# Identification of Calcium-Independent and Calcium-Enhanced Binding between S100B and the Dopamine D2 Receptor

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S *Supporting Information*

ABSTRACT: S100B is a dimeric EF-hand protein that undergoes a calcium-induced conformational change and exposes a hydrophobic protein-binding surface. Recently S100B was identified as a binding partner of the dopamine D2 receptor in a bacterial two-hybrid screen involving the third intracellular loop (IC3). The low *in vivo* calcium concentration in bacteria (100−300 nM) suggests this interaction may occur in the absence of calcium. In this work the calcium-sensitive ability for S100B to recruit the IC3 of the dopamine D2 receptor was examined, and regions in both proteins required for complex

formation were identified. Peptide array experiments identified the C-terminal 58 residues of the IC3 (IC3-C58) as the major interacting site for S100B. These experiments along with pull-down assays showed the IC3 interacts with S100B in the absence and presence of calcium. $^1H-^{15}N$ HSQC experiments were used to identify residues, primarily in helices III and IV, utilized in the IC3-C58 interaction. NMR titration data indicated that although an interaction between apo-S100B and IC3-C58 occurs without calcium, the binding was enhanced more than 100-fold upon calcium binding. Further, it was established that shorter regions within IC3-C58 comprising its N- and C-terminal halves had diminished binding to Ca^{2+}-S100B and did not display any observable affinity in the absence of calcium. This indicates that residue or structural components within both regions are required for optimal interaction with Ca^{2+}-S100B. This work represents the first example of an S100B target that interacts with both the apo- and calcium-saturated forms of S100B.
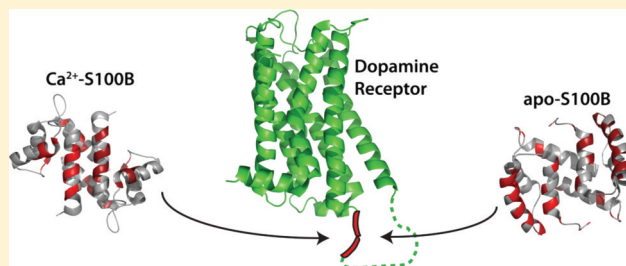
The S100 family consists of a group of about 25 dimeric, calcium-signaling proteins. Calcium binding to an S100 protein produces a conformational change that exposes a hydrophobic surface, allowing recruitment of other proteins that leads to a biological response. Each S100 protein is comprised of two EF-hand calcium-binding sites per protomer connected by a central linker. The first site, formed by helix I a calcium-binding loop and helix II, has relatively weak calcium binding ability.[1−3] The second motif contains helix III−loop−helix IV and has a higher affinity for calcium. The dimeric nature of the S100 proteins allows them to bind to two or more target molecules simultaneously. For example, S100B is a well-characterized member of the family that has been shown to bind a diverse array of targets, including annexin A6, p53, RAGE, and NDR kinase.[4−7] In addition, S100B has been shown to be a biomarker for brain injury[8] and is linked to neurological diseases such as Parkinson's and schizophrenia.[9,10]

Dopamine D2 receptors are G-protein coupled receptors that transmit the signal from extracellular dopamine to the cytoplasm in several neuronal cell types. Downstream targets affected by dopamine binding to the D2 receptor include adenylate cyclase,[11] inwardly rectifying potassium channels,[12] and voltage activated N-type calcium channels.[13] Like S100B, the D2 receptor has been implicated in Parkinson's disease and schizophrenia as well as drug addiction. The dopamine D2 receptor contains seven transmembrane domains and has the ability to dimerize.[14] There are several different types of

dopamine receptor that are classified based on the sequence of their third cytoplasmic loop (IC3) that connects the membrane spanning α5 and α6 helices. The dopamine D2 receptor IC3 is also the main site of interaction for several proteins including calmodulin[15] and GTP-coupled proteins giving rise to a variety of signal transduction events.

Calcium dependence is a defining characteristic of S100B interactions. For example, S100B binds to p53 in a calcium-dependent manner and inhibits p53 function, promoting cell survival.[5] The NMR solution structure of Ca^{2+}-S100B with a bound p53-derived peptide shows the p53 adopts an α-helical conformation and binds to helices III and IV of S100B along a flat hydrophobic pocket.[16] This same calcium-dependent interaction is also observed for NDR kinase[7,17] and TRTK peptides.[18,19] Recent work identified S100B as a potential binding partner of the dopamine D2 receptor using a bacterial two-hybrid assay.[20] This result was surprising as live-cell based two-hybrid assays typically have not been amenable to the search for binding partners of calcium-binding proteins.[21] This caveat is one of the major hurdles in studying calcium-signaling networks. In the case of the bacterial two-hybrid assay intracellular free calcium in *E. coli* is tightly regulated between

100 and 300 nM,[22,23] well below the reported calcium dissociation constants for S100B ($K_d \sim 20-60$ μM) and most other S100 proteins.[2,3,24] This suggests the interaction of S100B with the D2 receptor interaction could be calcium independent. It was also demonstrated that S100B could enhance D2 receptor downstream events in HEK293 cells,[20] although the role of calcium in this interaction is not yet fully understood. Resting calcium concentrations in mammalian cell types are also well below the S100B dissociation constant for calcium.[25] One suggestion is that S100B might be required for efficient surface presentation of receptors, like the dopamine D2 receptor, on the plasma membrane.[26] In a similar case S100A10 has been shown to bind to serotonin receptors and annexin A2, allowing them to be targeted to lipid domains in the plasma membrane. Interestingly, S100A10 knockout mice display depression like symptoms, a phenotype linked to problems in the serotonin-signaling pathway.[27]

In this work we used gel filtration, peptide array, and NMR experiments to understand the mechanisms of interaction between S100B and the dopamine D2 receptor. In particular, we examined the ability of calcium to modulate this interaction and identified the regions on both S100B and the D2 receptor required for complex formation. The work shows that S100B binding to the receptor is localized to the C-terminus of the IC3 loop. Interestingly, there is a clear, albeit weak association between S100B and the IC3 in the absence of calcium, and this association is strongly enhanced upon calcium binding to S100B.

