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Characterization of a novel weak interaction between MUC1 and Src-SH3 using nuclear magnetic resonance spectroscopy

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ABSTRACT

Breast cancer causes death through cancer cell migration and subsequent metastasis to distant organs. *In vitro*, the MUC1 mucin can mediate breast cancer cell migration by binding to intercellular adhesion molecule-1 (ICAM-1). This migration is dependent on MUC1 cytoplasmic domain (MUC1-CD) activation of the non-receptor tyrosine kinase, Src, possibly through competitive displacement of an inhibitory Src intramolecular SH3 binding. Therefore, we characterized the binding site and affinity of the MUC1-CD for Src-SH3 using multidimensional nuclear magnetic resonance (NMR) spectroscopy to monitor the titration of the ^{15}N labeled Src-SH3 domain with synthetic native and mutant peptides of MUC1-CD. The results revealed that the dissociation constant (K_d) for the interaction of the native MUC1-CD peptides and Src-SH3 domain was weak with a K_d of 2–3 mM. Notably, the SH3 residues most perturbed upon peptide binding were located outside the usual hydrophobic binding cleft in a previously described alternate binding site on the Src-SH3, suggesting that MUC1-CD binds to a non-canonical site. The binding characteristics outlined here suggest that the interaction between Src-SH3 and MUC1-CD represents a novel weak electrostatic interaction of the type which is increasingly recognized as important in transient and dynamic protein complexes required for cell migration and signal transduction. As such, this study forms the foundation for the design of specific inhibitors of this interaction which may target breast cancer metastases with exquisite specificity.

1. Introduction

In North American women, breast cancer is the second leading cause of cancer mortality [1] with death resulting from widespread metastatic disease. We have been investigating the mechanism of metastases in breast cancers and reported that MUC1, a mucin first isolated in breast milk and commonly overexpressed in breast cancer, binds to the common intercellular adhesion molecule, ICAM-1 [2] found on stromal cells and vessels. MUC1 is a type I transmembrane glycoprotein that lacks intrinsic kinase activity [3]. We subsequently published that binding to ICAM-1 initiates a Src dependent signal [4] that is followed by increased transmigration of MUC1 positive cells through an ICAM-1 positive monolayer [5,6].

Src contains SH2 (Src homology-2) and SH3 domains that bind to phosphorylated tyrosines or polyproline motifs, respectively. Src is usually maintained in a tightly folded inactive form stabilized by intramolecular SH2 and SH3 binding. Thus, one mechanism of Src activation is by displacing the inhibitory intramolecular SH3 binding interaction [7,8]. There is definitive *in vitro* GST pull down data showing that the cytoplasmic domain of MUC1 (MUC1-CD) can bind the Src-SH3 domain although the sequence has not been identified [9]. The existence of a functional SH3 recognition site suggests that MUC1 could recruit and then activate Src without involving a second kinase.

The Src-SH3 domain typically binds to ligands that form a left-handed polyproline type II conformation with a minimum consensus sequence PXXP (P = Proline; X = any residue) [10]. SH3 ligands form hydrophobic contacts that can bind in a plus orientation (Class I) with an N-terminal conserved Arginine (R*XXPXXP) or a minus orientation (Class II) with a C-terminal conserved Arginine (PXXPX*R). However, the Src-SH3 binding domain is notably promiscuous and also binds unconventional, non-PXXP motifs [11] partially or exclusively through tertiary electrostatic interactions [12].

There are no putative SH3 binding motifs in the MUC1-CD that are candidates for high affinity Src-SH3 binding. Identifying the residue-specific details of the binding interface are thus of interest in understanding the MUC1-CD and Src-SH3 interaction and thereby defining the trigger for MUC1-mediated cell migration.

Abbreviations: ICAM-1, intercellular adhesion molecule-1; CD, cytoplasmic domain; SH3, Src homology-3; NMR, nuclear magnetic resonance; K_d, dissociation constant; ^1H–^{15}N HSQC NMR, two-dimensional NMR spectra correlating the amide proton and nitrogen NMR chemical shifts.

