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Structural changes in the carboxyl terminus of the gap junction protein Connexin40 caused by the interaction with c-Src and Zonula Occludens-1*

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Abstract

c-Src can disrupt the Connexin43 (Cx43) and Zonula Occludens-1 (ZO-1) interaction leading to down-regulation of gap junction intercellular communication. Previously, we characterized the interaction of domains from these proteins with the carboxyl terminus of Cx43 (Cx43CT) and found that binding of the c-Src SH3 domain to Cx43CT disrupted the Cx43CT/ZO-1 PDZ-2 domain complex. Because Cx43 and Cx40 form heteromeric connexons and display similar mechanisms of pH-regulation, we addressed whether Cx40CT interacts with these domains in a similar manner as Cx43CT. NMR data indicate that Cx40CT is an intrinsically disordered protein. NMR titrations determined that PDZ-2 affected the last 28 Cx40CT residues and SH3 shifted numerous N-terminal Cx40CT residues. Finally, the Cx40CT/PDZ-2 complex was unaffected by SH3 and both domains interacted simultaneously with Cx40CT. This result differs from when the same experiment was performed with Cx43CT, suggesting different mechanisms of regulation exist between connexin isoforms, even when involving the same molecular-partners.

Keywords

Cx40; c-Src; ZO-1

Gap junctions are integral membrane proteins that enable the direct cytoplasmic exchange of ions and low molecular weight metabolites between adjacent cells. They provide a pathway for the propagation and/or amplification of signal transduction cascades triggered by cytokines, growth factors, and other cell signaling molecules involved in growth regulation and development. Gap junctions are formed by the apposition of connexons from adjacent cells, where each connexon is formed by 6 connexin proteins. Connexins are tetraspan transmembrane domain proteins with intracellular N- and C-termini. There are 21 different connexin genes in the human genome. The major divergence in primary structures, which allow specific regulatory properties for each isoform, occurs in the carboxyl terminal (CT) domain. Each of the connexins can form gap junctions by themselves, however, studies have demonstrated that many cells co-express more than one connexin isoform, giving rise to heteromeric connexons (i.e., more than one connexin isotype in the same single connexon; for review see (Koval, 2006)).

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Connexin43 (Cx43) and connexin40 (Cx40) co-express in several tissues, including cardiac atrial and ventricular myocytes, and vascular smooth muscle (He et al., 1999). The intercellular communication provided by Cx43 and Cx40 gap junctions (GJIC) is a principal determinant of myocardial conduction, and altered expression has been implicated in arrhythmogenesis (van Veen et al., 2006). Cx43 and Cx40 can hetero-oligomerize, leading to the formation of heterologous gap junctions. The physiological consequences of hetero-oligomerization on GJIC remain unclear, though functional studies demonstrate that these structures display biophysical properties that do not correspond to their homomeric forms (Burt et al., 2001; Cottrell and Burt, 2001; Cottrell et al., 2002; Gu et al., 2000b; Valiunas et al., 2001; Valiunas et al., 2000). Because the sensitivity of different homomeric gap junctions to biochemical modulators varies widely, the question arises as to what are the types of interactions that occur between connexins to integrate regulatory signals in heteromeric channels. Evidence supports involvement of the CT domains in regulation of these heteromeric channels.

Previous studies have shown that pH regulation, as well as the existence of the lower-conductance state of Cx43 and of Cx40 depend on the presence of the CT domain (Gu et al., 2000a; Gu et al., 2000b; Stergiopoulos et al., 1999). Moreover, replacing the Cx40CT with the CT domain of Cx43 rescues the behavior of the homologous channel, both when the fragments (i.e. the Cx40 truncated channel and the Cx43CT domain) are separately co-expressed and when both domains are covalently attached (Anumonwo et al., 2001; Stergiopoulos et al., 1999). Additionally, co-expression of Cx43 and Cx40 in the same cell enhances the susceptibility of the gap junction channel to acidification-induced uncoupling (Gu et al., 2000a; Gu et al., 2000b). These data support the notion that both Cx43 and Cx40: 1) interact within a heteromeric connexon in a synergistic manner, 2) have regulatory domains (e.g. the CT domain) that can specifically interact with a channel formed by another connexin, and 3) follow the “particle-receptor” hypothesis for pH gating. In this regard, studies from our laboratory have characterized the pH-dependent structural changes of the Cx43CT domain (Hirst-Jensen et al., 2007; Sorgen et al., 2004b), as well as the structural changes that occur upon binding known molecular partners involved in pH regulation (Sorgen et al., 2004a). However, from the standpoint of the Cx40CT, little information is available regarding molecular partner interactions and currently no structure is available.

