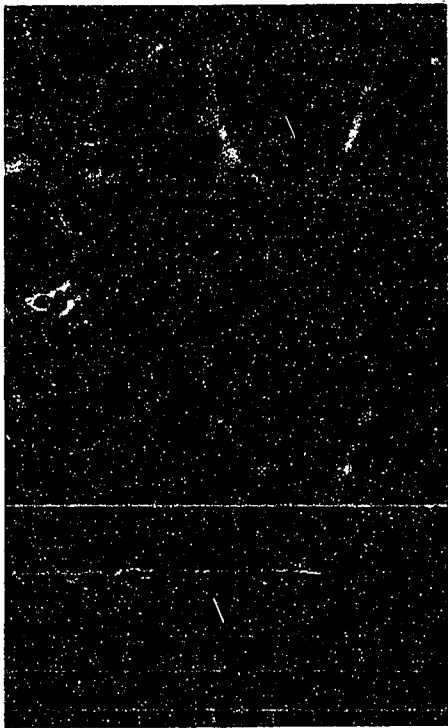
**A**



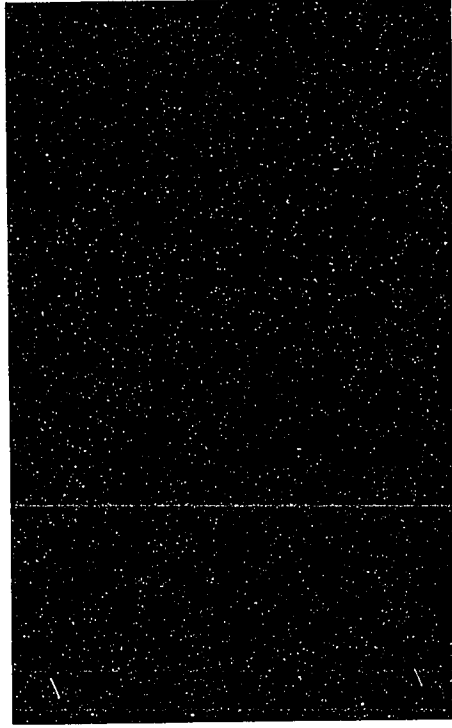
**B**



**C**



**D**

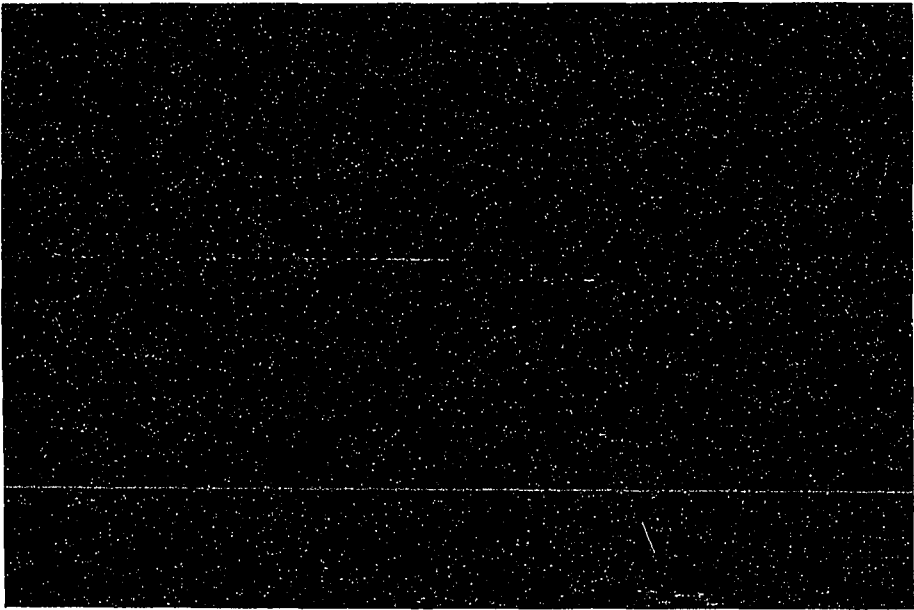


**Figure 3.3. Individual laser confocal microscopy images of pp60<sup>v-src</sup> and Cx43.** *v-src*-transformed fibroblasts were fixed, permeabilized, then examined by indirect immunofluorescent staining with pp60<sup>src</sup>-directed monoclonal antibody and TRITC-conjugated goat anti-mouse antibody, and Cx43 polyclonal antibody and FITC-conjugated goat anti-mouse antibody. The individual images were obtained with a BioRad MRC-600 laser scanning confocal microscope.

**pp60v-src**



**Cx43**



8 **Figure 3.4. Merged laser confocal microscopy images of pp60<sup>v-src</sup> and Cx43.**  
Individual confocal images of pp60<sup>v-src</sup> and Cx43 in *v-src*-transformed cells (Figure 3.3) were merged to demonstrate regions of overlap of the two proteins as indicated by arrowheads.



**CHAPTER FOUR**  
**RESULTS-**  
**CHARACTERIZATION OF BACULOVIRUS EXPRESSED**  
**CONNEXIN43**

**Expression of Cx43 in Sf-9 Cells**

The time course of Cx43 production in recombinant baculovirus-infected Sf-9 cells was examined by harvesting cells at the specified times after infection. Cell lysates prepared from an equal number of cells were examined by Western blotting with antibody against the C-terminal peptide of Cx43. We observed that Cx43 was produced in the infected cells as early as 24 hr post-infection at approximately 2.5 fold greater levels than detected in the Rat-1 fibroblast control (Figure 4.1A, lane 3 and 4.1B). Expression of Cx43 was even more dramatic at 48 and 72 hr post-infection with increases of 18- and 33- fold, respectively over Rat-1 fibroblast levels (Figures 4.1A, lanes 5 and 7 and Figure 4.1B). At these later times, multiple slower migrating forms of Cx43 were observed which are reminiscent of the phosphorylated Cx43 isoforms observed previously in mammalian cells (Crow et al., 1990; Lau et al., 1992). Uninfected Sf-9 cells did not express immunoreactive Cx43 (Figure 4.1A, lanes 2, 4, 6).

## **Subcellular Localization of Cx43**

Endogenously-expressed Cx43, like other members of the gap junction protein family, localize in the plasma membranes of mammalian cells or *Xenopus* oocytes microinjected with connexin mRNA (Swenson et al., 1990; Yancey, et al., 1989). To determine if Cx43 was also localized to the plasma membrane in recombinant baculovirus infected Sf-9 cells, biochemical subcellular fractionation of infected and uninfected Sf-9 cells was performed. Cytosolic and particulate proteins were separated by SDS-PAGE and Cx43 was detected by Western blot analysis. The particulate fraction of infected Sf-9 cells contained Cx43 with apparent molecular weights ranging from approximately 43-47,000 daltons (Figure 4.2A, lane 4). Cx43 was nearly undetectable in the soluble, cytosolic fraction (Figure 4.2A, lane 3) and it was not detected in either the particulate or cytosolic fractions of uninfected Sf-9 cells (Figure 4.2A, lanes 1 and 2).

To confirm these biochemical results and to identify the specific cellular membranes containing Cx43, immunofluorescence microscopy studies were conducted on Cx43 baculovirus-infected Sf-9 cells. Triton X-100 permeabilized Sf-9 cells were incubated with Cx43 C-terminal peptide antibody and the immune complexes were detected with fluorescein-conjugated secondary antibody. Infected cells demonstrated a strong Cx43 signal in the cell periphery with only a faint signal in the cytoplasm (Figure 4.2B, panel 3). These results are consistent with the notion that Cx43 is localized

predominantly to the plasma membranes of infected Sf-9 cells. Specificity of the Cx43 antibody reaction was demonstrated by the lack of a specific response in uninfected Sf-9 cells (Figure 4.2B, panel 1).

### **Phosphorylation of Cx43 in Sf-9 cells**

The postranslational modification of Cx43 by serine phosphorylation in various cells is associated with the modulation of channel gating activity and its membrane localization (Crow et al., 1990; Kanemitsu and Lau, 1993; Laird et al., 1991; Lau et al., 1991; Musil et al., 1990). Cx43 immunoprecipitated from fibroblasts migrates on SDS-polyacrylamide gels predominantly as the nonphosphorylated, 43 kDa form and two slower migrating bands of 45 and 47 kDa, each of which represents serine phosphorylated isoforms of Cx43 (Crow et al., 1990, 1992; Lau et al., 1992).