■ EXPERIMENTAL PROCEDURES

Protein Constructs and Purification. Expression and purification of unlabeled and uniformly ^{15}N-labeled S100B were completed as described previously.[28,29] The purified protein was thoroughly dialyzed against multiple changes of EDTA-containing buffer to remove all calcium, 50 mM KCl, 1 mM DTT, 5 mM EDTA, pH 8.0. The apo-S100B protein was then dialyzed into a 3 mM KCl solution at pH 8.0, lyophilized, and stored at -20 °C.

A construct containing residues R217-Q345 (IC3) from the D2S dopamine receptor was a generous gift from K. Neve (Oregon Health and Science University, Portland, OR).[20] This region, containing the entire IC3 as identified by TM-Finder,[30] was moved into a p11 plasmid (Structural Genomics Consortium-Toronto) containing a TEV protease cleavable His[6] purification tag. A synthetic gene construct covering residues S288-Q345 of the D2S receptor (IC3-C58) and containing a TEV protease cleavable His[6] tag was synthesized (DNA 2.0, Menlo Park, CA) and placed in a pJ404 plasmid under control of the T5 promoter. Synthetic peptides comprising residues R217-K241 (IC3-N25), S288-I315 (IC3-M28), and Q316-Q345 (IC3-C30) were purchased (Biobasic, ON, Canada). Purity of the peptides (>95%) was confirmed by HPLC and ESI-MS.

His[6]-IC3 and His[6]-IC3-C58 proteins were expressed and purified in identical manners. BL21 DE3 *E. coli* cells containing the appropriate construct plasmid were grown in Luria Broth with ampicillin at 37 °C to an OD[600] of 0.6. Protein expression was induced for 6 h with addition of 1 mM IPTG. Cells were harvested by centrifugation, and the cell pellet was resuspended in 50 mM NaH[2]PO[4] and 500 mM NaCl at pH 8.0 (Buffer A). Cells were lysed using a French pressure cell, and the lysate was clarified by centrifugation at 38 000 rpm for 1 h. The lysate was then applied to a HiTrap Ni^{2+} FastFlow column (5 mL)

(G.E. Healthcare, QC, Canada) equilibrated in Buffer A. The column was washed to a steady baseline with 10 column volumes of Buffer A supplemented with 62.5 mM imidazole. Proteins were step eluted with Buffer A containing 250 mM imidazole. Fractions containing purified proteins were pooled and TEV protease was added in a 1:100 ratio based on amount of protein in the pooled fractions. During TEV cleavage the protein was dialyzed overnight at 4 °C into Buffer A to remove imidazole. Protein solutions were reapplied to a HiTrap Ni^{2+} FastFlow column (5 mL) (G.E. Healthcare, QC, Canada), and the flow-through, containing the cleaved IC3 or IC3-C58 proteins, was collected. Further protein purification was achieved using a HiTrap S-HP column (5 mL) (G.E. Healthcare, QC, Canada) and a linear salt gradient. The final purified proteins were verified by SDS-PAGE and ESI-MS. From ESI-MS the mass obtained for the IC3 was 15 295.7 $\pm$ 0.7 Da, which corresponds closely to the calculated mass of 15 293.5 Da. The mass obtained for IC3-C58 was 6781.9 $\pm$ 0.1 Da, which corresponds well to the calculated mass of 6780.7 Da.

Pull-Down Assays. For pull-down experiments His[6]-IC3 was exhaustively dialyzed into 50 mM Tris-HCl, 200 mM NaCl, 0.2 mM tris(2-carboxyethyl)phosphine (TCEP) at pH 7.5. All buffers and protein solutions were treated with Chelex resin to ensure the complete removal of contaminating calcium. S100B and IC3 proteins (75 μM) were mixed in a 1:1 ratio (S100B protomer:IC3) in the presence and absence of 2 mM calcium and incubated at ambient temperature for 1 h. Ni^{2+}-NTA spin columns (Qiagen, ON, Canada) were equilibrated in dialysis buffer prior to addition of the S100B/IC3 mixture (600 μL). Spin columns were then washed three times with dialysis buffer and eluted with 600 μL of dialysis buffer supplemented with 500 mM imidazole. Samples were taken of the applied, unbound, and eluted fractions and run on SDS-PAGE gels, which were then stained with Coomassie blue dye.

Peptide Array Experiments. Lyophilized S100B protein (100 μM) was resuspended in 20 mM Tris-HCl and 150 mM NaCl at pH 7.0 (TBS). Solutions and proteins were supplemented with either 10 mM EDTA or 1 mM calcium depending on whether calcium-free (apo) or calcium-saturated S100B was needed. A G-25 desalting column (10 mL, GE Healthcare, QC, Canada) was washed with H[2]O and then equilibrated in TBS. The column was loaded with S100B (1 mL), and the two most concentrated fractions (0.5 mL) were pooled for further experiments. A solution of Alexa-Fluor 680 C2-maleimide (Invitrogen, ON, Canada) in methanol (5 mM, 100 μL) was prepared and mixed with 100 μL of TBS. A portion of this solution (100 μL) was transferred to the pooled S100B protein solution and gently mixed for 2 h at room temperature. The solution was passed through a G-25 desalting column; fractions containing the Alexa-S100B protein were collected and dialyzed exhaustively into TBS to remove any free Alexa-Fluor 680. The final concentration of Alexa-S100B was determined using the Alexa-Fluor 680 extinction coefficient ($\varepsilon^{679\,nm} = 184\,000$).

Peptide arrays were synthesized by FMOC chemistry using an Auto-Spot Robot ASP222 (Amimed) on nitrocellulose membranes.[31,32] Sequential peptides of the IC3 were spotted on each array membrane. Two arrays, using peptides 18 amino acid residues in length and stepping through the IC3 sequence by either one or two residues, were created producing two nonidentical but overlapping arrays. Each array was washed with methanol followed by TBS supplemented with 0.05% Tween-20 (TBS-Tween). Membranes were blocked using either TBS-Tween with 5% w/v skim milk powder (blocking buffer)

for Ca^{2+}-S100B experiments, or 1X Blocker (Pierce, ON, Canada) in TBS-Tween for apo-S100B experiments, followed by washing with TBS-Tween. Alexa-S100B (0.05 μM protomer) was diluted into blocking buffer and incubated with each array for 2 h at room temperature. In each case, the solution was removed and the array washed several times with TBS-Tween. Arrays were imaged using an Odyssey imaging system (LI-COR, Lincoln, NE).