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2. Materials and methods

2.1. Expression and purification of ^{15}N labeled Src-SH3 domain

The complementary DNA encoding the human c-Src-SH3 domain was subcloned from a recombinant pUASEMP_Src plasmid, into the pGEX-2T, GST fusion vector between EcoRI and BamHI sites. The recombinant human Src plasmid (pUASEMP_Src) was a generous gift from Dr. Tony Pawson, (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Ontario). The PCR primers with 6 flanking bases used to amplify the Src SH3 domain for insertion into the pGEX-2T vector are as follows (EcoRI and BamHI sites are underlined);

SH3_Forward => 5′-GGC CCG GGA TCC ATG GGT GGA GTG ACC ACC TTT-3′.
SH3_Reverse => 5′-GCG CCG GAA TTC TTA GGA GTC GGA GGG CGC CAC-3′.

The recombinant plasmids were sequenced to verify the insertion of the correct coding sequence with reference to Swiss-Prot sequence for human c-Src (Accession No. P12931).

The recombinant PGEX-2T-SH3 plasmid was transformed into BL21 (DE3) PlysS cells (*Escherichia coli* host). The recombinants were expressed in M9 minimal media enriched with $^{15}NH_4Cl$ to a cell density (Optical Density at λ_{600}) of 0.6–0.8 and induced with 1.0 mM IPTG. The cells were harvested after 3–4 h by centrifugation and the cell pellets were lysed according to standard protocols using an EmulsiFlex™ Homogenizer (Avestin Inc. Ottawa, Canada) and the lysates analyzed by Tris-Tricine SDS–PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis). The cell lysates were then purified using pre-packed GSTrapFF-1 ml columns (Pharmacia/GE Healthcare), cleaved with thrombin and desalted using a Sephadex-G25 column with 10 mM NH_4HCO_3. The SH3 domain (6.88 kDa) and GST (26 kDa) were then separated by size exclusion chromatography (Superdex-75, Pharmacia/GE Healthcare) and desalted again. The pure SH3 domain was lyophilized. The pure proteins were positively identified by mass spectrometry.

2.2. MUC1-CD synthetic peptides

The amino acid (aa) sequence of the synthetic MUC1 peptides is given in Fig. 1. A 69-residue full-length synthetic peptide of MUC1, without the CQC motif, was commercially obtained (GenScript Inc.). A shorter 23-residue synthetic MUC1-CD peptide that contained the SH2 binding motif as well as a control peptide (23-residue) with the same sequence but with a point mutation, $R^{34}A$, and a synthetic 48-residue-dimer MUC1 peptide (two 23-residue peptides linked *via* 2 cysteine residues, with one arm phosphorylated at Y^{46}) were obtained through the IBD (Institute of Biomolecular Design), University of Alberta. The amino acid sequences of all peptides were checked against the UniProtKB/Swiss-Prot sequence for human MUC1 (Acc No. P15941).

2.3. NMR titrations of Src-SH3 domain with MUC1-CD peptides

The NMR titrations of the Src-SH3 domain with the four synthetic MUC1-CD peptides were performed as follows: Calculated amounts of MUC1 peptide aliquots were titrated into 500 µl of 0.25 mM ^{15}N labeled Src-SH3 domain in NMR buffer [50 mM Na_2HPO_4, 100 mM NaCl, 10 µM EDTA, 1 mM Imidazole and 4.6 mM DSS (containing 0.196% NaN_3 and 98% v/v D_2O)]. The pH of the protein solution was kept within a range of 6.5–6.7 throughout the titration by adding 1 M NaOH as necessary. The initial protein and peptide concentrations were determined by weight/volume method but confirmed and adjusted by subsequent amino acid analyses. Changes in protein concentration due to the addition of peptide were recorded at each titration point and included in the calculation of subsequent volume of peptide solution.

