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Role of an Alternatively Spliced Form of αII-Spectrin in Localization of Connexin 43 in Cardiomyocytes and Regulation by Stress-Activated Protein Kinase

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Abstract

Decreases in the expression of connexin 43 and the integrity of gap junctions in cardiac muscle, induced by the constitutive activation of the c-Jun N-terminal kinase (JNK) signaling pathway, have been linked to conduction defects and sudden cardiac failure in mice [16,17]. We examined the membrane cytoskeletal protein, αII-spectrin, which associates with connexin 43, to learn if changes in its association with connexin 43 are linked to the instability of gap junctions. Several forms of αII-spectrin are expressed in heart, including one, termed αII-SH3i, which contains a 20-amino acid sequence next to the SH3 domain of repeat 10. In adult mouse heart, antibodies to all forms of αII-spectrin labeled the sarcolemma, transverse ("t-") tubules and intercalated disks of cardiomyocytes. In contrast, antibodies specific for αII-SH3i labeled only gap junctions and transverse tubules. In transgenic hearts, in which the JNK pathway was constitutively activated, αII-SH3i was lost specifically from gap junctions but not from t-tubules while other isoforms of αII-spectrin were retained at intercalated disks. Immunoprecipitations confirmed the decreased association of αII-SH3i with connexin 43 in transgenic hearts compared to controls. Furthermore, activation of JNK in neonatal myocytes blocked the formation of gap junctions by exogenously expressed Cx43-GFP fusion protein. Similarly, over-expression of the SH3i fragment in the context of repeats 9-11 of αII–spectrin specifically caused the accumulation of Cx43-GFP in the perinuclear region and inhibited its accumulation at gap junctions. These results support a critical role for the αII-SH3i isoform of spectrin in intracellular targeting of Cx43 to gap junctions and implicates αII-SH3i as a potential target for stress signaling pathways that modulate intercellular communication.

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Keywords

spectrin; connexin 43; gap junctions; cytoskeleton

INTRODUCTION

Proper cardiac function relies on the stability and coordinated organization of the various membrane domains of cardiomyocytes, including the sarcolemma, the transverse tubules, and the intercalated disks, yet the structures that organize and stabilize these membranes are poorly understood. Members of the spectrin superfamily of proteins are likely candidates for this role in striated muscle. Discovered decades ago in the human erythrocyte, spectrin and spectrin-like proteins (the "spectrin superfamily") have at least three major roles in muscle cells: (i) to provide structural support for cellular membranes, (ii) to organize integral membrane proteins into distinct domains, and (iii) to mediate force transduction across the plasma membrane. At least five different isoforms of spectrin have been identified in heart [21], with possible alternatively spliced isoforms of each [4]. The reasons for such diversity within the same cell may be explained by the ability of the spectrins to associate with and stabilize different structures. For example, in addition to their location at the sarcolemma, particular forms of αII-spectrin [1] and βII-spectrin [8] have been found at the transverse or "t" tubules in cardiac muscle.

The activities of the spectrin superfamily of proteins are profoundly influenced by phosphorylation of the spectrin subunits and their ligands. β-Spectrin contains several potential phosphorylation sites at its C-terminal domain, located near the head-to-head tetramerization site [19]. Phosphorylation in this region has been shown to lead to membrane fragility in erythrocytes, presumably due to de-stabilization of the spectrin network [15]. In addition, phosphorylation of the N-methyl-D-aspartate (NMDA) receptor subunit, NR2B, can alter its association with β-spectrin [22]. The ankyrins, which bind to β-spectrin, have been implicated in the localization of numerous other membrane proteins, including the Na,K-ATPase, the Na channel, ryanodine receptors, and SERCA. Hyperphosphorylation of ankyrin substantially reduces its binding to both β-spectrin and an integral membrane protein, the anion exchanger 1 [3,5].

Phosphorylation and dephosphorylation of contractile and membrane proteins play central roles in modulating cardiac function, and defects in these regulatory events can lead to arrhythmia or other cardiomyopathies. Heart failure is associated with abnormal activation of many signaling pathways, including the mitogen-activated protein (MAP) kinase pathways. There are three major classes of MAP kinases: extracellular signal-regulated kinase (ERK), c-Jun amino terminal kinase (JNK), and p38 kinase. Both the JNK and p38 pathways are activated by stress-related stimuli. Recent evidence reported by Petrich et al. [16,17] indicates that constitutive activation of JNK in heart by targeted expression of an upstream kinase, MKK7D, down-regulates the expression of connexin 43 (Cx43) and disrupts gap junctions in cultured myocytes and in intact mouse heart. Reports have previously linked the cytoskeletal protein, αII-spectrin, to the stability of gap junctions [20]. Here we report that connexin 43 is indeed linked to a particular alternatively spliced isoform of αII-spectrin containing a 20-amino acid insert adjacent to its SH3 domain in spectrin repeat 10. This isoform, termed αII-SH3i, is selectively lost from its normal location at gap junctions in transgenic cardiomyocytes in which JNK is constitutively activated. Activation of JNK or over-expression of the central region of αII-SH3i, but not of this region lacking the 20- amino acid insert, prevents the formation of gap junctions by exogenously expressed Cx43-GFP fusion protein in cultured cardiomyocytes. Our evidence suggests that the localization of connexin 43 to gap junctions requires the αII-SH3i form of αII-spectrin and that this activity is regulated by the JNK pathway.

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MATERIALS AND METHODS

Unless otherwise stated, all materials were the highest grade available from Sigma Chemical Co. (St. Louis, MO). All experiments were performed as described a minimum of three times.

Tissue Preparation

Heart tissue was collected from adult Sprague-Dawley rats (Zivic-Miller, Zelienople, PA) and adult control and JNK-activated, MKK7D mice [17]. Animals were anesthetized with a mixture of ketamine and xylazine and perfused through the left ventricle with phosphate-buffered saline (PBS) containing protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics Corp., Indianapolis, IN). All procedures complied with IACUC guidelines. The muscle tissue was excised and frozen in one of two ways. Tissue to be used for homogenization was immediately plunged into liquid nitrogen. Tissue to be used for frozen sections was snap frozen in a slush of liquid nitrogen, to reduce freeze damage. Neonatal rat cardiomyocyte cultures were prepared as described previously [12].

Antibodies

General antibodies recognizing multiple isoforms of α-fodrin (αIIΣ*-spectrin, or more simply, αII-spectrin) were prepared in rabbits using αII-spectrin purified from bovine brain as the immunogen. The antibodies were affinity purified as previously described [23] and were used at a concentration of 2 μg/ml for immunofluorescence experiments. Peptide-specific antibodies to the αII-SH3i form of spectrin were prepared in rabbits against the peptide, NH_2-TRITKEAGSVSLRMKQVEEL-COOH, synthesized as a MAP-peptide complex, and affinity purified. Two antibodies against connexin-43 (Cx43) were used in these studies, a monoclonal mouse antibody (Chemicon International, Temecula, CA) that was used for immunofluorescence and Western blotting, and a rabbit polyclonal antibody (Zymed, San Francisco, CA) that was used for immunoprecipitations and Western blotting. A polyclonal antibody to GFP (Clontech, Mountain View, CA) was used on Western blots and a monoclonal antibody to GFP (MBL International Corp., Woburn, MA) was used for immunoprecipitations. A control mouse IgG1 (MOPC21) and purified non-immune rabbit IgG were both purchased from Sigma Chemical Co. (St. Louis, MO). Secondary antibodies included Alexa Fluor 488 or 568 goat anti-rabbit IgG and Alexa Fluor 488 or 568 goat anti-mouse IgG (Molecular Probes, Inc. Eugene, OR). Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Jackson Immunoresearch Laboratories, Inc. (West Chester, PA).

