Recognition of the tumor suppressor protein p53 and other protein targets by the calcium-binding protein S100B

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1. Introduction

There are several members of the S100 family of EF-hand Ca²⁺-binding proteins that are distributed tissue-specifically in humans [1–3]. S100 proteins were given this name because they are soluble in 100% saturated ammonium sulfate [4]. One member, S100B, is a 21.5 kDa symmetric homodimer that is highly conserved (>97%) among mammals [1,4]. In general, low levels of S100B have trophic effects, and higher levels are problematic, resulting in uncontrolled cell growth [5–8]. Elevated levels of S100B are found in malignant melanoma [9–12], renal cell tumors [13], and malignant T-cells (such as doubly negative CD4−/CD8− adult T-cells in leukemia patients) [14]. Furthermore, S100B is up-regulated by cytokines that stimulate gliosis such as interleukin-1β and the basic fibroblast growth factor [15]. As is the case for S100B, a number of other S100 proteins are regulated in a tissue-specific manner [16]. S100A1, S100A6 (calcyclin), and S100B are elevated significantly in metastatic human mammary epithelial cells [17], and increased levels of S100A4 in transgenic mice induce metastatic mammary tumors [18]. In the case of S100A4 (mts1), protein levels are controlled in benign cell lines via a cis-acting element 1300 base pairs upstream of the rat S100A4 start site [18], and expression of antisense RNA to S100A4 suppresses metastatic potential for a high-metastatic Lewis lung carcinoma [19]. Protein levels of S100B, S100A4, and S100A6 correlate with malignant melanoma, so these S100
proteins are used as markers for this cancer [20–23]. In general, S100 antibodies are used clinically to identify and classify cancer in several tissues and cell types including brain, bladder, breast, cervix, head and neck, intestine, kidney, larynx, lung, lymph, mouth, skin, and testes among others [13,14,17,24–38]. More recently, S100B was shown to not only be a prognostic marker, but that it also contributes to cancer progression in malignant melanoma by interacting with p53 and inhibiting its function as a tumor suppressor [24–26].

p53 is a transcription activator that signals for the up-regulation of genes involved in cell cycle arrest and apoptosis [27,28] and plays a pivotal role in the maintenance and regulation of normal cellular functions. Its inactivation affects cell cycle checkpoints, apoptosis, gene amplification, centrosome duplication and ploidy [28–35]. If p53 is mutated, as found in many human cancers, then the cell cycle proceeds unregulated, cell growth proliferates, apoptosis pathways are not induced, and proliferating cells transform into cancerous ones [29,36]. On the other hand, if p53 levels are too high, then phenotypes associated with aging and problems with skin and bone occur [37]. Thus, p53 is highly regulated by numerous post-translational modifications and via interactions with other proteins (i.e. S100B, Hdm2, etc.) to regulate its activity [31,38–41].

In its most active form, p53 is a dimer of dimers to form a tetrameric X-type four-helix bundle in its C-terminus [42–44]. Upon binding specific DNA promoter sequences, the p53 tumor suppressor activates the transcription of numerous downstream target genes including a cyclin-dependent kinase inhibitor (p21WAF1/CIP1), cell cycle control proteins (cyclin G, GADD45), genes involved in apoptosis (i.e. Bax), and a gene product, Mdm2 (or Hdm2 for the human protein), which in turn negatively regulates p53 protein levels inside the cell as part of a feedback loop to keep p53 protein levels in check [27,28,45].

Hdm2 is an E3 ligase that down-regulates p53 by an ubiquitin-mediated pathway, which is dependent on the phosphorylation state of p53 in the N-terminal transactivation domain. The 3D structure of the N-terminal domain of Hdm2 somewhat resembles EF-hand proteins, despite the fact that it does not bind calcium [46]. A structurally related protein, Hdm4 (also called Mdmx or Mdm4 for the murine protein) also binds to the same region of p53 and inhibits its activity under certain conditions; however, unlike Hdm2, Hdm4 does not have significant ubiquitin ligase activity [47]. Directly C-terminal to the tetrameric domain on p53 is a basic region termed the “extreme C-terminus” or the “C-terminal negative regulatory domain”, which is unstructured when free in solution, but is helical when bound to the calcium-binding protein S100B [48]. If the negative regulatory domain is unmodified, p53 has lowered transcription activation activity (i.e. latent state). In fact, deletion of the last 30 residues, covalent modification, and/or binding of antibodies to this domain activates p53 by preventing non-specific DNA binding and/or perhaps by inhibiting S100B binding [49,50]. In this study, it is demonstrated that phosphorylation of the C-terminus does indeed inhibit S100B binding and is analogous to how p53 is protected from another known negative regulator of p53, namely Hdm2. Furthermore, it is shown that S100B interacts directly with Hdm2 in a calcium-dependent manner. Thus, it is possible that S100B and Hdm2 work concertedly to down-regulate p53, which is discussed.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents were ACS grade or higher and purchased from Sigma-Aldrich unless otherwise indicated. 15NH4Cl was purchased from Cambridge Isotope Laboratories (Woburn, MA). All buffers were passed through Chelex-100 resin to remove trace metals.