Here, we provide evidence that the Cx40CT is intrinsically disordered in structure and report the Cx40CT structural changes that occur upon binding to the SH3 domain of c-Src and the second PDZ domain (PDZ-2) of ZO-1. Our experiments identify selected groups of Cx40CT residues whose position in space is altered by the presence of the ligate. The regions of structural reorganization include but span beyond those expected from consensus SH3 and PDZ binding domains. While the overall Cx40CT structural changes mediated by the SH3 and PDZ-2 domains resemble that of the Cx43CT domain, potentially significant differences in the mechanism of ZO-1 dissociation from these CT domains are discussed.

Methods

Expression and Purification of Recombinant proteins

Cx40CT (residues 251–355 of Cx40 and 4 additional amino acids appended to the amino terminus; see (Duffy et al., 2002)), ¹⁵N-Cx40CT, the SH3 domain of c-Src, and the PDZ-2 domain of ZO-1 were expressed and purified as previously described (Sorgen et al., 2004a). All proteins were confirmed for purity and analyzed for degradation by SDS-PAGE and mass spectroscopy.

Nuclear Magnetic Resonance

Nuclear Magnetic Resonance (NMR) data were acquired at 7°C using a 600 MHz Varian INOVA NMR spectrometer outfitted with either a cryo-probe or penta-probe. Experimental data to determine backbone sequential assignments have been described (Bouvier, 2007). Connectivities were derived from nuclear Overhauser effects (NOEs) observed in ^{15}N -NOESY-HSQC and ^{13}C -NOESY-HSQC spectra, with mixing times of 125 ms and 150 ms, respectively. NMR spectra were processed using NMRPipe (Delaglio et al., 1995) and analyzed using NMRView (Johnson, 1994).

Gradient-enhanced two-dimensional $^1\text{H}^{15}\text{N}$ -HSQC experiments (Kay et al., 1992) were used to observe all backbone amide resonances in ^{15}N -labeled Cx40CT. Data were acquired with 1024 complex points in the direct dimension and 256 complex points in the indirect dimension. Sweep widths were 10,000 Hz in the proton dimension and 2,500 Hz in the nitrogen dimension. The buffer conditions for each protein (i.e., Cx40CT, c-Src SH3 domain, and ZO-1 PDZ-2 domain) were equilibrated in 1x phosphate-buffered saline (PBS) at pH 6.0 and 1 mM DTT, using NAP-5 columns (GE Healthcare). Binding isotherms from $^1\text{H}^{15}\text{N}$ -HSQC titration experiments were calculated with ORIGIN 7.0 software (Microcal Software Inc., Northampton, MA).

Results

Structure Prediction and Description of the Cx40CT

Our laboratory recently published the resonance assignments for the Cx40CT (Bouvier, 2007). We have now combined the information provided from the α - and β -carbon chemical shift values and NOE spectroscopy to predict the residues that may be present in stable secondary structures (Figure 1). A major factor affecting α - and β -carbon chemical shift values is the backbone geometry, which can cause differences of greater than ± 2 ppm from random coiled values for residues in α -helical and β -sheet conformations (Iwadate et al., 1999). Analysis of the Cx40CT found little to no chemical shift deviations from random coil for the α - and β -carbons (Figure 1A). Additionally, the ^{15}N -NOESY-HSQC and ^{13}C -NOESY-HSQC spectra contained little to no medium- or long-range NOE connectivities (i.e. $i, i+2$, $i, i+3$ or greater), which are commonly found in well-folded structures (Figure 1B). These observations, combined with the low chemical shift dispersion found in the $^1\text{H}^{15}\text{N}$ -HSQC (Bouvier, 2007), are consistent with the prediction of the Cx40CT as an intrinsically disordered protein.