Immunofluorescence Labeling

Frozen sections of control and transgenic cardiac muscle were cut at 20 μm on a Reichert-Jung cryostat (Cambridge Instruments, Deerfield, IL), collected on slides, and double labeled with rabbit antibodies to αII-spectrin-general or to the αII-spectrin-SH3i form, and monoclonal mouse antibodies to Cx43. Sections were first incubated in a solution containing 5% goat serum and 3% bovine serum albumin (BSA) in PBS for a minimum of 30 min, to reduce non-specific labeling. Samples were incubated with the primary antibodies, diluted in 1 mg/ml BSA in PBS, overnight at 4°C, washed with 1 mg/ml BSA in PBS, and incubated with secondary antibodies for 1-2 hr at room temperature.

Cultures of neonatal cardiomyocytes were fixed in ice-cold ethanol for 1 hr before immunolabeling. After washing, the cells were incubated with goat serum and BSA before labeling, as above.

All samples were mounted on slides with Vectashield anti-fade (Vector Laboratories, Inc., Burlingame, CA) to reduce photobleaching. Slides were examined with a Zeiss 410 confocal

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laser scanning microscope (Carl Zeiss, Inc., Tarrytown, NY) with pinholes set at 18 and a resolution in the *x* and *y* directions of 0.124 μm. Figures were prepared with Corel Draw 10 (Corel Corporation Limited, Ottawa, Ontario).

Immunoprecipitation

Homogenates were prepared from either adult rat or mouse cardiac muscle or from infected neonatal cardiocyte cultures, prepared as described below. The tissue was homogenized with a Brinkmann Polytron Homogenizer (Switzerland) at a w/v ratio of 0.05 in PBS containing 1% NP-40 with protease inhibitors (Complete Protease Inhibitor Cocktail Tablets, Roche Diagnostics), pH 7.2. The homogenates were incubated on ice for 1 hr before centrifugation at 12,000 × g and collection of the supernatant. Immunoprecipitations were performed from 1 mg of this homogenate with either sheep anti-rabbit M280 Dynabeads or goat anti-mouse M450 Dynabeads (Dynal, Lake Success, NY), according to the manufacturer's instructions, and either polyclonal rabbit antibodies to αII-spectrin or Cx43 or monoclonal antibodies to GFP. Parallel samples were routinely processed with control rabbit or mouse antibodies, as appropriate, to assess specificity.

Immunoblotting

Immunoprecipitates were heated for 10 min at either 42 or 65°C in SDS-PAGE sample buffer [10] and loaded onto a 4-12% gradient polyacrylamide gel. The proteins were transferred electrophoretically to nitrocellulose [2] and the blots were incubated in Blotto (3% dry milk solids in PBS containing 0.5% Tween 20 and 10 mM sodium azide) for 2 hr. The blots were probed with various antibodies (see above), followed by incubation with the appropriate secondary antibody conjugated to alkaline phosphatase. The bound antibody was detected by chemiluminescence (Western Light Detection, Tropix Laboratories, Bedford, MA). Immunoblots were quantitated using Metamorph (Molecular Devices Corp, Sunnyvale, CA) and the results were reported in relative intensity units.

RT-PCR and Preparation of Adenoviral Constructs

RNA was purified from hearts of adult rats with Trizol (Sigma Chemical Co., St. Louis, MO), following the manufacturer's directions. Aliquots containing 5 μg RNA were used in a First Strand cDNA Synthesis (Amersham Pharmacia Biotech UK Limited, Buckinghamshire England). Primers and Platinum Taq polymerase (Invitrogen Life Technologies, Carlsbad, CA) were added to the first strand reaction and placed in the thermocycler to generate the PCR products. Primers were designed to match the sequence for repeats 9-11 of the αII-spectrin from rat skeletal muscle, previously deposited in GenBank by our laboratory (GenBank Accession Number AF084186). The primers included: sense: 5'-GTCGAATTCTCTGGAGGACTCACTACAGG-3' and antisense: 5'-TTGCGGTACCCTACAGGAGCTGGCTACGTTC-3'. The product, $\alpha II_{9\text{-}11}$SH3i plus (+) or minus (−) the 20-amino acid insert after the SH3 domain, was cloned into pDsRed2-C1 (BD Biosciences, Palo Alto, CA) using the Eco RI and Kpn I restriction enzyme sites in the multiple cloning region. DsRed-$\alpha II_{9\text{-}11}$SH3i+ (i.e., DSRed fusion proteins carrying repeats 9-11 of αII–spectrin, including the 20-amino acid insert adjacent to the SH3 domain and DsRed-$\alpha II_{9\text{-}11}$SH3i− (i.e., the same construct, but lacking the 20-amino acid insert) were excised from the pDsRed2-C1 vector with Age I and Kpn I and cloned into the pAdlox vector [6] using the compatible Xma I and Kpn I restriction enzyme sites. Recombinant adenovirus expressing either the DsRED-$\alpha II_{9\text{-}11}$SH3i+ or DsRed-$\alpha II_{9\text{-}11}$SH3i−, driven by a cytomegalovirus promoter, were created and purified as previously described [9]. Adenoviral constructs expressing Cx43-GFP [11], MKK7D and LacZ [16] were described previously. All adenoviral constructs were used at a multiplicity of infection of 50-100 particles per cell. Cells were incubated for 48 hrs after infection before fixation with ice-cold ethanol. Cells were rehydrated

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with 1 mg/ml BSA in PBS before mounting on slides with Vecta-Shield and visualization by confocal microscopy. The results were analyzed and scored in two ways. First, we calculated the total number of images correctly identified as expressing Cx43-GFP and either DsRED-$\alpha II_{9\text{-}11}$SH3i+ or DsRed-$\alpha II_{9\text{-}11}$SH3i−. We used the FISHER EXACT test to analyze the significance. Second, we scored every cell in all of the images as either having normal gap junctions or impaired gap junctions. We then compared the percentage of normal junctions found in the two populations and used the Mann Whitney U test to determine the significance in the difference between the two percentages.

RESULTS

Expression of an alternatively spliced isoform of αII-spectrin in heart

We generated and affinity-purified rabbit antibodies to bovine brain αII-spectrin (αIIΣ*) and to a particular alternatively spliced sequence, αII-spectrin-SH3i. Antibodies to brain αIIΣ*–spectrin were extensively cross-adsorbed against other spectrin isoforms and were specific for this subunit [23]. In western blots of homogenates of rat heart, this antibody recognized multiple forms of αII-spectrin (Fig. 1B, lane 1). Antibodies to αII-SH3i, generated against the 20-amino acid, alternatively spliced sequence inserted into motif 10 of αII-spectrin, just after the SH3 domain (Fig. 1A), recognized a single band in western blots (Fig. 1B, lane 2). This band corresponded to the largest band recognized by the general antibodies to αII-spectrin. Both antibodies also reacted with a band at ~150 kDa, which corresponds to a well-characterized proteolytic fragment of αII-spectrin [7].