2.2. Peptides and S100B protein preparation

Unlabeled and 15N-labeled S100B were overexpressed and purified from E. coli as described previously [51,52]. Using phage display, Ivanenkov et al. defined a target sequence ([KK][L][L][x][x][x][x][L]) and identified a 12 residue peptide, termed TRTK-12, which binds S100B and most closely resembles a sequence (residues 265–276) in the actin capping protein, CapZ (62). By expanding protein database searches to include a larger number of protein sequences ([KK][L][L][P/S][N/D]/[W/L][L]/[S/O][L][x][L][L]/[L]), additional S100B targets were identified (Table 1). Peptides based on such searches were produced by Bio-Synthesis, Inc. (Lewisville, Texas) using solid-state peptide synthesis and their purity were >95% as determined by HPLC and mass spectrometry unless indicated otherwise (see Table 1 for the amino acid sequences). The concentration of peptides containing tryptophan were determined using the extinction coefficient for tryptophan (ε350 = 5600 cm⁻² M⁻¹), the concentration of the FITC labeled p53 peptide, FITC-p53367–393 was determined using the extinction coefficient for FITC (ε494 = 72,000 cm⁻² M⁻¹), while the concentration and composition of the other peptides were confirmed by amino acid analysis (Bio-Synthesis, Inc., Lewisville, Texas). The N- and C-termini were acetylated and amidated, respectively, for TRTK-12 (Biopolymer Lab, U. of Maryland, Baltimore, MD), P385Wp53367–393, RAGE, p53337–393, p53231–346, Hdm2235–45, Hdm425–43, and PAK125–271 peptides. The N-terminus of p53367–393 was acetylated as were the N-terminus of the phosphorylated serine/threonine version of that peptide, P376 Phos (p53367–393) phosphorylated at Ser-376), T377 Phos (p53367–393 phosphorylated at Thr-377), S378 Phos (p53367–393 phosphorylated at Ser-378), S392 Phos (p53367–393 phosphorylated at Ser-392), and acetylated lysine version of p53393–393 phosphorylated at Lys-382, while for the FITC-p53 peptide, the N-terminus of p53367–393 is conjugated to FITC. The C-terminus of RKLLW (Alexis Biochemical, San Diego, CA) was amidated. The termini of Nt, an Y11W mutant of neurotensin (residues 8–13), American Peptide, Sunnyvale, CA), XP-1, the xenopsin related peptide 1 (Bachem Inc.), PKCε393–393 (Sigma-Aldrich), and PKCβ255–271 (Sigma-Aldrich) were not modified at either the N- or the C-termini.

2.3. Fluorescence spectroscopy

The Ca²⁺-dependent interaction of peptides with wild-type and mutants of S100B were performed using fluorescence spectroscopy techniques on an SLM-Aminco Bowman series 2 (Thermo, Asheville, NC) or a Carey Eclipse (Varian Inc., Palo Alto, CA) fluorescence spectrophotometer with the temperature maintained at 37 °C. For low salt conditions listed in Table 1, the buffer contained 10 mM TES, pH 7.2, 15 mM NaCl, and 10 mM CaCl₂; the high salt buffer contained 100 mM KCl in addition to that listed in the “low salt” buffer. For the Trp containing peptides (P385Wp53367–393, TRTK-12, RAGE, RKLLW, XP-1, and Nt), increased fluorescence emission from the tryptophan residue at 338 nm was monitored upon titration of S100B with excitation at 295 nm. In all of the titrations, the titrant and reactant were prepared with equal concentrations of peptide (up to 4 mM), so no corrections for dilution were necessary. Fluorescent polarization measurements during competition binding studies was used to monitor the binding of peptides to S100B using the same low and high salt conditions except with 1 μM FITC-p53367–393. Polarization readings of FITC-p53367–393 were monitored as such assays using an SLM-Aminco series 2
| Table 1  |
|------------------|------------------|
| **Summary of S100B binding peptides** |