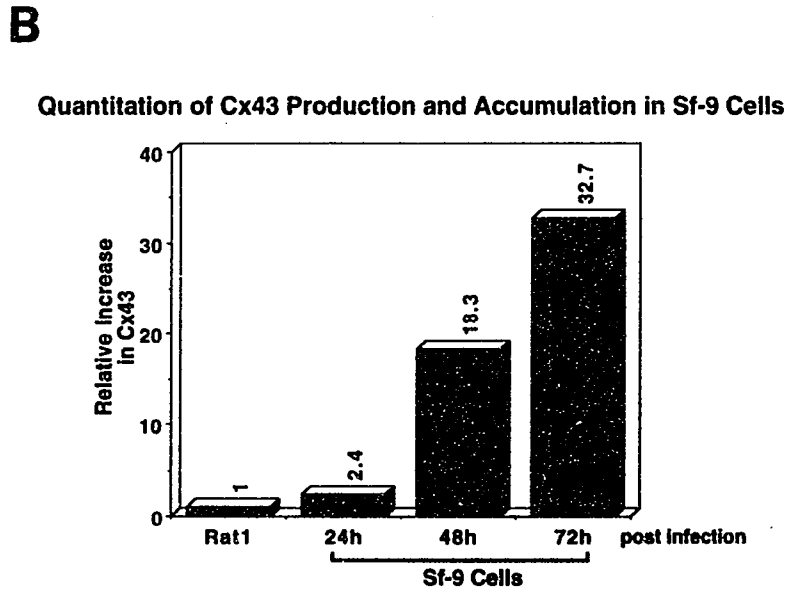
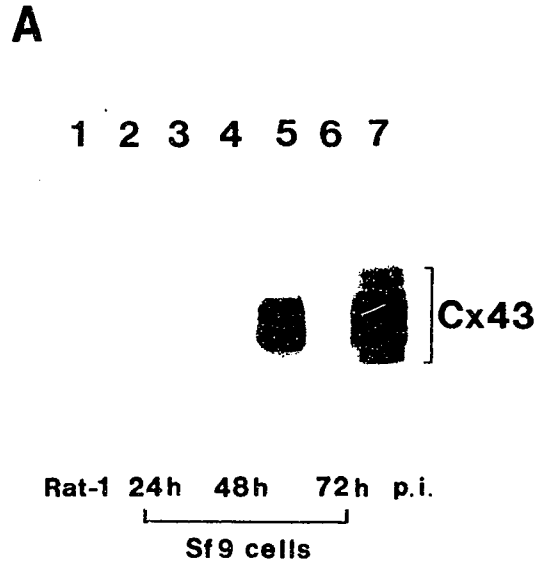
To determine if Cx43, expressed in recombinant baculovirus-infected Sf-9 cells, was also phosphorylated, [<sup>35</sup>S]- and [<sup>32</sup>P]-labeled Cx43 were immunoprecipitated from uninfected and infected Sf-9 cells and Rat-1 fibroblasts and resolved by SDS-PAGE. Five major forms of Cx43 with apparent molecular weights of 43, 44, 45, 47, and >47 kDa were isolated from [<sup>35</sup>S]-labeled infected, but not uninfected Sf-9 cells (Figure 4.3A, lanes 4 and 6). Immunoprecipitation of [<sup>32</sup>P]-labeled Cx43 from infected Sf-9 cells demonstrated that the slower migrating forms of Cx43 were phosphorylated (Figure 4.3B, lane 2). Four phosphoproteins with corresponding molecular weights of 44, 45, 47, and >47 kDa were detected, indicating that the Cx43

produced in the baculovirus expression system is phosphorylated in a manner similar to that observed in Rat-1 fibroblasts. However, there were differences in the extent of phosphorylation of the Cx43 isoforms isolated from the two cell types (Figure 4.3B, compare lanes 2 and 4). The predominant phosphorylated Cx43 in Rat-1 fibroblasts was the 45 kDa protein, whereas the 47 kDa form of Cx43 predominated in infected Sf-9 cells. Also, the >47 kDa band was readily detected in infected Sf-9 cells, but to a lesser extent in the Rat-1 fibroblasts. Phosphoamino acid analysis showed that Cx43 expressed in infected Sf-9 cells and Rat-1 fibroblasts contained predominantly phosphoserine (Pser) (Figure 4.3C).

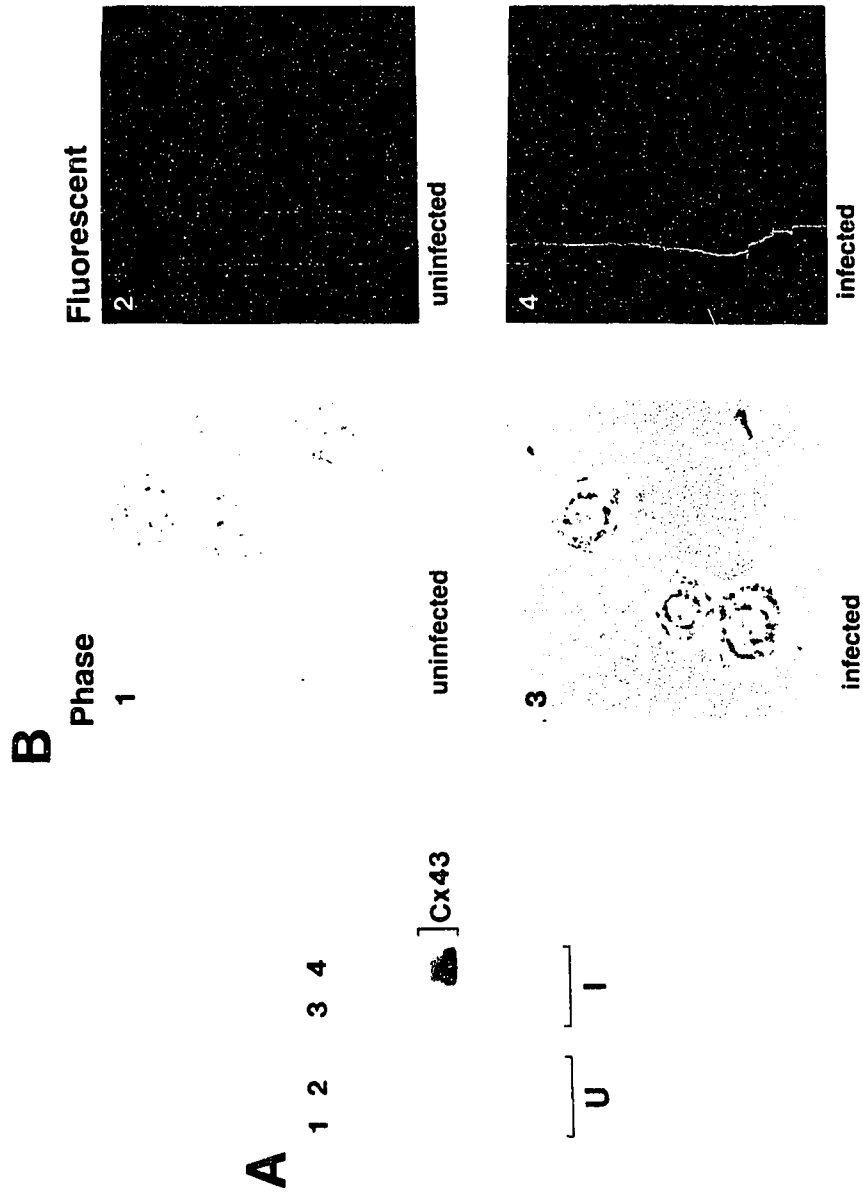
**Figure 4.1. Production and accumulation of Cx43 in recombinant baculovirus-infected Sf-9 insect cells.**

(A) Western Blotting of cell extracts from infected and uninfected Sf-9 cells harvested at the specified time points. Cell lysates from equal numbers of Rat-1 fibroblasts (lane 1), uninfected Sf-9 cells (lanes 2, 4, 6), and infected Sf-9 cells (lanes 3, 5, and 7) were resolved on a 12% SDS-polyacrylamide gel and transferred to an Immobilon-P membrane. The samples immunoblotted with Cx43 antibody and [<sup>125</sup>I]-goat anti-rabbit IgG, as described in the Materials and Methods.