Structural Changes in the Cx40CT Resulting from Binding of Cellular Partners

We have previously characterized the structural modifications brought about by the interaction of Cx43CT with two well-known molecular partners, c-Src and ZO-1 (Sorgen et al., 2004a). Because Cx43 and Cx40 are co-expressed throughout the cardiovascular system, form heteromeric connexons, and display similar mechanisms of pH regulation, we addressed whether Cx40 has the ability to interact with domains from these proteins in a similar manner as Cx43. The identification of the Cx40CT assignments allowed for an assessment of the modifications that result from its interaction with the SH3 domain of c-Src and the PDZ-2 domain of ZO-1 (Bouvier, 2007). Unlabelled SH3 or PDZ-2 fragments were diluted in a 1x PBS buffer solution (pH 6.0) containing ^{15}N -Cx40CT, and $^1\text{H}^{15}\text{N}$ -HSQC spectra were acquired. The $^1\text{H}^{15}\text{N}$ -HSQC is a 2D NMR experiment where each amino acid (except proline) gives one signal (or chemical shift) that corresponds to its N-H amide group. These chemical shifts are sensitive to the chemical environment and even small changes in structure and/or dynamics can change the chemical shift of an amino acid.

The addition of the PDZ-2 domain of ZO-1 affected the resonance peaks of the last 28 amino acids of the Cx40CT, a region larger than what was expected from a putative “PDZ binding domain” (Songyang et al., 1997). Indeed, as seen in Figure 2, the signal from residues G333-V355 completely disappeared (i.e. broadened beyond detection), while the amplitude of the peaks corresponding to residues H328-Q332 decreased in intensity. These changes can be explained by decreased tumbling rates caused by an increase in molecular weight and/or intermediate chemical exchange rates, both indicative of a structural modification caused by the association of the two molecules.

To calculate the dissociation constant (K_D) for the Cx40CT/ZO-1 PDZ-2 domain interaction, $^1\text{H}^{15}\text{N}$ -HSQC data were obtained upon titration of Cx40CT with different concentrations of PDZ-2 (Figure 3). The decreasing signal intensity for a subset of the Cx40CT residues affected as a result of the increasing PDZ-2 concentrations (Figure 3A; residues R349, S350, S354, and V355) were fit according to the nonlinear least squares method and the titration data afforded a K_D of $27.6 \mu\text{M} \pm 2.2 \mu\text{M}$ (mean $\pm$ SD; Figure 3B).

Addition of the SH3 domain strongly shifted (≥ 0.035 ppm) the resonance peaks of amino acids V255-N265, F276-S278, S281, G285-A294, and I310-H311 and weakly shifted (< 0.035 ppm) the resonance peaks of amino acids L254, Q266-L268, N270, D279-F280, G308, G315, K317, E319, S325, G327-H328, F330, Q332-H335, and R339 (Figure 4). Interestingly, amino acids H328, F330, Q332-H335, and R339 overlap with the residues involved in binding the ZO-1 PDZ-2 domain to Cx40CT. The K_D for the Cx40CT/c-Src SH3 domain interaction was also calculated by $^1\text{H}^{15}\text{N}$ -HSQC data obtained upon titration of Cx40CT with different concentrations of SH3 (Figure 5). For the SH3 domain, the change in chemical shifts for a subset of the Cx40CT residues affected as a result of the increasing SH3 concentrations (Figure 5A; residues Q256, G257, R287, and N289) were fit according to the nonlinear least squares method and the titration data afforded a K_D of $1.1 \text{ mM} \pm 0.2 \text{ mM}$ (mean $\pm$ SD; Figure 5B).