Intracellular localization of αII-spectrin-SH3i in wildtype and JNK-activated adult mouse cardiomyocytes

We used our two antibodies to αII-spectrin to label preparations of wildtype murine heart tissue, and tissue prepared from hearts in which the JNK signaling pathway was constitutively activated by targeted expression of its upstream activator MKK7D [17]. The MKK7D transgenic heart exhibits reduced conduction velocity and premature sudden death linked to a loss of connexin 43 (Cx43) protein, the major component of ventricular gap junctions [16, 17]. As Cx43 is normally linked to a membrane cytoskeleton composed in part of αII-spectrin, we investigated whether these proteins, too, would be altered in MKK7D hearts. The general antibodies to αII-spectrin label the t-tubules, sarcolemma and intercalated disks in both wildtype (Fig. 2A, arrows) and MKK7D hearts (Fig. 2D, arrows). As reported [17], labeling for Cx43 was decreased in the MKK7D transgenic mouse hearts compared to controls (Fig. 2E). Although this was accompanied by changes in the structure of the membrane at the intercalated disk, which appeared to be less tightly formed (compare insets in Fig. 2A and D), general labeling of αII-spectrin reliably marked the intercalated disk (Fig. 2D,F). By contrast, antibodies to αII-SH3i labeled only t-tubules and restricted portions of the intercalated disks which co-labeled with antibodies to Cx43 in the wildtype mouse (and rat: not shown) hearts (Fig. 2G-I). We do not find significant distributions of this protein in non-structural areas of the cytoplasm. In MKK7D hearts, immunolabeling for αII-SH3i was lost from gap junctions that were also depleted of Cx43, but staining at t-tubules was fully retained (Fig. 2J-L). Thus, the association of αII-SH3i with Cx43 at gap junctions, but not its association with t-tubules, is selectively regulated by the JNK pathway.

JNK activation inhibited Cx43 and αII-SH3i interaction in vivo

We performed immunoprecipitation experiments to confirm the linkage between Cx43 and αII-SH3i. We first examined homogenates of hearts of adult rats with antibodies to αII-spectrin-SH3i, general αII-spectrin, and control non-immune rabbit IgG (Fig. 3A). The results clearly showed that Cx43 was immunoprecipitated with the peptide-specific antibodies to αII-spectrin-SH3i but not with general αII-spectrin antibodies (Fig 3A). The control, prepared with

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preimmune-IgG was negative for Cx43. Immunoblots probed with antibodies to general αII-spectrin revealed a single band of spectrin in the precipitate prepared with antibodies to αII-SH3i (Fig. 3A), but multiple bands in the immunoprecipitate prepared with the general antibodies to αII-spectrin. This indicates that αII-SH3i was selectively associated with Cx43. In keeping with the results of our immunofluorescence experiments (Figure 2), this association was not evident in immunoprecipitates prepared from MKK7D transgenic hearts (Fig. 3B). Samples of homogenates from both wildtype and MKK7D heart were immunoblotted with antibodies to αII-spectrin-SH3i to show that the overall amount of αII-spectrin-SH3i was not significantly changed (Fig. 3C). This is consistent with our observation that αII-spectrin-SH3i is retained at the t-tubules of transgenic myocytes, at levels close to those seen in controls (Fig. 2G). Furthermore, we do not observe a distinct pattern of proteolysis that would indicate this as a possible mechanism for the results we obtained by immunofluorescence in Fig. 2.

As an additional test of the association between Cx43 and αII-SH3i, we used adenoviral vectors to express Cx43-GFP, DsRed-$\alpha II_{9\text{-}11}$SH3i+, and MKK7D in different combinations in cultured neonatal rat ventricular myocytes. Homogenates of these cells were immunoprecipitated with antibodies to GFP (Fig. 3D). The results show that, as expected, when cells are expressing both Cx43-GFP and MKK7D, the amount of Cx43-GFP protein in the pellet is approximately one-third less than that seen in cells expressing only Cx43-GFP. This reduction is also seen for the endogenous Cx43 (data not shown). Endogenous αII-spectrin and DsRed-$\alpha II_{9\text{-}11}$SH3i+ are both found in significant amounts in these immunoprecipitates. This further supports the association between αII-spectrin-SH3i and Cx43 and specifically identifies repeats 9-11 of αII-spectrin as the region involved in this association . The levels of endogenous αII-spectrin or DsRed-$\alpha II_{9\text{-}11}$SH3i+ in the immunoprecipitates do not appear to be significantly affected by the presence of MKK7D.

JNK activation modulates Cx43 targeting to gap junctions in vitro

The loss of gap junctions in JNK-activated heart suggested either that the expression of Cx43 is down-regulated or that its ability to localize at junctional membranes is impaired. Since our previous report showed that endogenous Cx43 was down-regulated in cultured cells similar to that found in the transgenic hearts upon JNK activation, we used adenoviral vectors to force the expression of a GFP-tagged form of Cx43 in cardiomyocytes in culture so that we could assess the levels and subcellular distribution of Cx43 in control and JNK-activated cells. Immunoblots showed that cardiomyocytes expressing Cx43-GFP produced considerably reduced levels of endogenous Cx43, whether or not JNK was selectively activated (Fig. 4A). This suggests that myocytes tightly regulate their overall levels of Cx43, presumably by post-translational mechanisms. Immunoblots, such as those in Fig. 4A, were quantified and the results are presented in Fig. 4B. These analyses show that the levels of endogenous Cx43 decrease significantly ($p<0.01$) when Cx43-GFP is expressed. These amounts remain low with the addition of MKK7D, while the levels of Cx43-GFP decrease significantly ($p<0.05$) with expression of MKK7D. Confocal imaging of transfected myocytes showed significant labeling of Cx43-GFP at gap junctions in cultured cells (Fig. 4C1). In cells where JNK was constitutively active, however, Cx43-GFP protein was expressed at lower levels (Fig. 4A) despite higher levels of mRNA transcript (data not shown) and failed to accumulate in gap junctions (Fig. 4C2). These results indicate that JNK activation diminishes the expression or stability of Cx43 protein, and prevents its accumulation in gap junctions.

Cx43-spectrin interaction in membrane targeting and gap junction formation

The results above suggest that Cx43 associates selectively with αII-SH3i. To further test this point, we co-infected cultured neonatal rat ventricular myocytes with adenoviral constructs expressing repeats 9-11 of αII-spectrin either plus ($\alpha II_{9\text{-}11}$SH3i+), or minus ($\alpha II_{9\text{-}11}$SH3i −) the 20-amino acid insert along with the Cx43-GFP. In cells expressing $\alpha II_{9\text{-}11}$SH3i+ (Fig. 5D),

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the Cx43-GFP did not form gap junctions but instead accumulated around the nucleus (Fig. 5E). By contrast, in cells expressing $\alpha II_{9\text{-}11}$SH3i − (Fig. 5A), the Cx43-GFP had normal localization at gap junctions with no obvious accumulation around the nucleus (Fig. 5B). We did a blind evaluation of 28 images from 3 separate experiments, with a total of more than 550 cells, to assess the statistical significance of our observations. Of the 28 images, we identified 22 correctly as either $\alpha II_{9\text{-}11}$SH3i− or $\alpha II_{9\text{-}11}$SH3i+ expressing cells based on Cx43-GFP localization pattern (p-value $<.005$, FISHER EXACT test). We obtained a similar significance when we compared the percent of cells expressing $\alpha II_{9\text{-}11}$SH3i+ that showed even partial formation of gap junctions in each image (43.5 ± 9.2, mean ± S.E., n=12) with those expressing the $\alpha II_{9\text{-}11}$SH3i − form (76.1 ± 4.8, mean ± S.E., n=16) ($p< .01$, MANN WHITNEY U test). Because some of our images had fewer cells than the average image, the difference in the absolute percents of cells with gap junctions was even larger (46 of 149, or 31%, for cells expressing the SH3i+ form of the protein, 315 of 404, or 78%, for cells expressing the SH3i− form). These results show that the effect of the 20 amino acid SH3 insert on the ability of Cx43 to form gap junctions is highly significant. This 20 amino acid insert is therefore likely to play a significant role in targeting and stabilizing the Cx43 in the membranes at gap junctions. Although the underlying mechanism is not clear, its regulation by stress-signal such as JNK provides a potential molecular mechanism for stress-induced loss of Cx43 from gap junctions and consequent defects in intercellular communication.