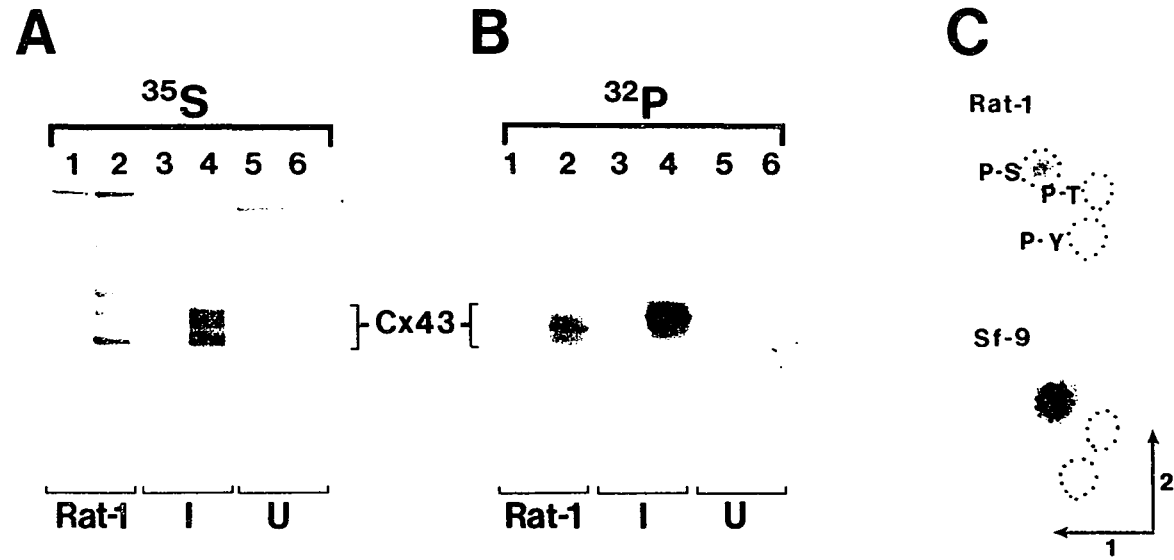
(B) Quantitation of Cx43 production in Sf-9 Cells. Immunoreactive protein bands were excised and quantitated with a Micromedics 4-channel Gamma Counter. The values were plotted as an increase of Cx43 in Sf-9 cells relative to the Cx43 level in Rat-1 fibroblasts.



**Figure 4.2. Localization of Cx43 in Sf-9 cells by biochemical subcellular fractionation and immunofluorescence microscopy.** (A) Cytosolic (lanes 1 and 3) and particulate (lanes 2 and 4) proteins of uninfected (lanes 1 and 2) and infected (3 and 4) Sf-9 cells 72 hr post infection were resolved on a 12% SDS-polyacrylamide gel, transferred to an Immobilon-P membrane and reacted with Cx43 antibody, as described in the Materials and Methods. (B) Uninfected (panels 1 and 2) and infected (panels 3 and 4) Sf-9 cells 48 hr post-infection were fixed, permeabilized, then examined by indirect immunofluorescent staining with Cx43 antibody and FITC-conjugated goat anti-rabbit antibody. Phase (panel 1 and 3) and fluorescent (panel 2 and 4) images were photographed with a Zeiss Axioplane Universal microscope with epifluorescence.



**Figure 4.3. Phosphorylation of Cx43 expressed in recombinant baculovirus-infected Sf-9 insect cells.** Rat-1 fibroblasts, uninfected (U), and infected (I) Sf-9 cells were labeled with either [<sup>35</sup>S]-Express (panel A) or [<sup>32</sup>P<sub>i</sub>] (panel B). Cx43 was immunoprecipitated from cell lysates with either nonimmune rabbit serum (odd numbered lanes) or antiserum against Cx43 (even numbered lanes) and resolved on 7.5-15% SDS-polyacrylamide gel as described in Materials and Methods. (C) Phosphoamino acid analysis of [<sup>32</sup>P]-Cx43 from the Rat-1 fibroblasts and infected Sf-9 cells. The positions of ninhydrin-stained unlabeled Pser (P-S), Pthr (P-T), and Ptyr (P-Y) are outlined. The direction of migration for the first dimension (pH 1.9) and second dimension (pH 3.5) on thin-layer cellulose plates are indicated by the arrows. All autoradiograms were prepared on Kodak XAR-5 film at -70°C.



**CHAPTER FIVE**  
**RESULTS-**  
***IN VITRO* PHOSPHORYLATION OF CONNEXIN43 BY**  
**pp60<sup>src</sup>**

**Immunoaffinity Purification of Cx43**

For *in vitro* phosphorylation studies, partial rapid purification of Cx43 was achieved by immunoaffinity chromatography using a monoclonal antibody against a Cx43 peptide (residues 241-254, clone D-7) covalently coupled to protein-G Sepharose. The solubilized, particulate fraction prepared from 72 hr post-infected Sf-9 cells was incubated with the immunoaffinity matrix (Figure 5.1, lane 1). Following extensive washing to remove nonspecific proteins, Cx43 was eluted from the matrix with a low pH buffer. Coomassie blue staining showed that the predominant protein isolated was the nonphosphorylated 43 kDa form of Cx43 (Figure 5.1, lane 5 and 6). The phosphorylated Cx43 isoforms were also present to a lesser extent in the purified preparations. The identity of this immunoaffinity product as Cx43 was confirmed by Western blotting utilizing a polyclonal antibody directed against C-terminal peptide of Cx43 (data not shown). Trace levels of non-Cx43 proteins were detected at 70 kDa and 31 kDa by Coomassie staining. The identity of these proteins is unclear at the present time.

### **pp60<sup>src</sup>-Mediated Phosphorylation of Cx43 *in vitro***

Previous studies have demonstrated that Cx43 is phosphorylated on tyrosine in mammalian cells transformed by pp60<sup>v-src</sup> (Filson et al., 1990, Crow et al., 1990; 1992) and in *Xenopus* oocytes microinjected with both Cx43 and pp60<sup>v-src</sup> mRNAs (Swenson et al., 1990). These studies implied that the tyrosine kinase responsible for phosphorylating Cx43 was pp60<sup>v-src</sup>. The availability of these purified proteins permitted us to examine the ability of pp60<sup>src</sup> to directly phosphorylate Cx43 in *in vitro* kinase assays. Several phosphorylated proteins were observed in these kinase reactions. One major phosphorylated protein of approximately 44-47 kDa comigrated with the Coomassie-stained, purified Cx43 (compare Figure 5.2A, lane 3 to Figure 5.2B, lane 4). The second major phosphorylated protein migrated around 60 kDa and represents autophosphorylated pp60<sup>c-src</sup>, since it comigrated with the major phosphorylated product present in reactions consisting only of pp60<sup>c-src</sup> (Figure 5.2A, lanes 1 and 3). Phosphoamino acid analyses of these *in vitro* phosphorylated products revealed only phosphotyrosine (Ptyr) (Figure 5.2C). In similar experiments, a baculovirus-expressed pp60<sup>c-src</sup>, purified independently under different conditions (Zhang et al., 1991; generously provided by Martin Broome, The Salk Institute), also phosphorylated Cx43 on tyrosine (data not shown). To determine if our Cx43 preparation contained endogenous kinases capable of phosphorylating Cx43, a kinase reaction was performed in the

absence of pp60<sup>c-src</sup>. No phosphorylated proteins were observed in this control reaction (Figure 5.2B, lane 2). These data are consistent with the idea that activated pp60<sup>src</sup> phosphorylates Cx43 directly *in vitro*.

To rule out the possibility that Cx43 was phosphorylated by an activated endogenous tyrosine kinase which was present in the partially-purified Cx43 preparation and activated by pp60<sup>src</sup> phosphorylation, we conducted a two stage kinase reaction in which pp60<sup>c-src</sup> was initially present, but then immunodepleted to reveal the activity of any activated endogenous tyrosine kinase(s). In this experiment, a complete kinase reaction, containing activated pp60<sup>c-src</sup> and Cx43, was first performed with unlabeled ATP (5 nM) to permit pp60<sup>c-src</sup> to phosphorylate and possibly activate any putative tyrosine kinase(s) present in the reaction mix. pp60<sup>c-src</sup> was then immunodepleted from this reaction mix by incubation with pp60<sup>src</sup> monoclonal antibody conjugated to protein A agarose beads and the kinase reaction was continued in the presence of 10  $\mu$ Ci of  $\gamma$ -[<sup>32</sup>P]-ATP to detect the activity of any putative, activated kinases. Under these experimental conditions, it was expected that any putative pp60<sup>src</sup>-activated tyrosine kinase might reveal itself in the second kinase reaction by phosphorylating Cx43 in the absence of pp60<sup>c-src</sup>. However, our results demonstrated that phosphorylation of Cx43 did not occur in pp60<sup>c-src</sup> immunodepleted reactions (Figure 5.3, lane 2). The absence of autophosphorylated pp60<sup>c-src</sup> in this reaction indicated that the immunodepletion of pp60<sup>c-src</sup> was effective (Figure 5.3, lane 2). Furthermore, when pp60<sup>src</sup> was added

back to the depleted reaction, Cx43 phosphorylation and pp60<sup>src</sup> autophosphorylation were observed which indicated that Cx43 was not altered or removed during the immunodepletion step and that the ability of the depleted reaction mix to support protein phosphorylation was not compromised. (Figure 5.3, lane 3). Thus, it is likely that the partially-purified Cx43 preparation did not contain any endogenous kinase(s) capable of phosphorylating Cx43. A control kinase reaction, performed in the presence of 5 nM unlabeled ATP with 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]-ATP, supported phosphorylation of Cx43 and pp60<sup>c-src</sup> (Figure 5.3, lane 1), which suggested that pp60<sup>c-src</sup> should have been capable of phosphorylating a putative tyrosine kinase present in the kinase reaction mix. The lower levels of radiolabeled Cx43 and pp60<sup>c-src</sup> observed in this reaction, compared to reactions containing 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]-ATP alone (see Figure 5.2B, lane 3), was probably because of dilution of the radiolabel by the unlabeled ATP.