Cx40CT Simultaneously Associates with Multiple Cellular Partners

c-Src can disrupt the Cx43/ZO-1 interaction, leading to down-regulation of gap junction intercellular communication (Duffy et al., 2004; Toyofuku et al., 2001). Our previous NMR studies demonstrated that binding of the c-Src SH3 domain to the Cx43CT disrupted the association of the Cx43CT/ZO-1 PDZ-2 domain complex (Sorgen et al., 2004a). Here, we determined if a similar mechanism exists in which SH3-induced structural modifications in Cx40CT affected the conformation of the region ascribed as the PDZ-2 binding domain (residues H328-V355; see Figure 2). ^{15}N -Cx40CT (Figure 6, black) was combined with unlabelled PDZ-2 in PBS buffer (pH 6.0) to a final 1:2 molar concentration (Figure 6, green); unlabeled SH3 was titrated into the complex (Figure 6, blue - 1:2:2 molar concentration, red - 1:2:4 molar concentration) and $^1\text{H}^{15}\text{N}$ -HSQC spectra were acquired. Clearly, addition of SH3 had no effect on the Cx40CT residues which were affected as a result of the Cx40CT/PDZ-2 interaction. It is worth noting that the presence of the PDZ-2 domain did not affect the shifts in the Cx40CT resonance peaks caused by the presence of the SH3 domain. Finally, a decrease in the intensity of the peaks was observed for Cx40CT residues R287-A294, which only shifted in the presence of SH3 alone. This observation is consistent with either an increase of the molecular weight of Cx40CT due to the binding of both the c-Src SH3 and ZO-1 PDZ-2 domains or the exchange rate of this Cx40CT region bound to the SH3 domain changed from fast to intermediate on the NMR time scale (i.e. higher binding affinity). Altogether, these data suggest that both c-Src and ZO-1 interact simultaneously with the Cx40CT and that c-Src alone may not be responsible for displacement of the Cx40CT/ZO-1 interaction, a departure from the observations made with the Cx43CT.

Discussion

We have characterized the structure of the carboxyl terminal domain of Cx40 by the use of NMR. Our data identified the structural modifications brought about by the interaction of Cx40CT with two well-known molecular partners and determined that those modifications are rigid, so that binding to one partner does not affect the interaction with the other. These studies further expand our structural knowledge of how connexin domains integrate in multi-molecular complexes and provide insight into the molecular mechanisms involved in the regulation of Cx40.

Comparison of the Cx40CT and Cx43CT Structures

The Cx43CT structure was determined to be predominately random coiled, but shows two short helical domains, which may be involved in protein interactions (Sorgen et al., 2002; Sorgen et al., 2004b). In this manuscript, we show that the Cx40CT, on the other hand, is completely unstructured according to standard NMR predictors (i.e. α - and β -carbon chemical shift values and NOEs connectivities). Our previous work showed that the Cx43CT domain undergoes pH-dependent homo-oligomerization; we also demonstrated that residues within the two helical domains are involved in the dimerization interface (Hirst-Jensen et al., 2007; Sorgen et al., 2004b). In contrast, sedimentation equilibrium and chemical cross-linking studies of the Cx40CT indicated only a small increase in dimerization upon acidification of the solvent (unpublished data). Together, these data support the notion that helical domains play an important role in the process of pH-dependent homo-oligomerization, which may occur in some, but not all connexin CT domains.

An interesting question that arises is, what is the physiological importance of an intrinsically disordered Cx40CT or Cx43CT domain? In general, intrinsically disordered proteins have been identified as playing important roles in molecular recognition, molecular assembly, protein modifications, and entropic chains (Dunker et al., 2002). In the case of membrane proteins, like Cx40 and Cx43, intrinsically disordered domains seem to play an important role in cell signaling events, acting as components of macromolecular processes and substrates for regulatory events such as phosphorylation (Dunker and Obradovic, 2001). We speculate that a disordered CT allows the connexin molecule to be a point of convergence for alternative regulatory pathways. Multiple alternative interactions of low affinity (even if of high specificity) allows a single molecule to rapidly transition from one molecular partner to another, thus acting as a “switchboard” for adapting the properties of cell communication to the environment prevalent in the intracellular space. Interestingly, the CT domains of Cx40 and Cx43 are not alone in their structural characteristics. Analysis of genomic sequence data strongly suggested that there is a high prevalence of intrinsically disordered domains. Indeed, an estimated 41% of human membrane proteins, including connexins, glutamate [NMDA] receptor subunit $\epsilon 4$, and voltage-dependent T-type calcium channel α -1G subunit (Minezaki et al., 2007); have intrinsically disordered regions with more than 30 consecutive residues, and these residues are preferentially localized at the cytoplasmic side.