Two-dimensional (2D) ^1H-^{15}N HSQC NMR spectra of MUC1-CD bound and unbound Src-SH3 domain were acquired at each titration point of MUC1 peptide at 30° C using Varian 500 or 600 MHz NMR spectrometers. The published ^1H and ^{15}N chemical shifts of the Src-SH3 available through the Biological Magnetic Resonance Data Bank (BMRB) were used to assign the HSQC spectrum.

2.4. Analyses of NMR spectral data

All ^1H-^{15}N HSQC NMR spectra acquired during a particular titration were equally processed using VNMRJ, NMRPipe [13], and NMRViewJ (One Moon Scientific, Inc.) software. The observed chemical shift changes per residue ($\Delta\delta$) in ^1H-^{15}N HSQC spectra of SH3 is calculated by the software, according to the equation, $\Delta\delta = [(\Delta\delta H)^2 + (\Delta\delta N/5)^2]^{1/2}$, where $\Delta\delta H$ is the chemical shift change in ppm in ^1H dimension and $\Delta\delta N$ is the chemical shift change in ppm in ^{15}N dimension. The coefficient of 0.2 is applied in the equation to compensate for the scaling differences between ^{15}N and ^1H chemical shifts.

2.5. Determination of dissociation constants of MUC1/Src-SH3 interaction

The interaction of MUC1 and Src-SH3 domain was assumed to follow a single site binding model with 1:1 stoichiometry given by, P + L↔PL (P = protein; L = ligand). *Xcrvfit* (in-house program)

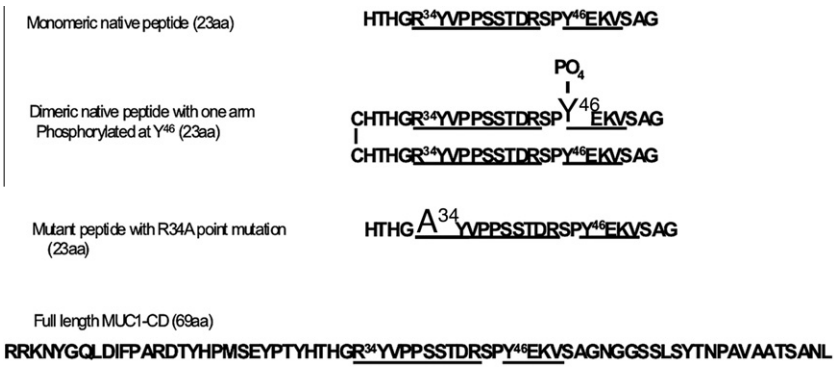

Fig. 1. The synthetic MUC1-CD peptides used for NMR titrations with the ^{15}N labeled Src-SH3 domain. The putative SH3 binding motif and the SH2 binding motif are underlined (Larger font: phosphorylated tyrosine residue in dimeric native peptide and mutated residue in R34A mutant; PO_4 = phosphate group).

software was used to fit an appropriate binding model for the observed chemical shift data based on all residues as well as those residues with total chemical shift change >0.04 ppm.

2.6. Chemical shift mapping of the MUC1-CD binding site on Src-SH3 domain

The NMR solution structure of Src-SH3 domain (1QWE) and the X-ray crystal structure of inactive Src molecule (2SRC) available through RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank (*pdb*) (http://www.pdb.org/pdb/home/home.do) were used to map the residues of Src-SH3 domain that showed the highest chemical shift changes (>0.04 ppm) upon addition of MUC1-peptides using the software, *MacPyMOL*.

3. Results

3.1. ^{1}H-^{15}N HSQC NMR spectra of MUC1-CD/Src-SH3 interaction

The two-dimensional (2D) ^{1}H-^{15}N HSQC NMR spectrum of the Src-SH3 domain was assigned using the published ^{1}H and ^{15}N chemical shift values available through BMRB [14]. All non-proline residues except G84, S126, S145 and T88, could be assigned.