To exclude the possibility that the tyrosine kinase, pp125<sup>FAK</sup> was associated with either pp60<sup>c-src</sup> or Cx43 during purification and that this tyrosine kinase was responsible for phosphorylating Cx43 *in vitro*. A Western blot of the purified samples was completed with polyclonal antibodies (BC3) against pp125<sup>FAK</sup> (Schaller et al., 1992). The results indicated that pp125<sup>FAK</sup> was not present in the purification samples, thus did not copurify with pp60<sup>c-src</sup> or Cx43 (data not shown).

We also constructed a recombinant plasmids to express GST-Cx43 and GST-Cx43CT (the carboxy-terminal tail region of the Cx43

molecule, amino acids 236-382) as fusion proteins in bacteria. In this construct, Cx43 or Cx43CT is fused to the carboxy-terminus of GST (glutathione S-transferase). The GST portion of the fusion protein facilitates rapid purification with glutathione agarose beads. Unfortunately, the expression levels of the GST-Cx43 fusion protein were very low. It was not readily detectable by Coomassie staining, but its presence was confirmed with Western blotting. Low expression levels may be due to the large size of the fusion protein (~70 KDa) and the extent of hydrophobicity (Smith and Corcoran, unpublished results). In contrast, expression levels for the GST-Cx43CT fusion protein were very high allowing for rapid purification of sufficient quantities of fusion protein for *in vitro* kinase reactions with activated pp60<sup>c-src</sup>.

The bacterially-expressed GST-Cx43CT protein was purified by absorption on glutathione-Sepharose. GST-Cx43 and phosphorylated by activated pp60<sup>c-src</sup> in an *in vitro* kinase reaction (Figure 5.4, lane 4). pp60<sup>c-src</sup> did not phosphorylate the GST portion of the fusion protein, indicating that phosphorylation events were occurring only on the Cx43CT portion (Figure 5.4 lane 3). A kinase reaction was complete in the absence of pp60<sup>c-src</sup> to rule out the possibility that endogenous kinases from the GST-Cx43 sample were phosphorylating the fusion protein. GST and GST-Cx43 were not phosphorylated in these reactions. (Figure 5.4, lanes 1 and 2).

## **Phosphotryptic Peptide Mapping of Cx43 Phosphorylated *in vivo* and in *in vitro* Kinase Reactions**

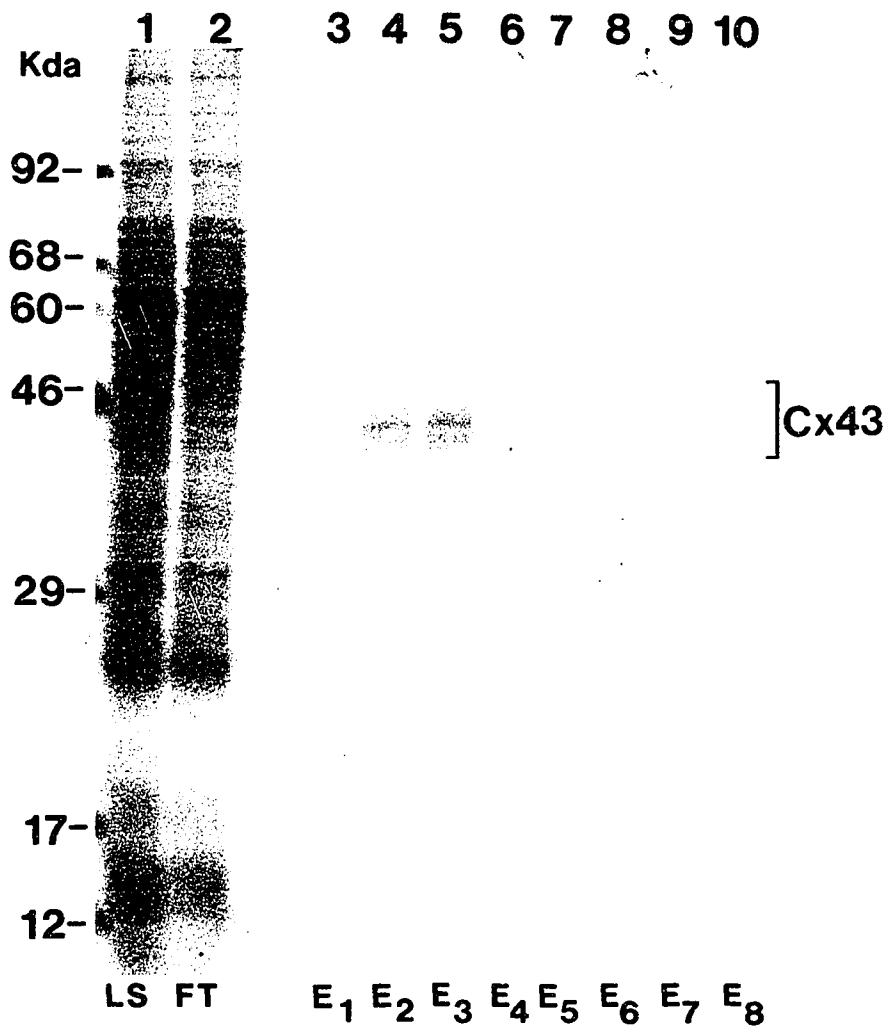
Two dimensional tryptic phosphopeptide analysis was performed to determine if the sites of Cx43 phosphorylation mediated by pp60<sup>c-src</sup> *in vitro* corresponded with sites of tyrosine phosphorylation on Cx43 from *in vivo* labeled *v-src*-transformed fibroblasts. Four phosphopeptides (labeled 1-4) of full length Cx43, purified from Sf-9 cells and phosphorylated *in vitro* by pp60<sup>c-src</sup>, migrated similarly to phosphopeptides from Cx43 radiolabeled with [<sup>32</sup>P<sub>i</sub>] in *v-src*-transformed cells (Figure 5.5, panels A and B). Analysis of a mixture of these two samples confirmed that the four phosphopeptides labeled *in vitro* comigrated with phosphopeptides from the *in vivo* labeled sample (Figure 5.5, panel C). Peptides 1 and 4 of Cx43 from *v-src*-transformed cells correspond to phosphotyrosine-containing peptides a and c previously reported by Kurata and Lau (1994). Phosphoamino acid analyses of peptides 2 and 3 revealed that they also contained P<sub>tyr</sub> (W. Kurata, unpublished observations).