Comparison of the Cx40CT and Cx43CT Interaction with the ZO-1 PDZ-2 Domain

Many studies have focused on the relationship between Cx43 and ZO-1 (for review see (Herve, 2007)). ZO-1 is important for maintaining cell polarity as well as for coupling the extracellular environment to intracellular signaling pathways and the cytoskeleton (Itoh et al., 1993). A Cx43/ZO-1 interaction has been identified in many cells (e.g. myocytes (Giepmans and Moolenaar, 1998)), however, ZO-1 binding is not essential for the formation of functional Cx43 channels (Dunham et al., 1992; Fishman et al., 1991). Data suggest that

this interaction may regulate the formation (Toyofuku et al., 1998) and size of the Cx43 gap junction plaque (Hunter et al., 2005), as well as the internalization and remodeling of Cx43 upon changes in the intracellular environment (Barker et al., 2002; Duffy et al., 2004).

We demonstrated that the PDZ-2 domain of ZO-1 affected residues H328-V355 of Cx40, a region larger than what was expected from a consensus “PDZ binding domain” (Doyle et al., 1996; Morais Cabral et al., 1996). The different dynamics between residues H328-Q332 and G333-V355 indicate a possible tighter association between residues G333-V355, which contains the consensus-binding motif for ZO-1. While this result is similar to what was observed when the PDZ-2 domain associated with the Cx43CT (Sorgen et al., 2004a), the Cx43CT and Cx40CT domains differed in the number of residues affected and in the dynamics of the interaction. Overall, the PDZ-2 domain affected the last 28 residues of the Cx40CT and 19 residues of the Cx43CT (S364-I382). If a comparison is made between the more “tightly” associating residues, only the last 11 Cx43CT residues were affected, while the last 23 Cx40CT were affected. This suggests an overall tighter association of the PDZ-2 domain with Cx40CT than Cx43CT.

Comparison of the Cx40CT and Cx43CT Interaction with the c-Src SH3 Domain

Many studies have demonstrated an association of Cx43 with c-Src, a plasma membrane-associated tyrosine kinase (Solan and Lampe, 2005). c-Src (and v-Src)-induced phosphorylation of Cx43 has been correlated with an inhibition of gap junction intercellular communication (GJIC). Src activation may lead to tyrosine phosphorylation (Lin et al., 2001) as well as indirectly serine phosphorylation (perhaps MAPK-mediated; (Zhou et al., 1999)). The mechanism of channel closure has been proposed to occur through a “particle-receptor” type of interaction, similar to that proposed for pH gating of Cx43 channels (Cottrell et al., 2003; Homma et al., 1998; Morley et al., 1996; Zhou et al., 1999), which results in a decrease in electrical coupling caused by a reduced channel open probability and possibly altered selectivity (Cottrell et al., 2003).

While many studies have determined that the SH3 domain of activated Src binds the CT domain of Cx43 near P280 (Calero et al., 1998; Kanemitsu et al., 1997; Sorgen et al., 2004a), to the best of our knowledge, no studies have demonstrated that c-Src can associate with Cx40. Our data indicate that SH3 binding induces major structural changes throughout the Cx40CT (V255-N265, F276-S278, S281, G285-A294, and I310-H311), unlike the Cx43CT, which was isolated to one specific region (K264-K287). The Cx40CT sequence does not contain a consensus PXXP SH3 binding motif; however, a number of peptides lacking a PXXP motif, but rich in prolines and/or positively charged residues, have been identified (Jia et al., 2005). Two of the Cx40CT domains affected by SH3 correspond to this description: V255-N265 contains 3 prolines and R287-L293 contains an arginine, lysine, and 2 prolines. Another difference between these CT domains upon binding the SH3 domain was that the Cx40CT residues shifted (exchange between the free and fully bound states of Cx40CT is fast on the chemical shift timescale), while Cx43CT residues K264-287 disappeared (exchange is intermediate on the chemical shift timescale), suggesting the SH3 domain has a tighter binding affinity for the Cx43CT. This observation is supported by the NMR data where the binding affinity for the SH3 interaction with Cx40CT ($K_D = 1.1$ mM) was significantly lower than those values calculated by SPR for the Cx43CT ($K_D = 4.5$ μ M; (Duffy et al., 2004)).