The overlay of ^{1}H–^{15}N HSQC spectra of Src-SH3 acquired during the NMR titrations with, (i) a 23-residue native peptide (Fig. 2A), (ii) a 48-residue-dimer peptide, and (iii) a 69-residue full-length MUC1-CD peptide all showed a very similar pattern of chemical shifts, in which the changes in chemical shift ($\Delta\delta$) were very small (<0.1 ppm). The residues with the highest chemical shift changes, ($\Delta\delta > 0.04$ ppm) in all three titrations were R98, E100, H125, T132, G130, Y134 and L103.

The overlay of ^{1}H-^{15}N HSQC spectra of Src-SH3 domain acquired during the titration of $R^{34}A$ mutant MUC1 peptide showed a reduction in chemical shift changes in some of the same residues (E100, H125, G130, Y134) that were perturbed during the titration of 23-residue peptide with the native sequence (Fig. 2B). Notably, there was the same degree of chemical shift perturbation of R98 in the mutant peptide titration compared to the native peptide titration.

3.2. The dissociation constant (K_d) of MUC1-CD/Src-SH3 interaction

The observed chemical shift data were fit to a 1:1 binding model. The dissociation constants (K_d) determined based on all residues (a global K_d), were similar for the three native peptides with, i) 2.9 mM for the 23-residue native peptide (Fig. 3A), ii) 2.3 mM for the 48-residue-dimer peptide (Fig. 3B) and (iii) 2.4 mM for the 69-residue full-length peptide (Fig. 3C). The residues with $\Delta\delta \geqslant 0.04$ ppm demonstrated a somewhat better fit to the model with a lower local K_d compared to the global K_d (data not shown). In contrast, the fitting for the $R^{34}A$ mutant did not converge, indicating a $K_d > 5$ mM for the $R^{34}A$ mutant peptide (Fig. 3D).

3.3. Mapping the MUC1-CD binding site on Src-SH3 domain

The chemical shift mapping clearly showed that, apart from the Y134 and D99, the residues of the canonical Src-SH3 binding site (W121, Y134, Y95, Y93, Y139 and D99) were not perturbed (Fig. 2) upon titration with MUC1-CD peptides. The residues with the highest chemical shift changes were mapped onto the following locations of the Src-SH3 domain [15]; R98 and E100 on the RT loop, H125 on the β-sheet-c, T132 and Y134 on the β-sheet-d and G130 on the distal loop (Fig. 4).

4. Discussion

SH3 domains can physically interact with a large number of diverse ligands through high affinity hydrophobic contacts or lower affinity contacts with highly charged residues outside the ligand binding site. Both types of interactions have been reported to be essential for assembly of numerous cellular signaling networks [16].

Titration of the 69aa MUC1-CD peptide into Src-SH3 did not show the classic chemical shift perturbations seen in SH3 domain-peptide complexes. Instead, many of the largest chemical shift changes observed on Src-SH3 delineate a surface that runs perpendicular to the classic binding cleft (see Fig. 4C) and then extends to an opposite face (which includes H125, G130, T132). This is consistent with the lack of a canonical Src-SH3 domain-binding sequence in MUC1-CD.

The 23aa peptide used in this study was found to duplicate the binding pattern of the 69aa peptide, indicating that the binding motif is fully contained within the shorter peptide. The R34A mutation abolished most of the larger chemical shift changes that occur during the NMR binding titration. (The notable exception to this is Src-SH3 R98, indicating that R98 is involved in a different even lower affinity interaction than the major one identified in this study.) The importance of MUC1-CD R34 and a number of hydrophilic Src-SH3 residues, E100, H125, G130, and T132, suggest that the MUC1-CD-Src-SH3 interaction is largely electrostatic in nature. The relatively small perturbations in chemical shift is further suggestive of superficial electrostatic interactions.