Gel Filtration Experiments. Purified S100B and IC3-C58 proteins were dialyzed into buffer containing 50 mM Tris-HCl, 150 mM NaCl at pH 7.4. S100B and IC3-C58 were mixed in a 1:1 ratio (50 μM protomer, 500 μL) and supplemented with either 10 mM EDTA or 1 mM CaCl$_2$ for calcium-free or calcium-saturated experiments. A Superdex G75 analytical gel filtration column (1 cm × 30 cm) (GE Healthcare, QC, Canada) was equilibrated with buffer prior to sample injections (500 μL) of the S100B/IC3-C58 solution. An elution rate of 0.5 mL/min was maintained, monitored at 280 nm. Since S100B and IC3-C58 proteins absorb poorly at 280 nm, the collected fractions (0.5 mL) were assessed by SDS-PAGE and stained with Coomassie blue dye.

NMR Titration Experiments of S100B and IC3. All NMR experiments were performed at 35 °C on a Varian INOVA 600 MHz spectrometer with pulsed-field gradient triple resonance probe. Protein solutions were prepared in 50 mM MOPS, 50 mM KCl, 1 mM TCEP, 1 mM DTT, and 5 mM NaN$_3$ at pH 7.0 in 90% H$_2$O/10% D$_2$O using DSS as an internal reference. In addition, imidazole was added as an internal pH standard to ensure the sample pH was maintained during titrations.[33] Samples contained either apo-S100B (200 μM protomer) or Ca^{2+}-S100B (100 μM protomer). Ca^{2+}-S100B experiments contained 6 mM calcium chloride. Apo-S100B experiments used EDTA-treated protein, and all buffers were treated with Chelex resin (BioRad, ON, Canada) to remove any free calcium. In all experiments concentrated stocks of peptides (IC3-C58, IC3-N25, IC3-M28, and IC3-C30) were made up in the appropriate buffer (with and without calcium), identical to that of the S100B protein. S100B protein concentrations were determined by amino acid analysis (The Hospital for Sick Children-Amino Acid Analysis Facility, Toronto) done on triplicate samples with peaks corresponding to alanine and leucine used to calibrate protein concentration.

Titration experiments were monitored using sensitivity-enhanced ^{1}H−^{15}N HSQC spectra collected for each addition of peptide.[34] Spectra were collected with 1024 complex points in the ^{1}H dimension and 64 increments in the ^{15}N dimension. Data were processed using NMRPipe[35] and analyzed using NMRViewJ.[36] Titrations were followed using additions of the appropriate peptide solution until a minimum of 2.0 equiv of peptide per S100B subunit had been added. In all cases, the volume of added peptide was less than 10% of the total starting sample volume (600 μL). The change in chemical shift for selected peaks in the Ca^{2+}-S100B spectrum with each addition of peptide was determined as $\Delta\delta = [(\Delta\ ^1H^2) + ((\Delta\ ^{15}N/5)^2)]^{1/2}$.[37] To reduce measurement errors in the relatively small apo-S100B chemical shifts, only the change in proton chemical shift was used during fitting. Titration binding data were fit according to the equation

$$Y = N\frac{(P_t + L_t + K_d) - \sqrt{(P_t + L_t + K_d)^2 - 4P_tL_t}}{2P_t} \quad (1)$$

where P_t is the total S100B protein concentration, L_t is the total IC3 ligand peptide concentration, K_d is the dissociation constant,

and N is the maximum chemical shift change upon ligand binding. A global K_d value was calculated for each peptide titration by simultaneously fitting the chemical shifts from six different residues in Ca^{2+}-S100B or ten different residues in apo-S100B to the above model.

■ RESULTS

In the dopamine receptor family the third cytoplasmic loop (IC3) defines the receptor subtype and is known to mediate cytoplasmic protein interactions between the dopamine receptor and the rest of the signaling pathway. There are two isoforms of the D2 receptor, which vary by a 29 amino acid insertion in the IC3, the D2S (short, IC3-129 residues), and the D2L (long, IC3-158 residues). Both the D2L and D2S variants of the IC3 have been shown to interact in a similar manner with S100B,[20] indicating that the insertion in the D2L receptor is not likely involved in the recognition of S100B. Therefore, for simplicity the D2S isoform of the IC3 region covering residues R217-Q345 of the transmembrane receptor was utilized in this study (Figure 1). Structural information about this region of the
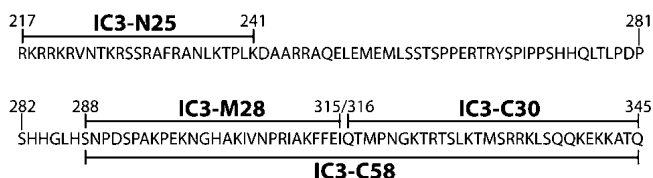

Figure 1. Schematic diagram of the dopamine peptides used for S100B binding experiments. The amino acid sequence for the third cytoplasmic loop of the dopamine D2S receptor (IC3) is shown (R217-Q345). Regions within the IC3 that were expressed from *E. coli* or synthesized as peptides are indicated by bars above the protein sequence near the N-terminus (IC3-N25) and C-terminus (IC3-C58, IC3-M28, IC3-C30).

D2 dopamine receptor is not available. The IC3 is highly basic and has a pI of 11.5. The crystal structure of the closely related D3 dopamine receptor does not contain electron density in the region of the IC3, and crystals were obtained only when the majority of the D3-IC3 was removed.[38] This suggests that the IC3 is present as a flexible loop and mediates protein interactions mostly through contiguous stretches in its sequence.

Apo- and Calcium-Bound S100B Interact with IC3. The hexahistidine-tagged IC3 (His$_6$-IC3) was expressed and purified from *E. coli* for structural analysis and interaction with S100B. The IC3 yield was low, and the protein was prone to proteolysis. Nevertheless, a sufficient quantity of purified protein was obtained to test the interaction between S100B and the IC3 using a Ni^{2+} pull-down assay. Given that S100B is a calcium-binding protein that interacts with its binding partners in a calcium-dependent manner, the binding of S100B with IC3 was examined in the absence and presence of calcium. His$_6$-IC3 was bound to Ni^{2+}-resin spin columns in the presence of apo- and Ca^{2+}-S100B. Bound proteins were eluted from the column and analyzed by SDS-PAGE (Figure 2). His$_6$-IC3 protein was found to coelute with Ca^{2+}-S100B from the Ni^{2+} column. Interestingly, even in the absence of calcium, apo-S100B bound to His$_6$-IC3, although to a lesser extent than Ca^{2+}-S100B. This indicates that both apo- and Ca^{2+}-S100B were able to bind with the IC3 region of the dopamine D2 receptor.