Comparison of c-Src mediated signaling via Cx40CT and Cx43CT

The binding of Cx43 to c-Src and to ZO-1 are interdependent. c-Src can disrupt the Cx43/ZO-1 interaction, leading to down-regulation of GJIC (Duffy et al., 2004; Toyofuku et al., 2001). Interestingly, the primary binding sites for ZO-1 (PDZ-2 domain) and c-Src (SH3

domain) correspond to Cx43 regions that are distant in primary sequence (~100 residues apart) (Sorgen et al., 2004a). Our NMR studies demonstrated that binding of the c-Src SH3 domain has global effects on the Cx43CT structure, extending upstream to the c-Src SH2 binding domain and downstream to the PDZ-2 binding domain of ZO-1; moreover, SH3 binding to the Cx43CT disrupted the association of the Cx43CT/ZO-1 PDZ-2 domain complex (Sorgen et al., 2004a). Surprisingly, in spite of the sequence differences between the Cx40CT and Cx43CT domains, the residues affected upon binding of the SH3 and PDZ-2 domains to Cx40CT are very similar to those observed for Cx43CT, suggesting a comparable signalling event mediated by c-Src (Figure 7). However, the Cx40CT/ZO-1 PDZ-2 domain complex is not affected by the addition of the c-Src SH3 domain. We have observed that an interaction between the ZO-1 PDZ-2 and c-Src SH3 domains exists and that this interaction may be involved in displacement of the Cx43CT/PDZ-2 binding (unpublished data). Therefore, we speculate that, if compared to the Cx43CT/PDZ-2 association, a combination of an increased affinity of Cx40CT for PDZ-2, and the increased number of Cx40CT residues affected by the PDZ-2 interaction (competitive inhibition), hinders the ability of SH3 to displace PDZ-2 from its Cx40CT binding site. Future studies will address the physiological role of c-Src in the context of binding and regulating homomeric and heteromeric Cx40 gap junctions, as well as determining whether Cx40CT phosphorylation or other molecular partner interactions are necessary to dissociate the Cx40CT/ZO-1 interaction.

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Figure 1.

Secondary structural analysis of the Cx40CT domain. A) Analysis of the chemical shift deviations from random coil for α - and β -carbon chemical shift. All residues located immediately N-terminal to a proline residue, except Q320, had α -carbon chemical shift values shifted approximately -2 ppm. B) Backbone NOEs used in the structural analysis.

Figure 2. $^1\text{H}/^{15}\text{N}$ -HSQCs of the Cx40 carboxyl terminus (Cx40CT) in the presence of the PDZ-2 domain of ZO-1. This experiment allowed for detection of the regions of Cx40CT that were structurally modified when in the presence of a binding domain from the molecular partner ZO-1. Panel A: Cx40CT was titrated with the PDZ-2 domain of ZO-1 to a 1:2 molar ratio. The control spectra, Cx40CT only (black), have been overlapped with spectra obtained when both molecules were present at a 1:2 molar ratio (red). The amino acid peaks that were affected have been labeled. Panel B: Close-up view from panel A (dotted box) to illustrate a sample of the affected amino acids. Panel C: Summary of the Cx40CT residues affected by the presence of the ZO-1 PDZ-2 domain. Residues which disappeared (green) and decreased in intensity (grey) have been labeled.

Figure 3.

Change in chemical shift intensities on Cx40CT binding to the ZO-1 PDZ-2 domain. Panel A: ^1H ^{15}N -HSQC titration of ^{15}N -Cx40CT with unlabelled PDZ-2 in which the Cx40CT:PDZ-2 concentration ratios ranged from 1:0 to 1:5. Each titration contained the same concentration of ^{15}N -Cx40CT ($20 \mu\text{M}$) with different concentrations of the PDZ-2 domain. The cross-peak color changes according to the concentration ratio (Cx40CT:PDZ-2 - 1:0 (black), 1:0.5 (red), 1:1 (green), 1:2 (blue), 1:2.5 (magenta), 1:3.5 (black), 1:5 (cyan)). The arrow points in the direction of the increasing PDZ-2 concentrations. The overlapped spectra have been offset for easier visualization of the decrease in signal intensity. A294 is the control demonstrating a Cx40CT residue that had no change in signal intensity. Panel B: The dissociation constant (K_D) for the Cx40CT/PDZ-2 interaction was estimated by fitting the observed decreases in signal intensity for Cx40CT residues R349, S350, S354, and V355 as a function of PDZ-2 concentration.