DISCUSSION

The spectrins are an increasingly diverse group of cytoskeletal proteins found in virtually every mammalian cell that play important roles in membrane stability, membrane domain organization and force transduction. More than one member of this family is typically found in the same cell and as many as five isoforms of spectrin have been found in heart cells [21]. In addition to the various genes producing these proteins, alternative splicing creates much of the diversity in this family of proteins. In this paper, we examine an alternatively spliced isoform of αII-spectrin that directs its localization to gap junctions and may serve to stabilize these critical structures.

We have produced two different antibodies to αII-spectrin that display very different patterns on Western blots and by immunofluorescent labeling of isolated cardiomyocytes. The first antibody was prepared against purified full-length bovine brain αII-spectrin and, after careful cross-adsorption against other, closely related, isoforms of spectrin, it recognizes multiple bands on Western blots of rat heart homogenates (Fig.1B). This strongly suggests that this antibody is able to recognize at least 3 different alternatively spliced forms of αII-spectrin. The immunofluorescence labeling pattern is also quite broad, with labeling of several membrane compartments including the sarcolemma, transverse tubules and the intercalated disk (Fig. 2A). Peptide-specific antibodies raised against the 20-amino acid, alternatively spliced product found in repeat 10, produce quite different results. On Western blots, the antibody recognizes a single form of αII-spectrin (Fig. 1) and by immunofluorescence, it labels two specific membranes: the transverse tubules and a specialized region of the intercalated disk containing gap junctions (Fig. 2G). We can therefore use this antibody to observe changes in a particular form of αII-spectrin.

Petrich et al. have previously described a line of transgenic mice that express a constitutively active form of MKK7 (MKK7D) which is an upstream activator of JNK in cardiomyocytes [17]. Under conditions of JNK-activation, several changes in the hearts of these mice were noted, including the dramatic remodeling and loss of connexin 43 at gap junctions. Previous work has implicated the spectrin cytoskeleton, together with ZO1, in stabilizing gap junctions in heart [20]. As expected, we see that αII-spectrin-SH3i loses its localization at the specialized regions of the intercalated disk when gap junctions are lost in the MKK7D transgenic mouse

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hearts (Fig. 2J). By contrast, ZO1 and cadherin retain their localization at the intercalated disk in the absence of gap junctions [17]. This suggests that, unlike other proteins at the intercalated disk (including other isoforms of αII-spectrin, see Fig. 2D), αII-spectrin-SH3i is closely linked to Cx43. In the current study, our immunoprecipitation experiments confirm this association (Fig. 3) by showing that Cx43 was efficiently co-immunoprecipitated by antibodies to αII-spectrin-SH3i but only minimally by antibodies recognizing more general isoforms of αII-spectrin (Fig. 3A). As we do not yet know the nature of the interaction between αII-spectrin-SH3i and Cx43, we believe that antibodies to the general form of αII-spectrin interact with a region near the binding site for Cx43, thereby promoting its dissociation during the immunoprecipitation experiment. In JNK-activated samples, Cx43 is undetectable in co-immunoprecipitation experiments using anti-αII-spectrin-SH3i, consistent with the immunofluorescent results in Fig. 2. Using adenoviral infections, we showed that the GFP-tagged Cx43 (Cx43-GFP) and the DsRed-tagged repeats 9-11 of αII-spectrin containing the 20-amino acid insert in repeat 10 (DsRed-$\alpha II_{9\text{-}11}$SH3i+) interact, which identifies the specific region of αII-spectrin-SH3i involved in this association (Fig. 3D).

By studying the expression of adenovirally expressed Cx43-GFP, we were able to address many questions about gap junctions. The results in Fig. 4A illustrate the tight control of expression of endogenous Cx43, which down-regulates in the presence of adenovirally produced protein. Further, when the Cx43-GFP is co-expressed with MKK7D, both forms of Cx43 down-regulate. Our previous work has shown that this results in impaired gap junctions [16,17]. Disruption of the targeting of Cx43 to gap junctions by overexpression of DsRed-$\alpha II_{9\text{-}11}$SH3i+ is likely to result in a similar impairment of function of gap junctions.

Additional experiments in cultured myocytes with adenoviruses expressing fragments of αII-spectrin show that over-expression of both Cx43-GFP and DsRed-$\alpha II_{9\text{-}11}$SH3i+ results in a dramatic decrease in gap junctions at the cell-to-cell junctions in neonatal rat cardiocyte cultures. Controls expressing DsRed-$\alpha II_{9\text{-}11}$SH3i− (repeats 9-11 of αII-spectrin *lacking* the 20-amino acid insert) do not show this result. It is also significant that the Cx43-GFP appears to be blocked in its translocation to the cell surface at the ER/Golgi apparatus when DsRed-$\alpha II_{9\text{-}11}$SH3i+ is over-expressed, as large aggregates of Cx43-GFP form just outside the nucleus. This is not seen in cells co-expressing $\alpha II_{9\text{-}11}$SH3i−, indicating that this phenomenon is specifically linked to the presence of the 20-amino acid insert. This is consistent with previous reports showing a linkage between the spectrin cytoskeleton and the Golgi apparatus in the sorting of specific proteins [18]. Our own unpublished observations in cultured neonatal myocytes reveals punctate staining of αII-SH3i around the nucleus, especially at early times after plating, consistent with a role of this form of spectrin in protein processing through the Golgi apparatus.

One way to account for our results is illustrated in Figure 6. We postulate that the αII-SH3i form of spectrin associates with Cx43 at the level of the Golgi apparatus and that it must remain associated if Cx43 is to traffic through the secretory pathway to the plasma membrane. Once at the plasma membrane, both αII-SH3i spectrin and C×43 would therefore be expected to accumulate at gap junctions, consistent with our observations. One potential hypothesis is that the association of αII-SH3i with C×43 is dependent on the SH3 insert in a phosphorylation-dependent manner. This phosphorylation could be directly mediated by JNK, or could be catalyzed by downstream kinases that are themselves regulated by JNK. The phosphorylation of βI-spectrin has been the subject of much research; recent reports now provide evidence for the phosphorylation of αII-spectrin as well [13,14]. Results of our recent preliminary experiments indicate that repeats 9-11 of αII spectrin can also be phosphorylated (unpublished observations; Zhang, Ursitti and Bloch). Thus, over-expression of αII-SH3i or changes in phosphorylation would each dissociate spectrin from C×43, preventing C×43 from moving to the plasma membrane, resulting in its accumulation in Golgi and other intracellular membranes.

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This, too, is congruent with our finding that over-expression of αII-SH3i (Fig. 5) and activation of JNK (Fig. 4) causes a similar decrease in the amount of C×43 found at cell-cell contacts. This model is also consistent with our preliminary observations that αII-SH3i concentrates at membranous structures in the perinuclear region of cardiomyocytes at early stages in culture. However, we cannot rule out the possibility that the β-spectrin subunit that associates preferentially with αIISH3i is the target of the phosphorylation cascade initiated by JNK.