Identification of IC3 Binding Regions with S100B. The IC3 loop from the dopamine D2 receptor is much larger than
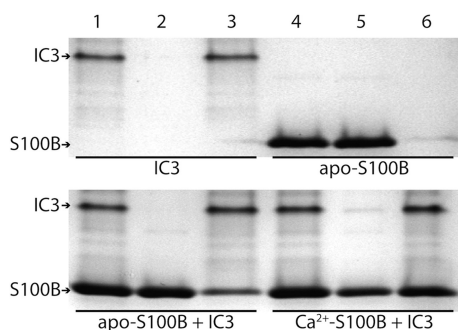
Figure 2. Interaction of IC3 with S100B from affinity chromatography. The SDS-PAGE gel in the top panel shows His_6-IC3 (lanes 1−3) and apo-S100B (lanes 4−6) loaded on the Ni^{2+} resin column (lanes 1, 4) and the wash fraction (lanes 2, 5) followed by elution with excess imidazole (lanes 3, 6). In the bottom panel purified apo-S100B (lanes 1−3) and Ca^{2+}-S100B (lanes 4−6) were incubated together with His_6-IC3 and applied to Ni^{2+} resin (lanes 1, 4), washed from the resin as unbound fractions (lanes 2, 5), and bound protein eluted (lanes 3, 6) with excess imidazole containing buffer.

typical S100B binding partners previously identified. Therefore, it is likely that a smaller portion of the IC3 is involved in the interaction with S100B and experiments were conducted to delineate the interaction site(s) more precisely. To determine the region of IC3 that binds to S100B peptide array experiments were conducted using a nested series of 18-residue sequences linked to a nitrocellulose membrane. The first peptide of the arrays corresponded to residues R217-A234 of the D2S receptor, the N-terminal 18 residues of the IC3. Two different arrays were constructed in which subsequent 18-residue peptides continued to step through the IC3 by either one or two amino acids. Both array types were probed for binding to either apo-S100B or Ca^{2+}-S100B, labeled with Alexa-Fluor 680 at position C84, previously shown to be benign to target protein binding when fluorescently labeled.[39] The results of the array that stepped through the IC3 region by two residues at a time (Figure 3) showed that both apo-S100B and Ca^{2+}-S100B were able to bind to the IC3 region of the D2 receptor. In addition, the pattern of peptides from the IC3 that bound to apo-S100B and Ca^{2+}-S100B was very similar. These observations were confirmed with arrays that stepped through the IC3 sequence by one residue. It is interesting that the peptide arrays did not show obvious differences in affinity between apo-S100B and Ca^{2+}-S100B that were observed in the pull-down experiments. This is likely due to the relatively high local concentrations of peptide on each spot and the concentrations of S100B protein needed to visualize a positive interaction. In combination with the different buffer conditions used in the apo-S100B and Ca^{2+}-S100B arrays it has been shown that this makes comparison of spot intensities as a means for relative affinities difficult.[40]

The most intense binding was observed for peptide F1 in both the apo-S100B and Ca^{2+}-S100B arrays. A closer analysis of the array data revealed two distinct binding regions in the IC3, which were selected for further study. The major region covered the C-terminal 58 amino acids (IC3-C58), denoted by peptides D6-F7. A second minor region identified from a single spot (A3) on the arrays included the N-terminal 25 residues of the IC3 (IC3-N25). A large area comprising residues K241-S288 (A6-D5) from the IC3 exhibited very little observable fluorescence for both apo- or Ca^{2+}-S100B, indicating this region of IC3 is not a major interacting site for S100B. Further analysis
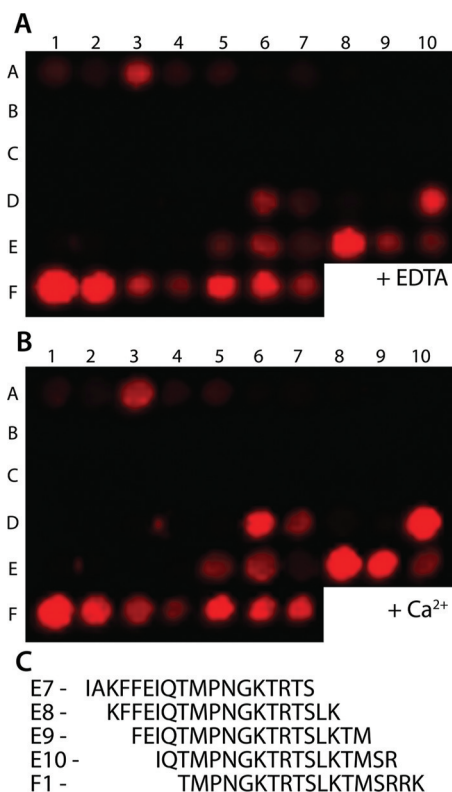

Figure 3. Representative peptide arrays of the IC3 regions probed with S100B. The 129-residue sequence of IC3 was arrayed on cellulose membranes and probed with fluorescently labeled S100B in the (A) absence and (B) presence of calcium. These arrays consist of 18-residue peptides synthesized at each spot on the membrane and stepping through the IC3 sequence two residues at a time. The coordinates of each spot on the arrays are identified by row (A, B, C) and column numbers (1, 2, 3) beside and above the arrays, respectively. (C) An example of the peptide sequence progression from spot to spot is shown using the array coordinates (E7−E10, F1). S100B was fluorescently labeled with Alexa Fluor 680 at position C84 and then used to treat the membranes in the presence of (A) EDTA or (B) calcium. Red fluorescence is indicative of bound S100B protein.

of the IC3-C58 region from the peptide array experiments showed that it could be subdivided into two smaller regions demonstrating more intense S100B binding, separated by a small region with weaker binding (E1-E5). The C-terminal 30 residues of the IC3 (IC3-C30) (E10-F7) and the 28-residue region adjacent to it (IC3-M28) (D6-E1) peptides split the IC3-C58 region close to the middle. Because the array sequences contain overlapping peptides, the region E2-E9 is represented in both IC3-C30 and IC3-M28 peptides. This series of four peptides (Figure 1) was used to identify the binding locations of IC3 on both apo- and Ca^{2+}-S100B as well as the strength of the interactions.