Bacterially-expressed GST-Cx43CT fusion protein was generated to determine if the putative pp60<sup>src</sup>-mediated phosphorylation of Cx43 occurred in this domain. The GST-Cx43 was phosphorylated only on tyrosine in the presence of activated pp60<sup>c-src</sup> (Figure 5.5, panel D, inset). The tryptic peptide map of phosphorylated GST-Cx43CT had a similar pattern consisting of the same four phosphopeptides observed in the *in vitro* phosphorylated

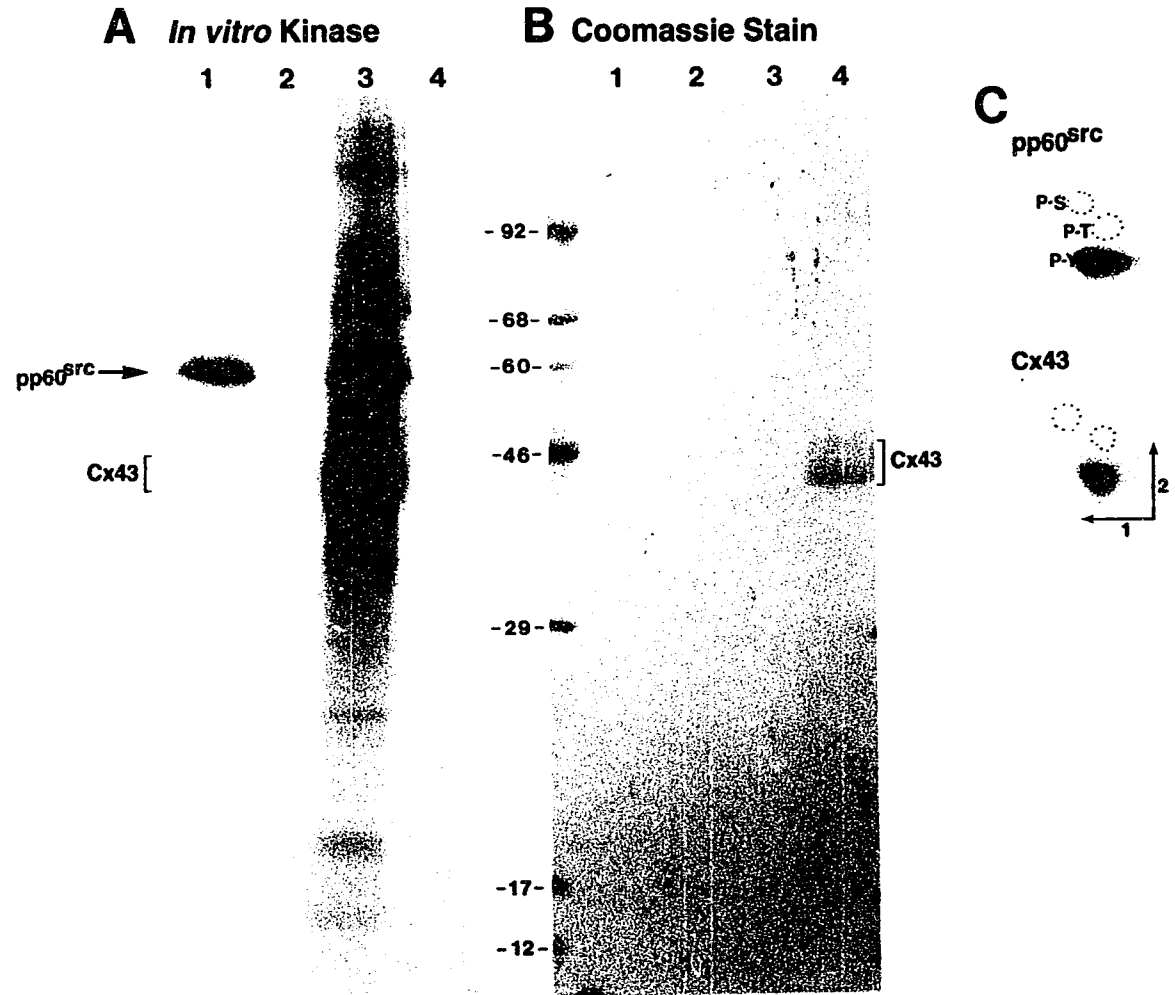
whole Cx43 isolated from Sf-9 cells (Figure 5.5, panel D). A mixture of the tryptic digests of the two samples demonstrated the comigration of the four phosphopeptides (Figure 5.5, panel E). However, the intensities of the phosphorylated peptides differed between the two samples. Peptide 4 represented the major phosphotryptic spot in the GST-Cx43CT protein, whereas peptides 1, 2 and 3 were the major phosphopeptides observed in full-length Cx43 isolated from the Sf-9 cells (Figure 5.5, compare panels B and D). Taken together, these results suggested that pp60<sup>src</sup>-mediated phosphorylation of Cx43 *in vitro* mimics similar phosphorylation events occurring in *v-src*-transformed fibroblasts and that the tyrosine phosphorylation of Cx43 may be occurring primarily in the carboxy tail region of the molecule.

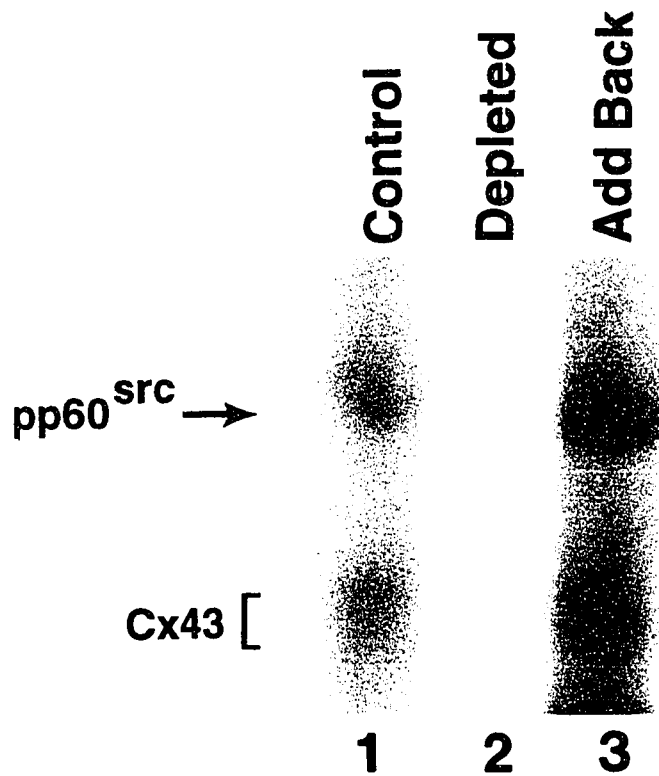
**Figure 5.1. Immunoaffinity purification of Cx43 from recombinant baculovirus-infected Sf-9 insect cells.**

Cx43 was purified from recombinant baculovirus-infected Sf-9 cells using a Cx43 monoclonal antibody chemically coupled to protein G Sepharose, as described in Materials and Methods. After extensive washing, Cx43 was eluted from the column with a low pH buffer. Samples were resolved on a 12% SDS-polyacrylamide gel and stained with Coomassie blue. Lanes 1 and 2 represent the loading sample (LS) and flow through (FT), respectively. Lanes 4-11 represent elution fractions 1-8 (E<sub>1-8</sub>). Cx43 was eluted predominantly in fractions 2 and 3 (lanes 4 and 5). The sizes of the molecular weight markers are as indicated at the left margin.

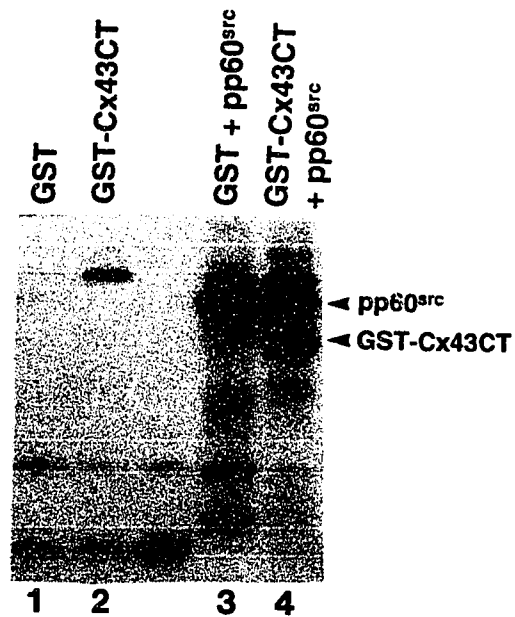


**Figure 5.2. Phosphorylation of Cx43 in pp60<sup>src</sup> kinase reactions.** pp60<sup>src</sup> and Cx43 were immunoaffinity-purified as specified in the Materials and Methods. (A) Results from the *in vitro* kinase assay with pp60<sup>src</sup> alone (lane 1), Cx43 alone (lane 2), and pp60<sup>src</sup> together with Cx43 (lane 3). (B) Coomassie stained SDS-polyacrylamide gel of the *in vitro* kinase reactions shown in panel B. Cx43 is indicated by the brackets. The samples were resolved on a 7.5-15% SDS-polyacrylamide gel. The gel was dried and autoradiographed at -70°C. The sizes of the molecular weight standards are indicated between panels A and B. Positions of phosphorylated pp60<sup>src</sup> and Cx43 are shown at the left. (C) Phosphoamino acid analysis of [<sup>32</sup>P]-pp60<sup>c-src</sup> and Cx43 from the *in vitro* kinase reaction. The positions of ninhydrin-stained, unlabeled Pser (P-S), Pthr (P-T), and Ptyr (P-Y) are outlined. The direction of migration for the first dimension (pH 1.9) and second dimension (pH 3.5) on thin-layer cellulose plates are indicated by the arrows.