The combined results of both in vivo and in vitro studies reveal a potentially critical role of selective isoforms of spectrin in stress-induced loss of C×43 protein and gap junction targeting in heart. The interaction between specific fragments of αII-spectrin and C×43, and its regulation by stress-kinase activity provide a previously unknown mechanism in stress induced cellular remodeling at gap junctions.

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REFERENCES

1. Bennett PM, Baines AJ, Lecomte MC, Maggs AM, Pinder JC. Not just a plasma membrane protein: in cardiac muscle cells alpha-II spectrin also shows a close association with myofibrils. J Muscle Res Cell Motil 2004;25:119–26. [PubMed: 15360127]
2. Burnette WN. “Western blotting”: electrophoretic transfer of proteins from sodium dodecyl sulfate--polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal Biochem 1981;112:195–203. [PubMed: 6266278]
3. Cianci CD, Giorgi M, Morrow JS. Phosphorylation of ankyrin down-regulates its cooperative interaction with spectrin and protein 3. J Cell Biochem 1988;37:301–315. [PubMed: 2970468]
4. Cianci CD, Zhang Z, Pradhan D, Morrow JS. Brain and muscle express a unique alternative transcript of alphaII spectrin. Biochemistry 1999;38:15721–30. [PubMed: 10625438]
5. Ghosh S, Cox JV. Dynamics of ankyrin-containing complexes in chicken embryonic erythroid cells: role of phosphorylation. Mol Biol Cell 2001;12:3864–3874. [PubMed: 11739786]
6. Hardy S, Kitamura M, Harris-Stansil T, Dai Y, Phipps ML. Construction of adenovirus vectors through Cre-lox recombination. J Virol 1997;71:1842–1849. [PubMed: 9032314]
7. Harris AS, Morrow JS. Proteolytic processing of human brain alpha spectrin (fodrin): identification of a hypersensitive site. J Neurosci 1988;8:2640–2651. [PubMed: 3074159]
8. Hayes NV, Scott C, Heerkens E, Ohanian V, Maggs AM, Pinder JC, Kordeli E, Baines AJ. Identification of a novel C-terminal variant of beta II spectrin: two isoforms of beta II spectrin have distinct intracellular locations and activities. J Cell Sci 2000;113:2023–34. [PubMed: 10806113]
9. Kontrogianni-Konstantopoulos A, Catino DH, Strong JC, Randall WR, Bloch RJ. Obscurin regulates the organization of myosin into A bands. Am J Physiol Cell Physiol 2004;287:C209–C217. [PubMed: 15013951]
10. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680–685. [PubMed: 5432063]
11. Lin JH-C, Yang J, Liu S, Takano T, Wang X, Gao Q, Willecke K, Nedergaard M. Connexin mediates gap junction-independent resistance to cellular injury. J Neurosci 2003;23:430–441. [PubMed: 12533603]
12. Lokuta A, Kirby MS, Gaa ST, Lederer WJ, Rogers TB. On establishing primary cultures of neonatal rat ventricular myocytes for analysis over long periods. J Cardiovasc Electrophysiol 1994;5:50–62. [PubMed: 8186877]
13. Nedrelow JH, Cianci CD, Morrow JS. C-Src binds αII Spectrin's Src homology 3 (SH3) domain and blocks calpain susceptibility by phosphorylating Tyr^{1176}. J Biol Chem 2003;278:7735–7741. [PubMed: 12446661]

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14. Nicolas G, Fournier CM, Galand C, Malbert-Colas L, Bournier O, Kroviarski Y, Bourgeois M, Camonis JH, Dhermy D, Grandchamp B, Lecomte M-C. Tyrosine phosphorylation regulates alpha II Spectrin cleavage by calpain. Mol Cell Biol 2002;22:3527–3536. [PubMed: 11971983]
15. Perrotta S, Del Giudice EM, Iolascon A, DeVivo M, DiPinto D, Cutillo S, Nobili B. Reversible erythrocyte skeleton destablization is modulated by beta-spectrin phosphorylation in childhood leukemia. Leukemia 2001;15:440–444. [PubMed: 11237068]
16. Petrich BG, Gong X, Lerner DL, Wang X, Brown JH, Saffitz JE, Wang Y. c-Jun N-terminal kinase activation mediates downregulation of connexin43 in cardiomyocytes. Circ Res 2002;91:640–647. [PubMed: 12364393]
17. Petrich BG, Eloff BC, Lerner DL, Kovacs A, Saffitz JE, Rosenbaum DS, Wang Y. Targeted Activation of c-Jun N-terminal Kinase in Vivo Induces Restrictive Cardiomyopathy and Conduction Defects. J Biol Chem 2004;279:15330–15338. [PubMed: 14742426]
18. Stankewich MC, Tse WT, Peters LL, Ch'ng Y, John KM, Stabach PR, Devarajan P, Morrow JS, Lux SE. A widely expressed betaIII spectrin associated with Golgi and cytoplasmic vesicles. Proc Natl Acad Sci U S A 1998;95:14158–14163. [PubMed: 9826670]
19. Tang HY, Speicher DW. In vivo phosphorylation of human erythrocyte spectrin occurs in a sequential manner. Biochemistry 2004;43:4251–62. [PubMed: 15065869]
20. Toyofuku T, Yabuki M, Otsu K, Kuzuya T, Hori M, Tada M. Direct association of the gap junction protein connexin-43 with Z0-1 in cardiac myocytes. J Biol Chem 1998;22:12725–12731. [PubMed: 9582296]
21. Vybiral T, Winkelmann JC, Roberts R, Joe E, Casey DL, Williams JK, Epstein HF. Human cardiac and skeletal muscle spectrins: differential expression and localization. Cell Motil Cytoskel 2001;21:293–304.
22. Wechsler A, Teichberg VI. Brain spectrin binding to the NMDA receptor is regulated by phosphorylation, calcium and calmodulin. EMBO J 1998;17:3931–3939. [PubMed: 9670010]
23. Zhou D, Ursitti JA, Bloch RJ. Developmental expression of spectrins in rat skeletal muscle. Molec Biol Cell 1998;9:47–61. [PubMed: 9436990]