The weak interaction identified in this study is unlikely, on its own, to mediate a stable MUC1-Src complex and constitutive Src

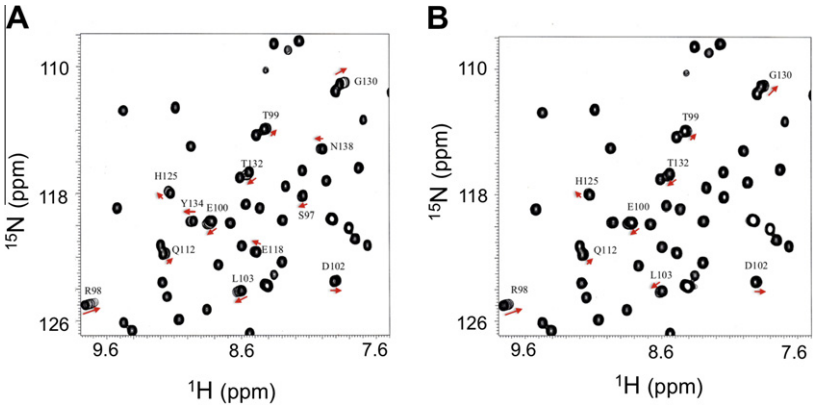

Fig. 2. Overlay of 2D ^{1}H-^{15}N HSQC NMR spectra of Src-SH3 obtained by titrating the 23-residue monomer (A) and the 23-residue R34A Mutant (B) MUC1 peptides into Src-SH3.

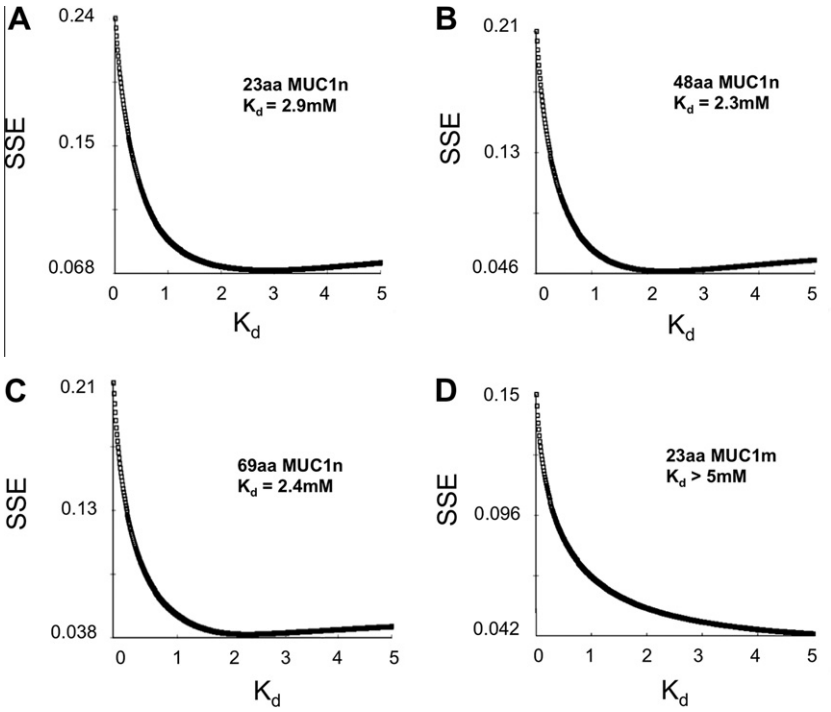

Fig. 3. The estimated K_d values *vs.* sums of squares of error (SSE) based on the chemical shift changes of all residues are shown for (A) 23aa native MUC1 peptide (B) 48aa dimer MUC1 peptide (C) 69aa full length MUC1-CD peptide and (D) 23aa R34A mutant MUC1 peptide.