**Figure 5.3. Phosphorylation of Cx43 in kinase reactions depleted of pp60<sup>src</sup>.** Control kinase reaction performed with pp60<sup>c-src</sup> and Cx43 in the presence of 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]-ATP and 5 nM ATP (lane 1). Kinase reaction performed first with pp60<sup>c-src</sup> and Cx43 in the presence of 5 nM ATP, pp60<sup>c-src</sup> was then immunodepleted, and the reaction was continued with 10  $\mu$ Ci  $\gamma$ -[<sup>32</sup>P]-ATP (lane 2). Kinase reaction performed as described for lane 2 but pp60<sup>c-src</sup> was added back to the depleted reaction (lane 3). Samples were resolved on a 10% SDS-polyacrylamide gel. The gel was dried and autoradiographed at -70°C. The positions of pp60<sup>c-src</sup> and Cx43 are indicated at the left margin.



**Figure 5.4. Phosphorylation of GST-Cx43CT in pp60<sup>src</sup> kinase reactions.** GST, GST-Cx43CT and pp60<sup>c-src</sup> were isolated as described in Materials and Methods. *In vitro* kinase reactions were performed on GST (lane 1) and GST-Cx43CT (lane 2) in the absence of pp60<sup>c-src</sup>. *In vitro* kinase reactions were performed on GST (lane 3) and GST-Cx43CT (lane 4) in the presence of pp60<sup>c-src</sup>. Samples were resolved on a 12% mini-gel, Coomassie stained, dried, and autoradiographed at -70°C. The positions of pp60<sup>c-src</sup> and GST-Cx43CT are indicated in the right margin.

**Figure 5.5. Two-dimensional tryptic phosphopeptide maps of [<sup>32</sup>P]-labeled Cx43.** Cx43 from radiolabeled *v-src*-transformed fibroblasts was immunoprecipitated with Cx43 CT368 peptide antiserum and resolved on a SDS-polyacrylamide gel as described in the Materials and Methods . Partially purified, full length Cx43 from infected Sf-9 cells and the GST-carboxy-tail Cx43 fusion protein were phosphorylated *in vitro* in the presence of pp60<sup>c-src</sup> as described in the Materials and Methods. Phosphotryptic peptides of: A, full length Cx43 phosphorylated *in vitro*; B, Cx43 from *v-src*-transformed fibroblasts; C, mixture of *in vivo* labeled Cx43 and full length Cx43 phosphorylated *in vitro*; D, GST-Cx43CT phosphorylated *in vitro* E, mixture of GST-Cx43CT phosphorylated *in vitro* and full-length Cx43 phosphorylated *in vitro*. Origins are indicated by the arrowheads. The direction of migration are indicated by the arrows. The inset in panel D shows the phosphoamino acid analysis results of the phosphorylated GST-Cx43CT fusion protein. The positions of the unlabeled Pser (P-S), Pthr (P-T), and Ptyr (P-Y) standards are outlined.

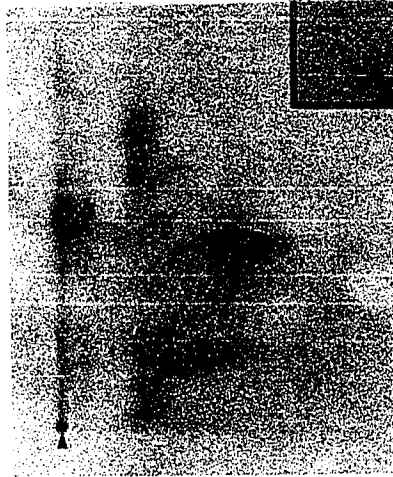
**A** *in vitro* Cx43



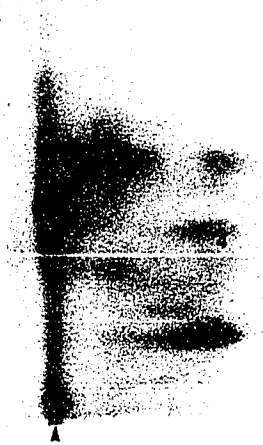
**B** *in vivo* Cx43



**D** *in vitro* GST-Cx43CT



**C** *in vivo* Cx43  
*in vitro* Cx43



**E** *in vitro* GST-Cx43 CT  
*in vitro* Cx43



**CHAPTER SIX**  
**RESULTS-**  
**PHOSPHORYLATION OF CONNEXIN43 IN Sf-9 CELLS**  
**COINFECTED WITH pp60<sup>v-src</sup>**

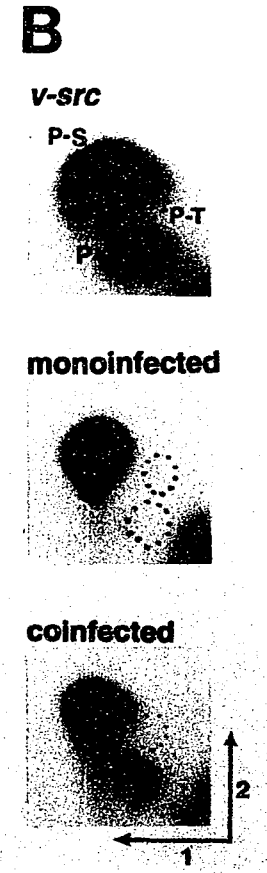
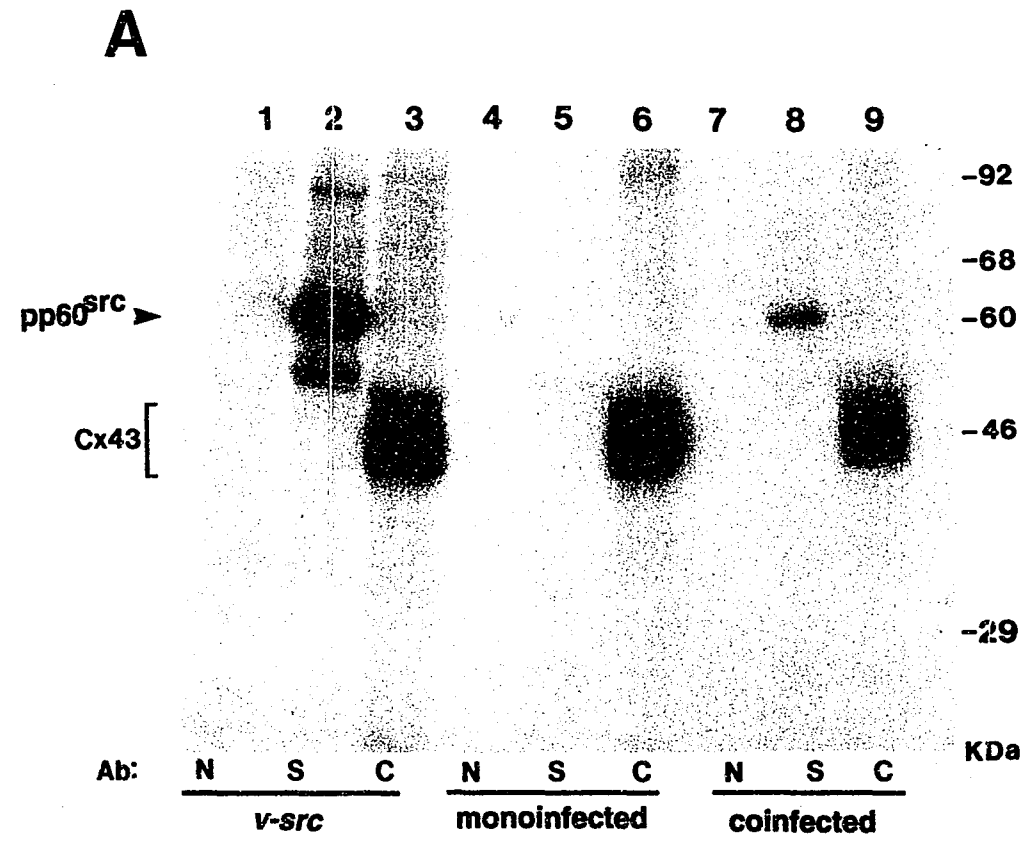
**Phosphorylation of Connexin43 in Sf-9 Cells  
Coinfected with pp60<sup>v-src</sup> and Connexin43  
Recombinant Baculoviruses**