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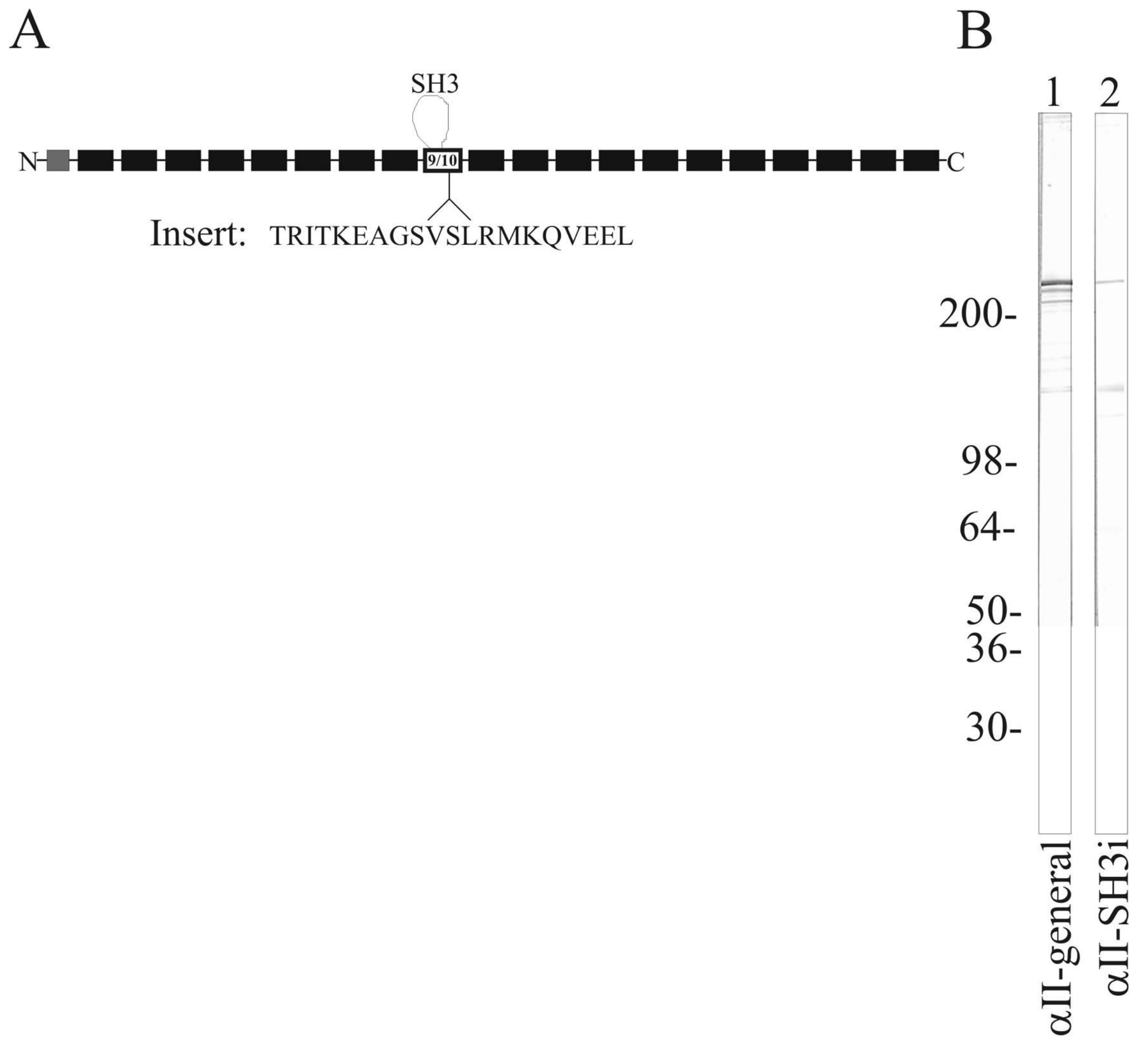

Figure 1. Specificity of antibodies to αII-spectrin.

(A) Diagram of αII-spectrin with the sequence of the alternatively spliced portion of αII-spectrin in motif 10, present in αII-SH3i, indicated below the molecule. Black rectangles represent the 20 triple helical motifs in αII-spectrin while the gray square represents the first partial motif called α'. The looping structure depicts the SH3 domain that interrupts motifs 9 and 10, which together form a single triple helical motif. **(B)** Two antibodies were generated in rabbits, one a general antibody against αII-spectrin from bovine brain, the other a peptide-specific antibody to the alternatively spliced sequence of αII-SH3i. Homogenates of hearts from adult rats (50 µg per lane) were separated by SDS-PAGE on 4-12 % acrylamide gels and blotted with the general antibodies to αII-spectrin (lane 1) or with the peptide-specific antibodies to αII-SH3i (lane 2). The results show that heart expresses several different forms of αII-spectrin, but that only the largest form contains the peptide insert.

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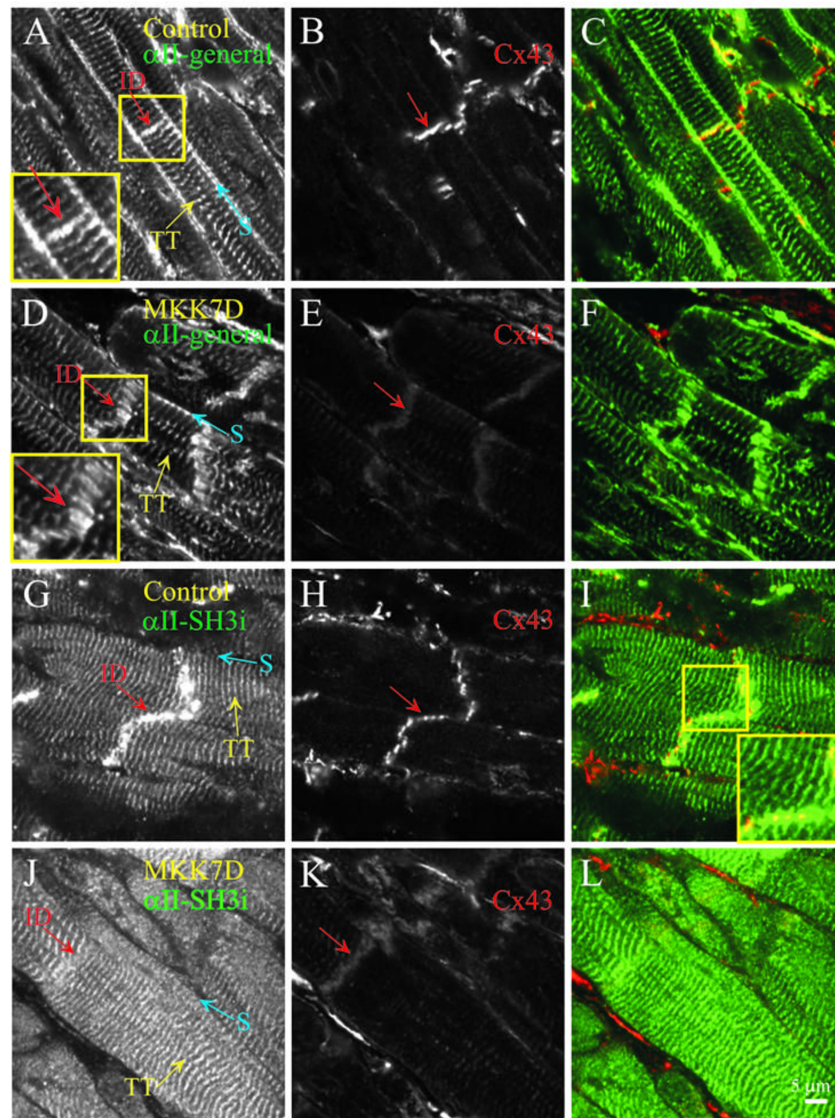

Figure 2. Immunofluorescent labeling of frozen sections of control and transgenic hearts.
Wildtype control (A-C, G-I) and MKK7D transgenic (D-F, J-L) mouse hearts were collected and prepared as described in *Materials and Methods*. Frozen sections were cut at a thickness of 20 μm and incubated with monoclonal mouse antibodies to C×43 (B, E, H, and K) and rabbit antibodies to either bovine brain αII-spectrin (A and D) or to αII-SH3i (G and J), followed by species-specific secondary antibodies. **(A-F)** The general antibody to αII-spectrin labeled the sarcolemmal membrane (blue arrows, S) and the transverse tubules (yellow arrows, TT) as well as the intercalated disk (red arrows, ID) in both control (A-C) and transgenic (D-F) cells. Inserts in panels A and D are magnified 1.7X to show that the antibodies label all along the membrane and that the membrane appears to be more deeply folded at the intercalated disk in the MKK7D cells. **(G-L)** Antibodies specific for αII-spectrin-SH3i labeled only the transverse tubules and gap junctions in control cells (G). Note the absence of label at the sarcolemmal membrane (blue arrow, S) and the more restricted distribution of label at the intercalated disk (red arrow, ID) that closely co-localizes with the gap junctions (I, insert magnified 1.7X to show co-label in overlay). In transgenic cells (J-L), immunolabeling for αII-spectrin-SH3i was

retained at the transverse tubules but, like C×43, was lost from the gap junctions at intercalated disks.