Disordered IC3-C58 Interacts with S100B. The IC3-C58 peptide was expressed, purified, and analyzed by circular dichroism (CD) spectropolarimetry. The resulting spectrum showed one region of stronger ellipticity near 200 nm, typical of a disordered protein (data not shown). In addition, a ^1H−^{15}N HSQC spectrum collected using ^{15}N-labeled IC3-C58 showed the majority of the peaks were very intense and collapsed into a small central region centered between 7.1 and 8.0 ppm (^1H shifts, data not shown). These data show that

IC3-C58 is disordered in solution and lacks regular secondary structure.

The formation of complexes between IC3-C58 and both apo-S100B and Ca²⁺-S100B was examined using gel filtration chromatography. Mixtures of S100B and IC3-C58 proteins were applied to an analytical gel filtration column in the presence of either EDTA (apo-S100B) or excess CaCl₂. Since S100B and IC3-C58 have poor absorbance characteristics at 280 nm, fractions were collected from the gel filtration column and examined by SDS-PAGE gel electrophoresis, providing a clear image of the proteins and potential complexes (Figure 4).
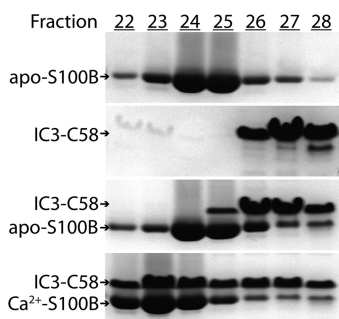

Figure 4. Identification of the S100B-IC3 complex by gel filtration. Bacterially expressed and purified IC3-C58 was preincubated with either apo-S100B or Ca²⁺-S100B and loaded on an analytical Superdex-75 gel filtration column. Eluted fractions were collected and analyzed by SDS-PAGE gel electrophoresis. The top two panels show the individual apo-S100B and IC3-C58 proteins. The bottom two panels show the mixture in the presence of 10 mM EDTA (apo-S100B) or 1 mM CaCl₂ (Ca²⁺-S100B).

The data showed that S100B, in the absence of IC3-C58, eluted at an earlier volume than the D2 receptor protein, consistent with the larger dimeric nature of S100B (21 kDa) compared to IC3-C58 (6.5 kDa). When the IC3-C58 and Ca²⁺-S100B proteins were combined a notable shift to a higher molecular weight was noted for both proteins consistent with complex formation between the proteins. In the absence of calcium, a reproducible shift to higher molecular weight was also observed although this was not as obvious as with IC3-C58 and Ca²⁺-S100B. As with the peptide array experiments, these results indicate that apo-S100B can interact with the C-terminal 58 residues of the D2 IC3 region. However, the interaction is considerably stronger upon calcium binding to S100B.

IC3-C58 Binds to Ca²⁺-S100B with High Affinity. The peptide array experiments identified distinct regions near the N-terminus (IC3-N25) and C-terminus (IC3-C58) in the IC3 that interact with S100B. In order to verify these interactions and determine the strengths of the interactions with S100B, titrations of the individual peptides with apo- and Ca²⁺-S100B were monitored by NMR spectroscopy. These experiments also allow detailed information about the regions of S100B involved in the interaction with IC3 to be identified. Figure 5 shows that in the absence of calcium several residues in S100B underwent small chemical shift changes (<0.1 ppm) upon addition of IC3-C58. These included residues V52, K55, V56, M57, and T59 in helix III and M79, V80, A83, E89, and E91 in helix IV. All chemical shifts observed were in the fast exchange regime. Using a global fitting approach for 1:1 binding between IC3-C58 and an S100B protomer, changes in chemical shift were fit simultaneously to eq 1 to obtain a K_d of 274 ± 58 μM (Figure 5C).
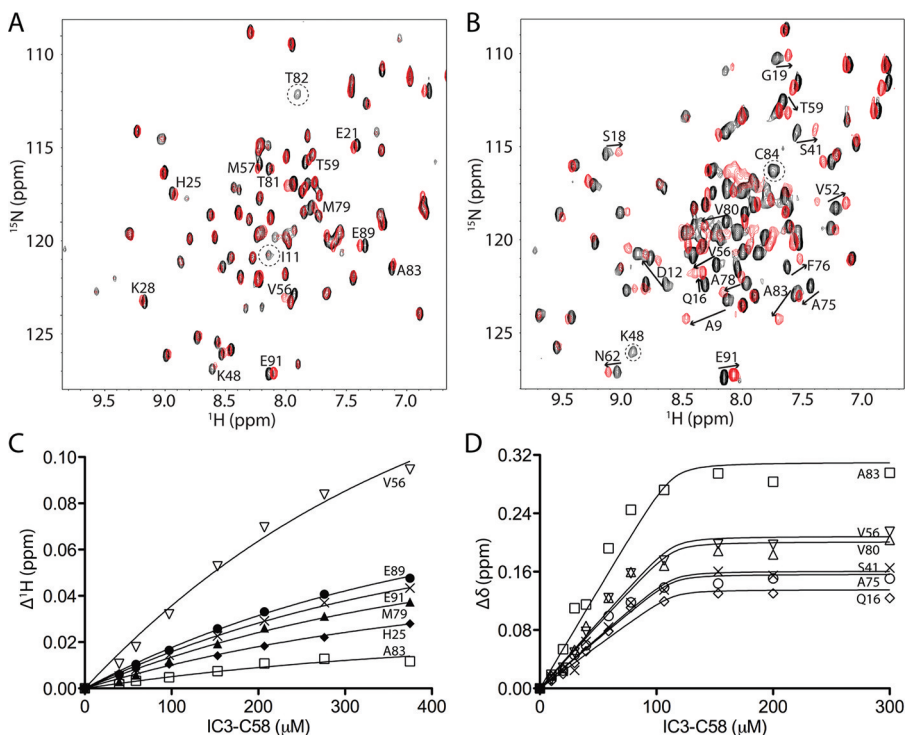