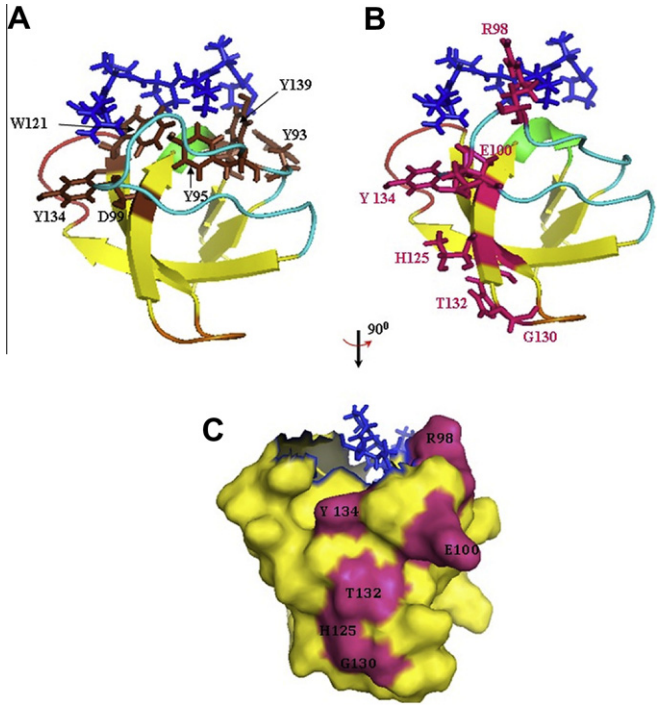

Fig. 4. (A) A ribbon diagram of Src-SH3 domain complexed with a class II ligand (blue), generated using the solution NMR structure (pdb code: - 1QWE) [15]. The *XP* dipeptidyl moieties of the class II ligand binds to the hydrophobic clefts formed by conserved aromatic residues (brown) that are located in between RT (cyan) and n-Src (red) loops. (B) Chemical shift mapping of the MUC1-CD binding site on Src-SH3 domain based on the residue-shifts >0.04 ppm (magenta) obtained by titrating the 23-residue, 69-residue and 48-residue MUC1 peptides into ^{15}N-Src-SH3. (C) Molecular surface representation of Src-SH3 domain (rotated 90° clockwise with respect to *a* and *b*). The same residues with total chemical shift >0.04 ppm (magenta) are mapped onto the surface.

activation. Nonetheless, there is precedent for physiologically important SH3 domain-ligand interactions with an affinity of 3.0 mM. [12]. Cellular signaling pathways are often dynamic and must be activated and inactivated quickly. This is especially true for SH3 domains which participate in assemblies of molecules that operate as transient but specific switching between multiple interaction partners with fast on and off rates [17]. Thus the MUC1-CD may exhibit low affinity interactions as a scaffold protein that has evolved to bind multiple molecules with fast on/off rates.

Recent data has shown the importance of MUC1-CD dimers to MUC1's tumorigenic function [18,19]. However, in the current study there is no difference in the chemical shift pattern or affinity between the 23aa "monomeric" peptide and the 48aa "dimer" peptide. This does not necessarily exclude MUC1-CD dimers as the preferred Src-SH3 binding partner, since it is yet possible that the intermolecular cysteine bridge used to dimerize MUC1-CD in the 48aa construct over-constrains the system and interferes with dimer binding. Nevertheless, the similarity of binding curves between the 23aa monomer, 69aa monomer, and 48aa dimer is consistent with a monomeric interaction between MUC1-CD and Src-SH3. Future studies with isotope-labeled MUC1-CD will clarify the issue.

Besides the interaction identified in this study, the p-Y^{46}EKV motif of MUC1-CD is known to bind to Src-SH2 domain, and the tyrosine residue of the motif is itself a potential target for Src kinase. The cross-talk between these interactions, how they depend on dimerization and clustering, and how they contribute to Src activation is the subject of further investigation.

A protein–protein interaction between one structured and one unstructured partner is thought to be a druggable feature [20], particularly as a target for a small molecule that would show tighter binding to the structured partner (Src) than the weak interaction by the disordered molecule (MUC1-CD). Since the binding specificity of MUC1-CD and Src-SH3 domain is unique, as revealed by this study, it can be a potential target of higher-affinity small molecules that would uniquely inhibit the MUC1-Src-SH3 interaction.

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