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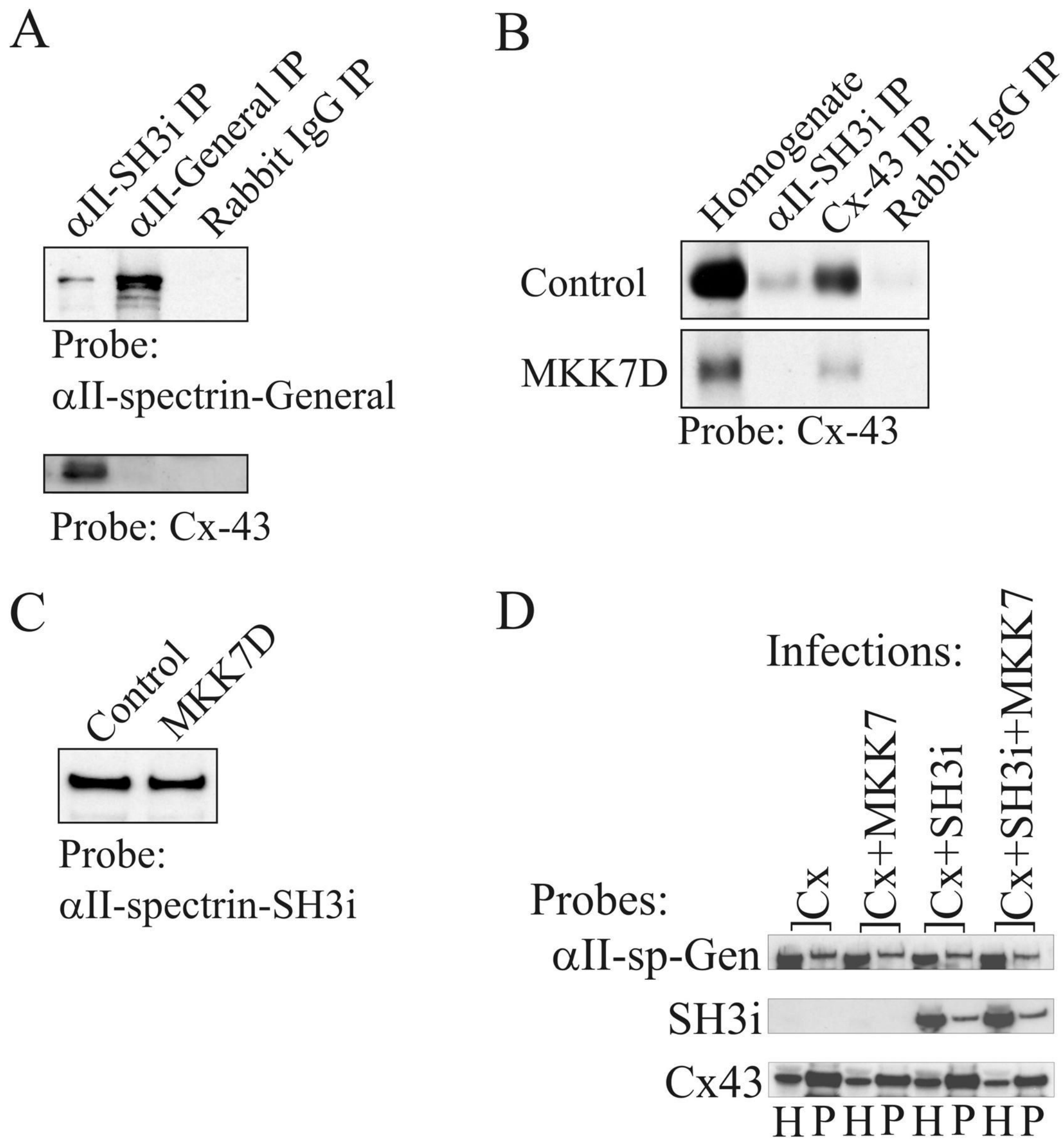

Figure 3. Co-immunoprecipitation of connexin 43 and αII-spectrin-SH3i from control, transgenic, and infected tissues.

(A) Immunoprecipitates from adult rat cardiac muscle were prepared with rabbit antibodies to either αII-SH3i or to the general isoforms of αII-general, as well as with a control non-immune rabbit IgG. Immunoprecipitated proteins were separated by SDS-PAGE, blotted, and probed with antibodies to αII-spectrin-SH3i or C×43. The precipitates prepared with anti-αII-spectrin-SH3i that were blotted with the αII-spectrin-general antibody showed a single band. The precipitates prepared with the general antibody to αII-spectrin contained at least 3 distinct bands, consistent with the results of Fig. 1. Much more C×43 specifically co-immunoprecipitated with αII-spectrin-SH3i than with general forms of αII-spectrin. **(B)** Homogenates of hearts from either control or transgenic, MKK7D, mice were subjected to immunoprecipitation with antibodies to αII-SH3i or connexin 43. Equal amounts of protein in the homogenates and immunoprecipitates were separated by SDS-PAGE, blotted, and probed with a monoclonal mouse antibody to connexin 43. Homogenates of control hearts showed approximately 5-fold more Cx43. They also yielded much more Cx43 in the

immunoprecipitates generated with both antibodies. Cx43 could not be detected in the immunoprecipitates generated from transgenic hearts with αII-spectrin-SH3i. **(C)** Blots of the homogenates shown in **(B)** were also probed with antibodies against αII-spectrin-SH3i. The results show that the amount of αII-SH3i is similar in both control and transgenic hearts. **(D)** Cultured neonatal cardiocytes were infected with adenoviral constructs expressing Cx43-GFP, DsRed-$\alpha II_{9\text{-}11}$SH3i+, and MKK7 in the combinations indicated above each lane. The infected cells were homogenized and immunoprecipitated with monoclonal antibodies to GFP, to concentrate Cx43-GFP and associated proteins. The immunoprecipitates were separated by SDS-PAGE, blotted and probed with antibodies to general αII-spectrin, SH3i, and Cx-43 (Cx43-GFP is shown).

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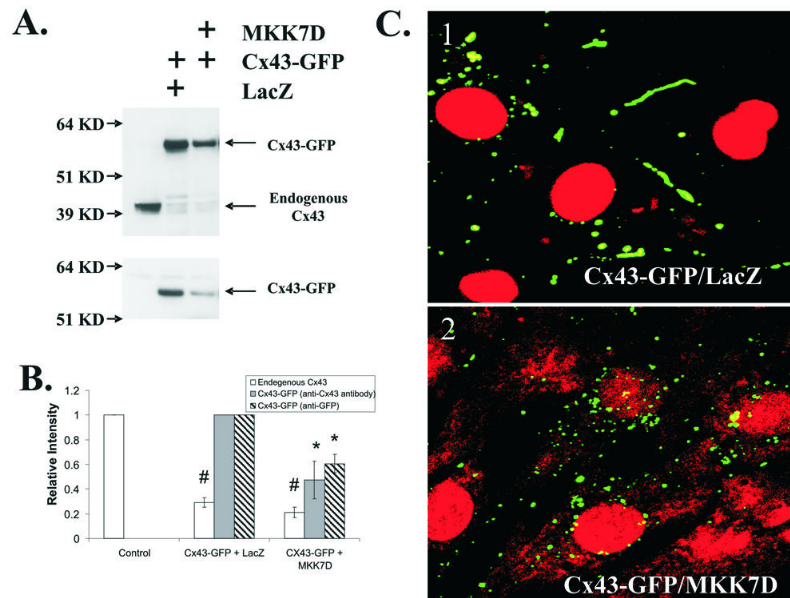

Figure 4. Effect of JNK activation on Cx43 targeting to gap junctions in cardiomyocytes.