Several studies have utilized Sf-9 cells coinfecting with recombinant baculoviruses to demonstrate protein kinase-substrate interactions (Park et al., 1992; Piwnica-Worms et al., 1990; Vik et al., 1990). We also examined the ability of pp60<sup>v-src</sup> to phosphorylate Cx43 in coinfecting Sf-9 cells. Phosphorylated forms of both Cx43 and pp60<sup>v-src</sup> were immunoprecipitated from these cells (Figure 6.1A, lanes 8 and 9). Detectable levels of pp60<sup>src</sup> were immunoprecipitated from the *v-src*-transformed cells and the cells the coinfecting Sf-9 cells but not the monoinfecting Sf-9 cells. This indicated that the coinfecting Sf-9 cells were expressing pp60<sup>v-src</sup> and that the monoinfecting Sf-9 cells were not. Cx43 from the coinfecting cells was phosphorylated primarily on Pser, but also contained levels of P<sub>tyr</sub> (Figure 6.1B) that was comparable to the phosphorylated Cx43 expressed in *v-src*-transformed Rat-1 fibroblasts (Figure 6.1a, lane 3 and Figure 6.1B). In contrast, Cx43 expressed in Sf-9 cells infected with the Cx43 baculovirus alone was phosphorylated only on serine residues as described previously

(Figure 6.1A, lane 6 and Figure 6.1B). Thus, these coinfection experiments further support the conclusion that a direct kinase-substrate interaction between pp60<sup>v-src</sup> phosphorylates Cx43 exists.

**Figure 6.1. Phosphorylation of Cx43 in Sf-9 insect cells coexpressing pp60<sup>v-src</sup>.**

(A) Rat-1 *v-src* transformed fibroblasts, monoinfected (Cx43 recombinant baculovirus), and coinfecting (Cx43 and pp60<sup>v-src</sup> recombinant baculoviruses) Sf-9 cells were labeled with [<sup>32</sup>P<sub>i</sub>]. Cell lysates were immunoprecipitated with either nonimmune rabbit serum (lanes 1, 4, and 7), MAb 327 monoclonal antibody specific for pp60<sup>src</sup> (lanes 2, 5, and 8), or antiserum directed against Cx43 (lanes 3, 6, and 9). Immunoprecipitates were resolved on a 7.5-15% SDS-polyacrylamide gel and autoradiographed. The positions of pp60<sup>v-src</sup> and Cx43 are shown at the left. Molecular weight standards are shown at the right. (B) Phosphoamino acid analysis of [<sup>32</sup>P]-Cx43. The positions of ninhydrin-stained, unlabeled Pser (P-S), Pthr (P-T), and P Tyr (P-Y) are outlined. The direction of migration of the first dimension (pH 1.9) and second dimension (pH 3.5) are indicated by the arrows.



## CHAPTER SEVEN

### DISCUSSION AND CONCLUSIONS

Our laboratory and others have previously demonstrated that Cx43 is phosphorylated on tyrosine in Rous sarcoma virus transformed fibroblasts (Crow et al., 1990; Filson et al., 1990). This phosphorylation event is tightly associated with the kinase activity and membrane localization of pp60<sup>src</sup>. In cells expressing temperature-sensitive pp60<sup>v-src</sup>, phosphorylation of Cx43 on tyrosine correlated closely with activation of the pp60<sup>v-src</sup> kinase activity and the reduction of gjc (Crow et al., 1992). Moreover, tyrosine phosphorylation of Cx43 and disruption of its function were not observed in cells containing kinase-active, but nonmyristylated pp60<sup>src</sup>, which does not localize to plasma membranes (Crow et al., 1992; Filson et al., 1990). In this study, we present data which strongly suggests that pp60<sup>src</sup> is one of the tyrosine kinases responsible for directly phosphorylating Cx43 in *src*-transformed fibroblasts. In support of this conclusion, we have demonstrated that: 1) Cx43 and pp60<sup>v-src</sup> are in physical proximity in regions such as gap junction plaques of *v-src*-transformed cells. 2) Purified, activated pp60<sup>c-src</sup> phosphorylates Cx43 in *in vitro* kinase reactions. 3) The tryptic phosphopeptides of *in vitro* phosphorylated Cx43 represent a subset of those obtained from Cx43 metabolically-labeled in *v-src*-transformed cells. 4) pp60<sup>src</sup> phosphorylated Cx43 at the carboxy-terminal tail region. 5) Cx43 is phosphorylated on tyrosine

in Sf-9 cells coinfecting with both Cx43 and pp60<sup>v-src</sup> recombinant baculoviruses.

The confocal microscope was used to determine if Cx43 and pp60<sup>v-src</sup> were in physical proximity for their potential interaction. The confocal microscope, unlike standard epifluorescence, is capable of partitioning the cell into optical sections of approximately 1-2  $\mu\text{m}$  in height. Cx43 and pp60<sup>v-src</sup> appeared to be present in overlapping regions in the plasma membrane (Figure 3.4). The regions of overlap occur in or near the gap junction plaques, where Cx43 is present. These observations are consistent with the electron microscopic results of Willingham et al. (1979), which demonstrated that immunocytochemically-labeled pp60<sup>v-src</sup> was present in the gap junction-like structures.

We chose the baculovirus eucaryotic expression system for the purification of Cx43 because it has been used successfully to express high levels of native, post-translationally-modified protein (Alnemri et al., 1991; Morgan et al., 1989; Ramer et al., 1991). Cx43 was expressed in the recombinant baculovirus-infected Sf-9 cells at levels 33 times greater than those observed with Rat-1 fibroblasts (Figure 4.1). Biochemical subcellular fractionation and immunofluorescence microscopy demonstrated that Cx43 was found in the plasma membranes of the Sf-9 cells (Figure 4.2). We also observed that the expressed Cx43 was postrationally-modified by endogenous serine kinases in insect cells (Figure 4.3).

Baculovirus-expressed Cx43, partially purified with a monoclonal antibody immunoaffinity matrix, was used in *in vitro*

kinase assays with immunoaffinity-purified, activated pp60<sup>c-src</sup> to demonstrate that Cx43 is phosphorylated on tyrosine in the presence of activated pp60<sup>src</sup> (Figure 5.1). Several control reactions assured us that the tyrosine kinase responsible for phosphorylating Cx43 *in vitro* was indeed pp60<sup>src</sup>. First, kinase reactions performed with the partially-purified preparation of Cx43 alone did not result in the phosphorylation of Cx43 (Figure 5.2B). Second, because it was possible that another tyrosine kinase, which required activation by pp60<sup>src</sup>, was present in either the pp60<sup>src</sup> or Cx43 preparations. We performed a two-stage kinase reaction in which the putative tyrosine kinase was first activated by pp60<sup>src</sup>. Its activity was then measured in the second stage in the absence of pp60<sup>src</sup>. This experimental protocol failed to produce *in vitro* phosphorylated Cx43. However, Cx43 from the pp60<sup>src</sup>-depleted reaction mixture could be phosphorylated when pp60<sup>src</sup> was added back (Figure 5.3). Therefore, these results suggest that Cx43 is phosphorylated directly by pp60<sup>src</sup> in these *in vitro* assays.

This conclusion was reinforced by our demonstration of pp60<sup>src</sup>'s ability to directly phosphorylate a GST-Cx43CT fusion protein expressed in bacteria. This substrate, purified from bacterial cell lysates on glutathione-Sepharose, is unlikely to contain endogenous tyrosine kinases capable of phosphorylating Cx43 *in vitro* because bacteria such as *E. coli* do not possess endogenous tyrosine kinases.

To examine whether the ability of pp60<sup>src</sup> to phosphorylate Cx43 *in vitro* reflected its ability to do so in intact *v-src*-transformed

cells, we compared the phosphotryptic peptides of *in vitro* and *in vivo* phosphorylated Cx43 by two-dimensional peptide mapping. These experiments demonstrated that four of the Cx43 peptides phosphorylated *in vitro* represented a subset of those obtained from Cx43 phosphorylated in *v-src*-transformed fibroblasts (peptides 1-4, Figure 5.4). The corresponding four phosphopeptides of *in vivo* labeled Cx43 contained phosphotyrosine (unpublished observations). Peptides 1 and 4 (Figure 5.4) were identified in a previous study characterizing the phosphorylation of Cx43 in *v-src*-transformed cells (labeled as peptides a and c in Kurata and Lau, 1994). Peptides 2 and 3 (Figure 5.4) were also detected in this previous study, but at a reduced level of phosphorylation (Figure 3 of Kurata and Lau, 1994). The reasons for the differences in phosphorylation efficiency of peptides 2 and 3 are not clear at this time.