- **K$_D$ (µM)**

<table>
<thead>
<tr>
<th>Peptide</th>
<th>-K$_D$ (µM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High salt (125-130 mM)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hdm4 (25-43)</td>
<td>0.4±0.02</td>
<td>New Data</td>
</tr>
<tr>
<td>Hdm2 (25-43)</td>
<td>3.1±0.35</td>
<td>New Data</td>
</tr>
<tr>
<td><strong>TRTK-12 c</strong></td>
<td>17.2±2.31</td>
<td>[88, 89]</td>
</tr>
<tr>
<td>F385W p53 (367-388)</td>
<td>17.3±2.54</td>
<td>New Data</td>
</tr>
<tr>
<td>p53 (367-393)</td>
<td>99.2±7.71</td>
<td>New Data</td>
</tr>
<tr>
<td>p53 (325-335)</td>
<td>112.0±7.00</td>
<td>[90]</td>
</tr>
<tr>
<td>p53 (325-339)</td>
<td>172.0±4.00</td>
<td>[90]</td>
</tr>
<tr>
<td>p53 (340-351)</td>
<td>302.0±7.00</td>
<td>[90]</td>
</tr>
<tr>
<td>p53 (305-322)</td>
<td>0.25±0.05</td>
<td>[90]</td>
</tr>
<tr>
<td>p53 (399-393)</td>
<td>46.0±2.00</td>
<td>[90]</td>
</tr>
<tr>
<td><strong>Modified p53 (367-393)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S376 Phos</td>
<td>239.9±6.80</td>
<td>New Data</td>
</tr>
<tr>
<td>T377 Phos</td>
<td>92.7±44.0</td>
<td>New Data</td>
</tr>
<tr>
<td>S378 Phos</td>
<td>41.3±14.2</td>
<td>New Data</td>
</tr>
<tr>
<td>S392 Phos</td>
<td>41.5±5.60</td>
<td>New Data</td>
</tr>
<tr>
<td>K382 Ac</td>
<td>218.3±21.9</td>
<td>New Data</td>
</tr>
<tr>
<td><strong>Other peptides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKC (19-36)</td>
<td>ND</td>
<td>New Data</td>
</tr>
<tr>
<td>p53 (321-346)</td>
<td>ND</td>
<td>New Data</td>
</tr>
<tr>
<td>ROS-C1 (962-981)</td>
<td>0.198±0.11</td>
<td>[91]</td>
</tr>
<tr>
<td>p53 (319-393)</td>
<td>0.025±0.01</td>
<td>[92]</td>
</tr>
<tr>
<td>Mu p53 (321-346)</td>
<td>ND</td>
<td>[92]</td>
</tr>
<tr>
<td>Mu p53 (357-381)</td>
<td>ND</td>
<td>[92]</td>
</tr>
<tr>
<td>PKCA258 (19-36)</td>
<td>ND</td>
<td>[93]</td>
</tr>
<tr>
<td>Nm (37-53)</td>
<td>ND</td>
<td>[93]</td>
</tr>
<tr>
<td>MARCKS (152-172)</td>
<td>ND</td>
<td>[94]</td>
</tr>
<tr>
<td>Tau (337-370)</td>
<td>ND</td>
<td>[95]</td>
</tr>
<tr>
<td>Melittin</td>
<td>ND</td>
<td>[95]</td>
</tr>
</tbody>
</table>

* a: New Data
b: 15-30 mM

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- **Binding in low salt (15-30 mM)**
- **Modified p53 (367-393)**
- **Other peptides**
fluorescence spectrophotometer at 37 °C with an excitation wavelength of 85 nm and an emission wavelength of 520 nm. The binding data were fit using a single site binding model with Origin software (OriginLab Corp., Northampton, MA) and one peptide bound per S100B subunit. For fluorescent intensity competition assays with the FITC-S100B peptide, non-fluorescent peptide was used to compete for the S100B with the upper limit for the dissociation constant (K_D) being determined using the Cheng–Prusoff equation; K_D = [I]_0 / (1 + [FITC-S100B]K_D) where [I]_0 is the concentration of the unlabeled peptide at 50% inhibition and [FITC-S100B] is the dissociation constant of the S100B-FITC complex (in low salt S100B-FITC = 3.9 ± 0.3 μM; Table 1) [53]. For fluorescence polarization competition assays, an equation derived by Nikolovska-Coleska et al. was used for determination of the K_D, as previously described [54], using: K_D = [I]_0 /([I]_0/[FITC-S100B]K_D + [P]_0/[FITC-S100B]K_D + 1) where [I]_0 is the concentration of the unlabeled peptide at 50% inhibition, [I]_0 is the concentration of the free FITC-p53 at 50% inhibition, [P]_0 is the concentration of the free protein at 0% inhibition, and [FITC-S100B]K_D is the dissociation constant of the S100B-FITC complex (in high salt S100B-FITC = 99.2 ± 7.7 μM; Table 1) [54].