(A) Neonatal myocytes in culture were infected with adenoviral vectors expressing LacZ, MKK7D and Cx43-GFP as indicated. The expression of endogenous Cx43 and Cx43-GFP fusion protein were detected on immunoblots of extracts prepared from whole cell lysates. **(B)** The relative intensities of the Cx43 and Cx43-GFP signals were detected on immunoblots by assessing the levels of either anti-Cx43 or anti-GFP as indicated (see Materials and Methods). Error bars represent standard deviation for $n>3$. $p<0.01$ (#) for levels of endogenous Cx43 in untreated cells; $p<0.05$ (*) for Cx43-GFP/LacZ vs. Cx43-GFP/MKK7D in treated cells. **(C)** Representative immunofluorescent images of neonatal cardiomyocytes in culture co-infected with Adv-Cx43 and Adv-LacZ (B1, top) or Adv-Cx43 and Adv-MKK7D (B2, bottom). Cx43-GFP protein is shown in green and nuclei, labeled by propidium iodide, are shown in red. Note that Cx43-GFP is concentrated at gap junctions (arrows) in control cells (B1) but remains associated with intracellular structures upon JNK activation (B2).

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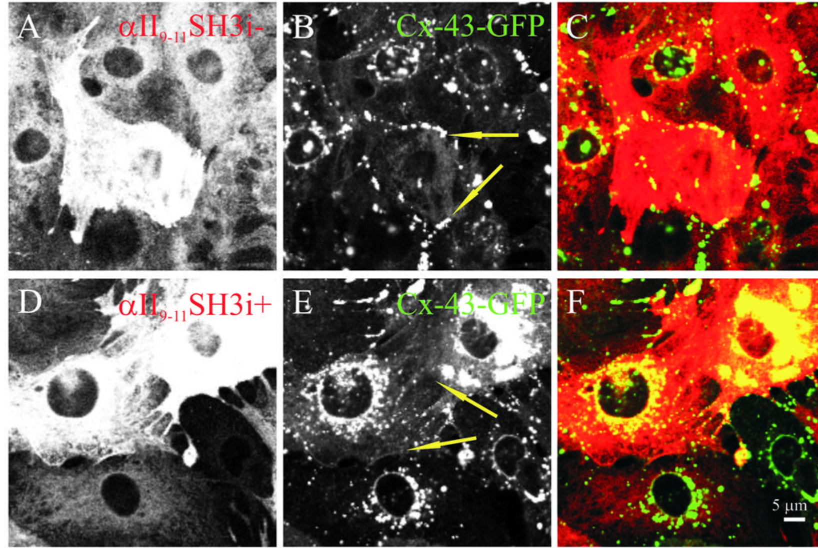

Figure 5. Effect of adenoviral expression of the SH3i sequence on gap junctions in cultures of neonatal rat cardiomyocytes.

Cultures of neonatal rat cardiomyocytes were infected with adenovirus encoding Cx43-GFP and DS-Red fusion proteins of repeats 9-11 of αII–spectrin either with ($\alpha II_{9\text{-}11}$SH3i+) or without ($\alpha II_{9\text{-}11}$SH3i−) the 20-amino acid insert adjacent to the SH3 domain of repeat 10. Cultures were fixed and observed under confocal microscopy to identify infected cells, indicated by the presence of DS-Red, and to determine the distribution of exogenous Cx43, labeled with GFP. (A-C) Cardiomyocytes infected with virus expressing $\alpha II_{9\text{-}11}$SH3i− (red, in C) showed normal levels of Cx43 (green, in C) at gap junctions. (D-F) Cardiomyocytes infected with virus expressing $\alpha II_{9\text{-}11}$SH3i+ (red, in F) showed few gap junctions containing Cx43-GFP (green, in F), which instead accumulated in the perinuclear region. Structures shown in yellow in C and F are labeled with either of the DS-Red fusion proteins and Cx43. Arrows in B and E point to regions of cell-cell contact.

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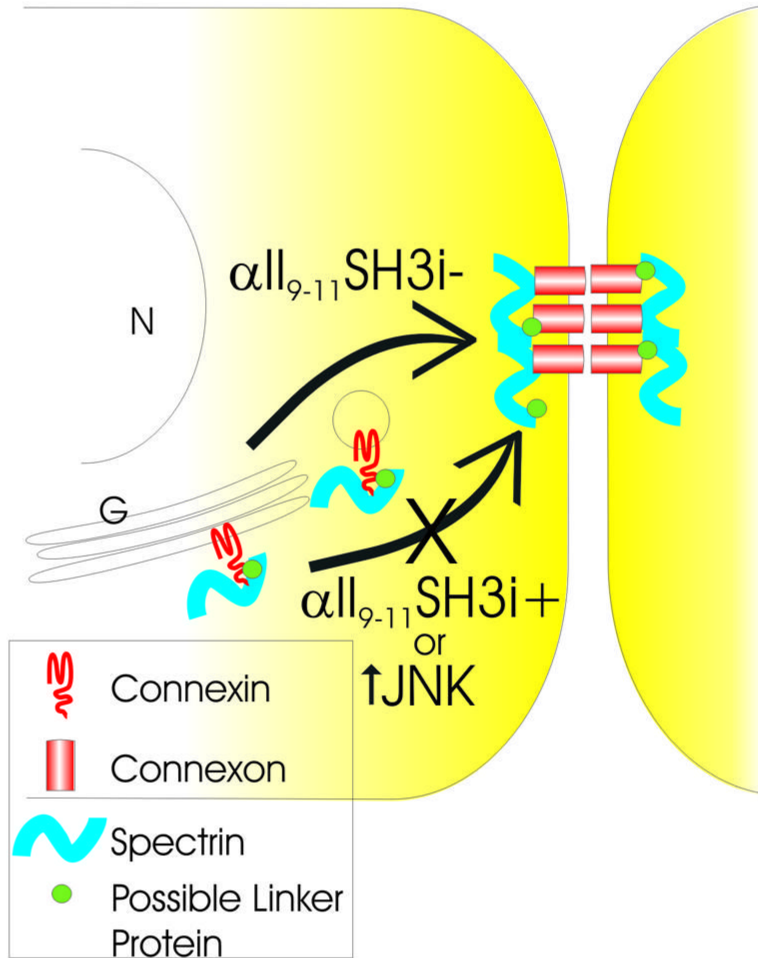

Figure 6. Model of the translocation of connexin 43 to gap junctional membrane through association with spectrin.

This model depicts the possible mechanism by which Cx43 is transported to the membrane in cardiac muscle. The role of the spectrins at the Golgi apparatus has been proposed previously by Stankewich et al. [18]. As our data do not differentiate between the direct or indirect linkage of Cx43 and αII-spectrin, we have shown a potential unknown protein linking the two proteins (green circle).