Figure 5. Interaction of IC3-C58 with apo-S100B and Ca²⁺-S100B identified by NMR spectroscopy. ¹H−¹⁵N HSQC spectra of (A) ¹⁵N-labeled apo-S100B (200 μM) in the absence (black) and presence (red) of IC3-C58 peptide and (B) Ca²⁺-S100B (100 μM) in the absence (black) and presence (red) of IC3-C58 peptide. In both spectra residues affected by the addition of IC3-C58 are labeled. Dotted circles indicate residues that have shifted from their original positions but could not be located in the S100B-IC3-C58 complex. Chemical shift changes for selected residues in (C) apo-S100B and (D) Ca²⁺-S100B plotted as a function of IC3-C58 concentration and globally fit to provide dissociation constants (K_d) of 274 ± 58 and 1 ± 1 μM, respectively.
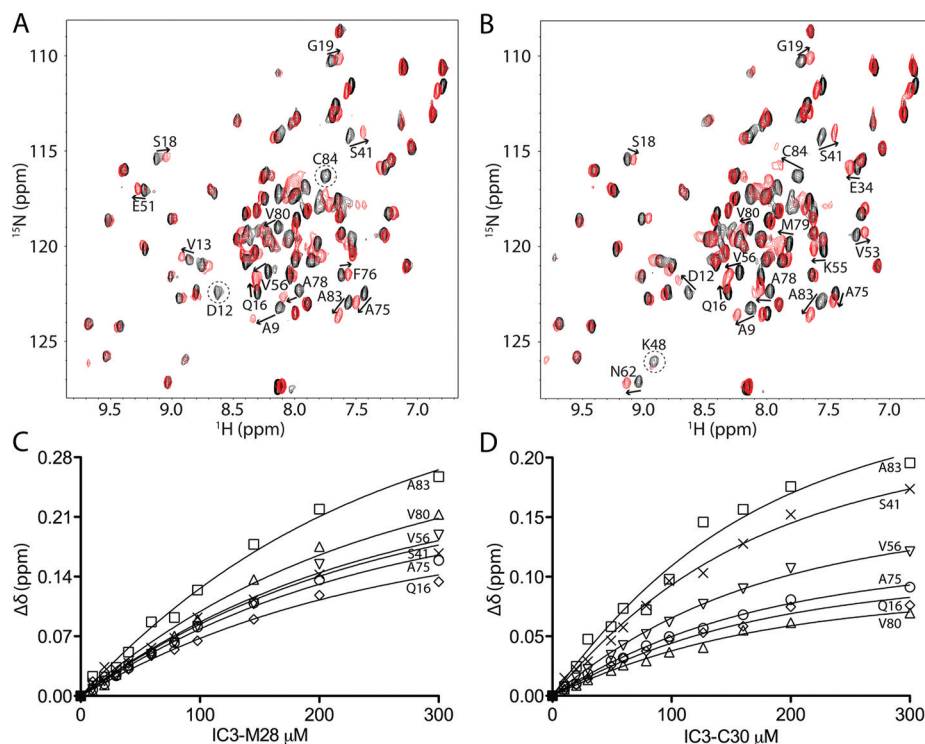
Figure 6. Interaction of IC3-M28 and IC3-C30 with Ca^{2+}-S100B identified by NMR spectroscopy. ^{1}H−^{15}N HSQC spectra of ^{15}N-labeled Ca^{2+}-S100B (100 μM) in the absence (black) and presence (red) of (A) IC3-M28 and (B) IC3-C30 peptides. Residues most affected by the addition of the IC3 peptides are labeled. Dotted circles indicate residues that have shifted from their original positions but could not be located in the S100B−peptide complex. Chemical shift changes ($\Delta\delta$) for indicated residues in Ca^{2+}-S100B were plotted as a function of (C) IC3-M28 and (D) IC3-C30 concentrations. Global fitting was completed for 1:1 binding using the residues indicated between each peptide and the S100 protomer as described in the Experimental Procedures section. This yielded dissociation constants (K_d) of 149 ± 26 μM for IC3-M28 and 98 ± 12 μM for IC3-C30. Attempts to fit the data for 2:1 binding (2 peptides per protomer) yielded similar results with larger errors.

In contrast to the interaction between apo-S100B and IC3-C58, titrations between IC3-C58 and Ca^{2+}-S100B led to much larger chemical shift changes in the ^{1}H−^{15}N HSQC spectra (Figure 5B). The largest chemical shift changes in Ca^{2+}-S100B were noted for A9, D12, Q16, and S18 (helix I), V52, V56, and T59 (helix III), and A75, F76, A78, V80, and A83 (helix IV). Similar to apo-S100B, the changes in chemical shift for Ca^{2+}-S100B appeared in the fast or fast-intermediate exchange regime allowing assignment of Ca^{2+}-S100B bound to IC3-C58 to be completed based on previous assignments.[41] In addition, all changes in chemical shifts appeared to be linear, suggesting only a single binding event was occurring (Supporting Information, Figure S1). Global fitting of the titration data for several residues found in different regions (helix I, linker, helices III, IV) of the S100B protein yielded an approximate K_d near 1 μM for the interaction of IC3-C58 with each Ca^{2+}-S100B protomer (Figure 5D). This indicates that the affinity for IC3-C58 is ~300-fold higher for Ca^{2+}-S100B compared to apo-S100B.

Titrations of IC3-N25 into either apo-S100B or Ca^{2+}-S100B resulted in no significant changes in the ^{1}H−^{15}N HSQC spectra (data not shown). Although peptide array experiments were suggestive of an interaction with S100B the titration indicates that the affinity is very weak. It is important to note that only a single peptide spot displayed observable binding in the IC3-N25 region of peptide arrays (for example, Figure 3), even with a large excess of free S100B used in the array experiments, while large contiguous peptide regions were observed for IC3-C58 binding to S100B. These results indicate that the C-terminal

portion of the IC3 for the dopamine D2 receptor is the major site of interaction with S100B, and this interaction is accentuated upon calcium binding to the S100 protein.