2.4. Isothermal titration calorimetry (ITC)

Heat changes during the titration of the Hdmb2–45, Hdm425–43, PAK132–271, and F385Wp53367–393 peptides into Ca2+-S100B was measured using a VP-ITC titration microcalorimeter (MicroCal, Inc., Northampton, MA) as done previously [55]. For each titration the sample cell (1.4 ml) contained 10 mM TES, pH 7.2, 15 mM NaCl, and 10 mM CaCl2, 100 mM KCl, and 0.1 mM S100B while the reference cell contained water with all solutions being degassed under vacuum and equilibrated at 37 °C prior to titration. Upon equilibration, a 1.25–1.8 μM peptide solution prepared in the same buffer without S100B was injected in 29×5 μl aliquots using the default injection rate with a 300 s interval between each injection to allow the sample to return to baseline. The resulting titration curves were corrected for the protein free buffer control and analyzed using the Origin for ITC software supplied by MicroCal (Northampton, MA).

2.5. NMR spectroscopy

NMR spectra were collected at 37 °C with either a Bruker DMX600 NMR spectrometer (600.13 MHz for protons) or a Bruker AVANCE 800 NMR spectrometer (800.27 MHz for protons) equipped with four frequency channels and a triple-resonance z-axis gradient 5 mM cryoprobe. All protein chemical shifts are reported with respect to the H2O or DHO signal taken to be 4.658 ppm relative to external TSP (0.0 ppm) at 37 °C. The 15N chemical shifts were indirectly referenced using the following ratios of the zero-point frequencies at 37 °C: 0.10132905 for 15N [56–58]. Uniformly 15N-labeled S100B was used to collect the 2D 1H, 15N-fast HSQC [59] during the titration of peptides into Ca2+-S100B and changes in S100B backbone 15N and 1H were monitored and readily assigned. Typical NMR samples contained 0.05–0.50 mM S100B, 0–50 mM peptide, 10 mM TES, pH 7.2, 15 mM NaCl, 10 mM CaCl2, 0.34 mM NaN3, 5–10% D2O.

2.6. Lambda2Hybrid protein association assay

Under normal conditions, double infections of E. coli by lambda phage do not occur; however, expressing protein binding partners fused to the C-terminus of phage D protein enables the two phages to associate and simultaneously infect a cell. By using modified lambda phage, which contains different antibiotic resistances, the interaction and formation of multi-lysogens will result in double antibiotic resistant colonies (Cmlr/Kanr). Such colonies can then be quantified as the formation of specific protein–protein complexes; furthermore, disruption of such protein–protein interactions are also performed in inhibition assays since the interaction between the two phages is done in vitro. Construction of the S100B (JD-S100Bc), wild type human p53 (JD-p53h), dimeric p53 mutant (JD-p53ΔM08) [60] and Hdm2 (2H-D-hm2) display phages were done according to the method of Bair, et al. (in preparation). For the association assay, the phage fusions being tested are first diluted separately then combined at a ratio of 1:1 in the reaction tube, mixed gently and incubated for 5 min at RT. To this, 100 μL SABE buffer (20 mM Tris, pH 7.4, 10 mM CaCl2, 10 mM MgSO4, 100 mM NaCl, and 5 mM EDTA) with or without inhibitor is added with gentle mixing and incubated for 10 min at room temperature. At that time, 1×10^7 mid-log phase E. coli C600

Notes to Table 1:

a. The underlined amino acids are within 6 Å of residues in S100B based on the observance of NOE correlations for determining the NMR based structures of p53367–393 [86], TRTK [88], and Ndr [63] peptides bound to Ca2+-S100B. The short hand notation NB was used to indicate “No binding” to the peptide was observed, and ND was used to indicate that the peptide binds to S100B but that the exact K_D is “not determined”.