We also examined the phosphorylation of Cx43 in Sf-9 cells coinfecting with the Cx43 and pp60<sup>v-src</sup> baculoviruses. Phosphoamino acid analysis demonstrated that the Cx43 expressed in these coinfecting cells contained both P<sub>tyr</sub> and P<sub>ser</sub>. In contrast, Cx43, expressed in Sf-9 cells infected with only the Cx43 recombinant baculovirus, contained only P<sub>ser</sub> (Figure 6.1). Our attempts to measure an alteration of the gating activity of Cx43 in Sf-9 cells, expressed in the presence or absence of pp60<sup>v-src</sup>, were unsuccessful due to the decreased adhesiveness of the cells following baculovirus infection.

Taken together, these data demonstrated that Cx43 is phosphorylated directly by pp60<sup>src</sup> in *in vitro* kinase reactions and

support the hypothesis that Cx43 is phosphorylated by pp60<sup>v-src</sup> in intact *v-src*-transformed fibroblasts. However, because of the existence of enzymes like pp125<sup>FAK</sup>, which is associated with pp60<sup>v-src</sup>, we cannot exclude the possibility that Cx43 may also be phosphorylated *in vivo* by other tyrosine kinases besides pp60<sup>src</sup>.

The apparent ability of pp60<sup>src</sup> to phosphorylate four phosphotryptic peptides of full length Cx43 suggested the possibility of multiple Tyr sites in Cx43. These results differ from those published previously which indicated that phosphorylation of tyrosine 265 in Cx43 expressed in *Xenopus* oocytes was sufficient to disrupt junctional conductance (Swenson et al., 1990). Because these multiple phosphopeptides may possibly result from events such as incomplete tryptic cleavage or differential serine phosphorylation, we are directly identifying the Tyr sites present in these four Cx43 tryptic peptides. The same phosphopeptides also resulted from pp60<sup>src</sup>'s phosphorylation of the GST-Cx43CT fusion protein (Figure 5.4), which contains the C-terminal cytoplasmic region of Cx43. Therefore, the potential phosphorylation sites of interest must be located between amino acids 236-382 of Cx43. Thus, one of the four phosphopeptides from Cx43, phosphorylated in *v-src*-transformed fibroblasts, may also contain phosphorylated tyrosine 265.

Site-directed mutagenesis studies will help us to clarify which tyrosines are being phosphorylated. This will be accomplished by mutating candidate tyrosines to phenylalanines in the GST-Cx43CT construct. The mutated GST-Cx43CT protein will then be used in *in vitro* kinase assays with kinase-active, purified pp60<sup>src</sup> to determine

if tyrosine phosphorylation events continue to occur in the absence of the respective tyrosine(s).

We demonstrated that the site(s) of phosphorylation occur in the carboxy-terminal tail region of Cx43. This region is less conserved among the members of the gap junction family of proteins and this divergence is believed to function by facilitating differential regulation of the various gap junction channels. Since the carboxy-terminal tail is thought to regulate the gating of the channel, it is conceivable that pp60<sup>v-src</sup> downregulates gjc by directly phosphorylating Cx43 on tyrosine residue(s) in this region.

An increased serine phosphorylation on Cx43 has also been observed in *v-src*-transformed cells (Filson et al., 1990; Kurata and Lau 1994). It is possible that pp60<sup>v-src</sup> controls gating activity by phosphorylating Cx43 and by initiating a cascade of signals which activate other kinases to phosphorylate Cx43. *v-src* has been demonstrated to activate protein kinase C and thus may induce serine phosphorylation of Cx43 by this mechanism (Qureshi et al., 1991; Spangler et al., 1989). pp60<sup>v-src</sup> also associates with Shc (McClade et al., 1992; Rozakis Adcock et al., 1992) and activates the *ras-raf* signalling pathway which may result in mitogen-activated protein (MAP) kinase-mediated phosphorylation of Cx43 (Brott et al., 1991; Bouton et al., 1991). Additional *v-src* dependent serine kinases may also be involved but are yet to be identified. Serine phosphorylation of Cx43 induced by the *ras* oncogene or EGF is correlated with down-regulation of gjc (Brissette et al., 1991; Kanemitsu and Lau, 1993; Lau et al., 1992). EGF's effects on Cx43

phosphorylation are independent of PKC, but may involve activated MAP kinase (Kanemitsu and Lau, 1993). The function of the observed increase in serine phosphorylation in *v-src*-transformed cells is at present unknown. It is possible that a combination of serine and tyrosine phosphorylation of Cx43 contribute to the regulation of the gap junction channel.

Attempts to understand the role of pp60<sup>src</sup>-mediated tyrosine phosphorylation on Cx43 can be addressed with experiments utilizing site-directed mutants possessing tyrosine to phenylalanine mutations. The mutated Cx43 and pp60<sup>v-src</sup> can then be cotransfected into cells to determine if gjc is downregulated in the absence of phosphotyrosine. If tyrosine phosphorylation is a critical parameter for downregulating gjc, then site-directed mutants of Cx43 should maintain similar communications levels in the presence or absence of pp60<sup>v-src</sup>. These experiments will also elucidate the role of serine phosphorylation in regulating gjc. As mentioned above, serine phosphorylation on Cx43 increases in *v-src*-transformed cells. For example, if we observe that gjc remains down regulated in the absence of phosphotyrosine on Cx43, then would be reasonable to conclude that serine phosphorylation events are directly responsible for the decrease in gjc. The control of gating activities in *v-src*-transformed cells may in fact require a critical balance of both serine and tyrosine phosphorylation. Experiments with these site-directed mutants will yield very critical information regarding the role of both tyrosine and serine phosphorylation on Cx43 in *v-src*-transformed fibroblasts.

An alternative approach to understand the effects of tyrosine phosphorylation on gjc involves the reconstitution of hemichannels in lipid bilayers. This *in vitro* method facilitates a controlled environment. Gating activities can be measured in the presence or absence of a purified kinase and it has the added benefit of reconstituting the hemichannel with wild type or mutated forms of Cx43.

It is difficult to speculate how tyrosine phosphorylation could potentially control the gating activities of the gap junction channel because the system may involve a complex array of interactions involving the Cx43 molecules or auxiliary molecules which associate and interact with the connexon. However, one possibility may be that the phosphorylation events result in a conformational change, such as a difference in folding at the carboxy-terminal tail which prevents flow through the channel. Another possibility could be that the combination of the inherent structure of tyrosine, which contains a phenyl side chain and the negative charge from the phosphate on tyrosine serves to repel the passage of certain ions and small molecules. This may not be achieved with phosphorylated serine residues because serine does not possess the protruding phenyl side chain that tyrosine has to potentially hinder the flow through the channel. Alternatively, the tyrosine phosphorylation sites on Cx43 may serve to dock SH2 containing proteins at the hemichannel, physically obstructing the flow through the channel. We hope to gain a better understanding of the mechanisms of tyrosine phosphorylation on Cx43 with continued use of the reagents and

information developed in this thesis effort. Ultimately, we intend to dissect the events which contribute to the phenomenon of cellular transformation by pp60<sup>v-src</sup>.

Cx43 is one of multiple pp60<sup>src</sup> substrates which have been identified in *src*-transformed cells. Substrates such as cytoskeletal proteins (Reynolds et al., 1989; 1992; Turner et al., 1990; Wu and Parsons 1993) the epidermal growth factor receptor (Wasilenko et al., 1991), focal adhesion kinase (p125<sup>FAK</sup>) (Guan and Shalloway 1992; Schaller et al., 1992), and a RNA binding protein (Fumagalli et al., 1994; Taylor and Shalloway 1994). The identification of pp60<sup>src</sup> substrates is critical to the understanding of the mechanisms involved in pp60<sup>src</sup>'s effects on growth and cellular transformation. It is possible that the phosphorylation of only one or two substrates may be sufficient for cellular transformation. Alternatively, multiple pp60<sup>src</sup> substrates may work in concert to initiate and maintain the different properties associated with altered cell growth and behavior. Several studies have suggested that the maintenance of intercellular *gjc* is one parameter critically involved in normal growth control (Mehta et al., 1986; Trosko et al., 1990). pp60<sup>v-src</sup>-mediated tyrosine phosphorylation of Cx43 and the associated disruption of *gjc* could be one mechanism by which pp60<sup>v-src</sup> mediates some aspects of cellular transformation and loss of growth control.

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