Evidence for Multiple Binding Regions in IC3 for Ca^{2+}-S100B. Structures of different S100 proteins bound to peptides representing protein target-binding regions vary from 12 (TRTK-12) to 30 (Siah1-interacting protein, SIP) residues.[18,42] At 58 residues, the IC3-C58 region would, therefore, be considered an unusually large S100B binding region. Since peptide array experiments suggested two separate binding areas (IC3-M28, IC3-C30) might exist within the IC3-C58 region, we used peptides for both regions (Figure 1) to determine how these might contribute to binding with S100B. In both cases, no significant chemical shift changes were observed in apo-S100B despite addition of excess IC3-M28 or IC3-C30 peptides (data not shown).

The titration of IC3-M28 to Ca^{2+}-S100B showed several changes in the ^{1}H−^{15}N HSQC spectrum (Figure 6A and Supporting Information Figure S2) although in general the magnitude of the chemical shift changes were smaller than observed for IC3-C58. Titrations with the IC3-C30 peptide and Ca^{2+}-S100B produced very similar results (Figure 6B and Supporting Information Figure S3). Interestingly many of the affected residues in Ca^{2+}-S100B were the same for both IC3-C30 and IC3-M28 peptides including A9, D12, S41, V56, A78, A83, and C84. The chemical shift changes for multiple residues in each of these peptides were globally fit to determine dissociation constants of ~98 and ~148 μM for binding of IC3-C30 and IC3-M28, respectively, to Ca^{2+}-S100B (Figure 6).
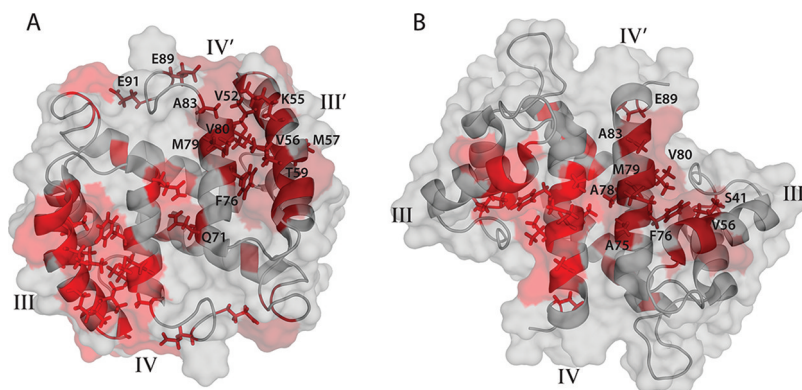
Figure 7. Potential binding surfaces for the IC3 region of the dopamine D2 receptor mapped to the surfaces of apo-S100B and Ca^{2+}-S100B. Residues in the dimeric structures of (A) apo-S100B (2PRU)[67] and (B) Ca^{2+}-S100B (1MQ1)[29] that shifted more than 0.25 standard deviations above the average chemical shift change in IC3-C58 titrations are indicated in different shades of red to denote the two protomers in the S100B dimer. Only residues for one protomer are labeled.

These values are nearly 2 orders of magnitude higher than observed for IC3-C58. Since the overall trends for residues that shifted in Ca^{2+}-S100B were very similar for IC3-C58 and its subpeptides IC3-M28 and IC3-C30 (Supporting Information Figure S4), attempts were also made to fit the data for 2:1 stoichiometry (i.e., two IC3-M28 peptides to each Ca^{2+}-S100B protomer) assuming that each peptide might bind to two different regions akin to the intact IC3-C58 peptide. Attempts to fit to this model yielded similar affinities with poor fits and large errors likely a result of the overall weak affinity of these individual peptides for Ca^{2+}-S100B. Nevertheless, the low affinity of IC3-M28 and IC3-C30 suggest that regions within both peptides contribute to the higher affinity binding of IC3-C58.

Identification of the IC3 Binding Surface on S100B. The magnitudes of the chemical shift changes for binding of IC3-C58 to Ca^{2+}-S100B and apo-S100B were used to identify the surface of interaction for the IC3 with S100B (Figure 7). For apo-S100B the only contiguous area of residues experiencing relatively large chemical shift changes was located on helices III and IV. However, several of these residues are not surface accessible in the apo-S100B structure including V52, V56, F76, V80, and A83. It may be possible that apo-S100B is undergoing a ligand-induced conformational change upon presentation of IC3-C58, a finding noted previously for the interaction of apo-calmodulin with the cardiac sodium channel Na$_V$1.5[43] or myosin.[44] The discontinuity of the surface for IC3-C58 binding to apo-S100B is in agreement with the lower binding affinity observed.

The interaction of IC3-C58 with Ca^{2+}-S100B shows a contiguous interaction surface is formed at the junction of helices III and IV of S100B, typical for many S100 target–protein interactions. Some of these residues, including V56 (helix III) and F76, M79, V80, A83, and E89 (helix IV), were also affected in apo-S100B upon IC3-C58 interaction. In addition, there is a smaller region of interaction on the opposite face of Ca^{2+}-S100B, running along the solvent exposed side of helix I composed of residues A9, I11, D12, and Q16. This region on helix I is found in each of the Ca^{2+}-S100B IC3-peptide titrations (IC3-C58, IC3-C30, IC3-M28) and is similar to a binding surface found on Ca^{2+}-S100A6 for Siah 1 interacting protein (SIP) binding.[42] The two distinct regions of chemical shift changes on Ca^{2+}-S100B may indicate that IC3 utilizes two binding sites.

■ DISCUSSION

Characterization of S100B-IC3 Binding. A variety of three-dimensional structures have shown that calcium-binding to most S100 proteins results in a conformational change that exposes a hydrophobic binding pocket used to recruit target protein partners.[16,17,29,42,45,46] Although S100B has been observed to interact with a wide variety of proteins, most of these have been identified from *in vitro* experiments. *In vivo* interactions derived from two-hybrid or co-immunoprecipitation experiments tend to work poorly for S100B and most other EF-hand containing proteins due to the calcium-binding requirement.[21] The recent identification of the dopamine D2 receptor as an S100B target protein using a bacterial two-hybrid assay[20] presents one of the first cases where a binding partner for S100B has been identified using these methods. Since intracellular free calcium is tightly regulated near 100–300 nM in *E. coli*[22,23] and 100–200 nM in humans,[25] well below the calcium dissociation constants for most S100 proteins, this suggested that S100B might be interacting with the dopamine D2 receptor in the calcium-free state.