Figure 4. $^1\text{H}/^{15}\text{N}$ -HSQCs of the Cx40 carboxyl terminus (Cx40CT) in the presence of the SH3 domain of c-Src. This experiment allowed for detection of the regions of Cx40CT that were structurally modified when in the presence of a binding domain from the molecular partner c-Src. Panel A: Cx40CT was titrated with the SH3 domain of c-Src to a 1:4 molar ratio. The control spectra, Cx40CT only (black), have been overlapped with spectra obtained when both molecules were present at a 1:4 molar ratio (red). The amino acid peaks that were affected have been labeled. Panel B: Close-up view from panel A (dotted box) to illustrate a sample of the affected amino acids. Panel C: Summary of the Cx40CT residues affected by the presence of the c-Src SH3 domain. Residues which shifted ≥ 0.035 ppm (red) and < 0.035 ppm (grey) have been labeled.

Figure 5.

Change in chemical shift position on Cx40CT binding to the c-Src SH3 domain. Panel A: ^1H - ^{15}N -HSQC titration of ^{15}N -Cx40CT with unlabelled SH3 in which Cx40CT:SH3 concentration ratios ranged from 1:0 to 1:32. Each titration contained the same concentration of ^{15}N -Cx40CT (100 μM) with different concentrations of the c-Src SH3 domain. The cross-peak color changes according to the concentration ratio (Cx40CT:SH3 - 1:0 (black), 1:1 (green), 1:7 (blue), 1:13 (red), 1:32 (black)). The arrow points in the direction of the increasing SH3 concentrations. Titrations of 1:0.6, 1:4, 1:10, 1:16, and 1:24 were also collected and used for calculating the dissociation constant (panel B), but were not included for easier visualization of the chemical shift changes. N300 is the control demonstrating a Cx40CT residue that did not shift. Panel B: The dissociation constant (K_D) for the Cx40CT/SH3 interaction was estimated by fitting the change in chemical shift ($\Delta\delta$) for Cx40CT residues G257, Q256, R287, and N289 as a function of SH3 concentration. The $\Delta\delta$ was calculated according to the formula $\Delta\delta_{\text{av}} = \sqrt{(\Delta\delta_{\text{HN}})^2 + (\Delta\delta_{\text{N}/5})^2}$.

Figure 6.

$^1\text{H}/^{15}\text{N}$ -HSQCs demonstrating the Cx40CT interacts simultaneously with the ZO-1 PDZ-2 and c-Src SH3 domains. The Cx40CT residues which disappeared due to the Cx43CT/PDZ-2 interaction (G333-V355; green) and shifted ≥ 0.035 ppm due to the addition of the SH3 domain (V255-N265, F276-S278, S281, G285-A294, and I310-H311; black) have been labeled. Colors correspond to $^1\text{H}/^{15}\text{N}$ -HSQC spectra of Cx40CT alone (black), Cx40CT combined with unlabelled PDZ-2 (green), ^{15}N -Cx40CT/ZO-1 PDZ-2/c-Src SH3 (1:2:2 molar ratio; blue), and 4) ^{15}N -Cx40CT/ZO-1 PDZ-2/c-Src-SH3 (1:2:4 molar ratio; red). Each sample contained the same concentration of ^{15}N -Cx40CT (100 μM).

Figure 7. Comparison of the Cx40CT and Cx43CT residues affected by the interaction with the ZO-1 PDZ-2 and c-Src SH3 domains. Data for the Cx43CT interaction with the ZO-1 PDZ-2 and c-Src SH3 domains was obtained from (Sorgen et al., 2004a).