Peptide array, gel filtration, and NMR titration experiments identified the C-terminal portion of the dopamine D2 receptor IC3 loop (IC3-C58) as the major interacting site with both apo- or Ca^{2+}-S100B. Sequence alignments of IC3-C58 with S100B binding sequences from p53, NDR kinase, and TRTK-12 showed there are no clear sequence similarities in any of these peptides other than a basic pI and several hydrophobic residues in the sequences. A TRTK consensus sequence[18,47] at the N-terminus of the IC3[20] is apparent, although this region (IC3-N25) showed little binding to S100B. This observation is consistent with the ability of S100B to bind multiple, diverse targets with little sequence similarity.

A Unique Interaction of IC3 with Apo-S100B. The affinity of the interaction between apo-S100B and the IC3-C58 region of the dopamine D2 receptor was ~274 μM. This binding affinity is similar to those measured for some other apo- and Ca^{2+}-S100 protein interactions including apo-S100A4 and F-actin (~550 μM),[48] apo-S100P and melittin (residues 1–26, ~200 μM),[49] apo-S100A12 and the receptor for advanced glycated end-products (RAGE) (residues 129–336, ~140 μM),[50] Ca^{2+}-S100B with the N-terminus of p53 (residues 1–29, K_d ~ 76 μM), and Ca^{2+}-S100A2 with p53 (residues 362–393, K_d ~ 106 μM).[51] In addition, several well-studied protein–protein interactions have been identified for calmodulin, an EF-hand

protein structurally distinct from S100B, which in the calcium-free state often binds to proteins that contain short IQ motifs (consensus − $(I/L/V)Q(X)_3R(X)_4(R/K)$, X = any residue).[52] These apo-calmodulin interactions include an IQ motif in neurogranin ($K_d \sim 60$ μM),[53] the IQ motif in Purkinje cell protein 4 (PEP-19) ($K_d \sim 13$ μM),[54] and the IQ motif from the cardiac sodium channel Na$_V$1.5 (~ 0.2 μM).[55] The solution structure of CaM bound to the Na$_V$1.5 IQ motif[43] reveals that IQ binding has partially opened the CaM binding site to an intermediate position and the IQ motif has moved into the hydrophobic binding pocket. This ligand induced conformational change may also be occurring when IC3-C58 binds to apo-S100B, as indicated by the chemical shift changes of buried residues, particularly in helices III and IV. A search of the D2-IC3 sequence did not reveal any obvious sequence similarity to the IQ motif. These observations indicate that the dopamine D2 receptor likely interacts with apo-S100B in a unique manner from the IQ motif interaction with calmodulin and support a calcium-free recruitment suggested from bacterial two-hybrid experiments.[20]

Calcium Enhancement of IC3 Binding to S100B. Gel filtration and NMR experiments indicated that calcium-binding to S100B leads to an ~ 300-fold increase in affinity for IC3-C58 compared to the apo-state. The affinity ($K_d \sim 1$ μM) is similar to interactions of Ca^{2+}-S100B with SIP ($K_d \sim 5$ μM),[42] TRTK-12 ($K_d \sim 0.3$ μM),[56] myelin-associated glycoprotein ($K_d \sim 7$ μM),[57] and guanylate cyclase ($K_d \sim 2$ μM).[58] Separation of IC3-C58 into two smaller regions, IC3-C30 and IC3-M28, reduces this binding by ~ 100-fold, indicating that at least a portion of each of these regions is required for optimal interaction. This observation is similar to that observed by Lee and co-workers for the interaction of Ca^{2+}-S100A6 with the C-terminus of SIP.[42] In this structure, SIP forms a tandem two-helix arrangement where one helix (helix A) binds in the hydrophobic surface between helix III and helix IV of Ca^{2+}-S100A6, while the second helix (helix B) makes contacts with helix I on the opposite face of S100A6. Both helices of SIP are needed for maximal binding since helix A shows reduced binding to Ca^{2+}-S100A6 and helix B does not have any observable binding. The surface of Ca^{2+}-S100B displays similar features as SIP for the interaction with IC3-C58 having the largest changes in chemical shift near helices I, III, and IV. This ability of Ca^{2+}-S100B to function in complex formation with more than one protein region may also be important for the recruitment of multiple target proteins. For example, S100A10 and S100A11 can bind annexin A1 and A2 in combination with membrane spanning proteins dysferlin, NS3, or TASK-1 to function in membrane repair.[59−62] The current observations suggest that the S100B interaction with the dopamine D2 receptor may fall into this class of S100 binding.

The recognition of the IC3 loop from the dopamine D2 receptor by apo-S100B may represent a new type of calcium-insensitive target interaction for an S100 protein. For dopamine signaling this association may provide an rapid response to calcium and enhanced formation of the S100B-IC3 complex. For example, it has been shown that the calcium affinities of S100B[2,3] and S100A1[63,64] can be increased in the presence of a target protein. Alternatively, it is possible that calcium binding to S100B results in inactivation of the dopamine receptor through altered binding since S100B can increase D2 receptor stimulation of the dopamine-signaling pathway in HEK293 cells[20] at resting calcium concentrations. This idea fits well with the observation that calcium-activated calmodulin can interact with the IC3-N25 region of the dopamine D2 receptor,

resulting in reduced signaling.[15] Calcium is also known to reduce intracellular cAMP, which is normally up-regulated when dopamine binds to the D2 receptor, by inhibiting adenyl cyclase type V and activating CaM-sensitive phosphodiesterases.[65,66] The current work identifies the region of the dopamine D2 receptor recruited by both apo- and Ca^{2+}-S100B and paves the way for future biochemical studies to elucidate the mechanism of these interactions in the signaling pathway.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

^{1}H−^{15}N HSQC spectra showing the titrations of Ca^{2+}-S100B with IC3-C58, IC3-M28, or IC3-C30 peptides and the resulting chemical shift perturbation plots. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

DTT, dithiothreitol; DSS, 4,4-dimethyl-4-silapentane-1-sulfonic acid; EDTA, ethylenediaminetetraacetic acid; HSQC, heteronuclear single quantum coherence; NDR kinase, nuclear Dbf2-related kinase; TEV, tobacco etch virus; SIP, Siah1 interacting protein.

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