b. Binding affinities in “low salt” conditions were determined by fluorescence spectroscopy or with isothermal titration calorimetry (ITC; for TRTK-12) with 15 mM TES, pH 7.2, 10 mM CaCl2, 15 mM NaCl, at 37 °C except for Ndr that was determined by Bhattacharya et al. by plotting the chemical shift changes determined by NMR in 20 mM Tris, pH 7.5, 5 mM CaCl2, 30 mM KCl, and 10 mM DTT at 37 °C [63].

c. The TRTK-12 peptide is derived from residues 265 to 276 of the actin capping protein CapZ peptide, which was discovered by Dimlich et al. [62]. Previously, McClintock et al. report that TRTK-12 binds S100B with a K_D = 0.27 ± 0.03 μM in 50 mM Tris, 50 mM KCl, and 1 mM CaCl2 at pH 7.2 [65,88,89].

d. The buffer conditions were 10 mM TES, pH 7.2, 10 mM CaCl2, 15 mM NaCl, 100 mM KCl, at 37 °C.

e. Fernandez-Fernandez et al. [90] reported binding for p53325–355, p53325–359, p53340–341, p53305–32, and p53293–393 in 25 mM Tris, pH 7.5, 10 mM CaCl2, 99.2 mM NaCl, 1 mM DTT. This group also determined the binding affinity for p53367–393 under these conditions, which gave the same affinity for Ca2+-S100B (K_D = 0.27 ± 0.3 μM) as reported here for the “high salt” buffer.

f. The domain encompassing the tetramerization and C-terminal regulatory region of p53 was synthesized and two S100B binding sites were detected; these are the likely tetramerization site (residues 325 to 355) and in the C-terminal regulatory region (residues 367 to 393) [90].

g. The p53367–393 peptide was covalently modified in the C-terminal region of p53 to mimic covalent modifications that occur in vivo such as phosphorylation (Phos) and acetylation (Ac), which are italicized.

h. NMR experiments show the PKCα39–40 and p53321–346 bind S100B in 10 mM TES, pH 7.2, 10 mM CaCl2, 15 mM NaCl, at 37 °C, but the exact K_D was not determined.

i. A peptide from rod outer segment membrane guanylate cyclase type 1 (ROS-GC1; residues 962 to 981) was immobilized to a sensor chip via the cytoine thiol group, from a Cy3 residue attached to the N-terminal of the peptide, and surface plasmon resonance (SPR) was used to determine the binding affinity [91].

j. The C-terminal domain of human p53 (residues 319–393) was immobilized to a sensor chip by random coupling of its amine groups and SPR was used to determine the binding affinity to S100B [92]. Subsequently, two shorter peptides derived from mouse p53, Mu p53325–314 and Mu p53357–381, were added to compete off S100B; the K_D for this competition was not reported (ND). While the tetramerization domain peptide, Mu p53357–381, was not able to displace S100B at concentrations as high as 1 μM. Note that the sequence of the mouse p53 peptide, Mu p53357–381, differs from the human p53 peptide, p53367–393 at three positions (L383M, residue colored in gray; R379H, F385K, human numbering), which are important for binding S100B. The Mu p53357–381 peptide was mutated at two positions (Y321W, F322W, human numbering).

k. S100B inhibits the PKCα phosphorylation of these peptides as it does the p53367–393 peptide by binding the peptide [93].

l. The MARCKS peptide (residues 152 to 172) contains a PKCα phosphorylation site whose phosphorylation is inhibited due to binding to S100B but the affinity is undetermined [94].

m. Baudier et al. showed the interaction of Tau peptide (residues 337 to 370) with S100B and the Tau37–370 peptide contains a PKCα phosphorylation site. In addition, these authors also report that Melittin, a 26 amino acid long peptide from bee venom, also binds S100B, but for both of these peptides, no K_D is reported [95].
Incubated overnight at 32 °C, after which time, the number of colonies are counted.

### 2.7. GST-Hdm2 pull down assay

The GST-Hdm2 expression vector was prepared in the laboratory of Allan M. Weissman (NCI, NIH, Bethesda, MD 20892) and provided by Shengyun Fang (The University of Maryland School of Medicine, Baltimore, MD). This vector was used to produce glutathione S-transferase (GST) fusions of Hdm2 with a N-terminal GST tag in E. coli BL-21 (DE3) star cells (Novagen Inc.) that were grown at 37 °C until mid-log phase (i.e. OD_{600} \sim 0.8–1.0), induced with 0.84 mM IPTG for 1–1.5 h after which the cells were centrifuged 5000 \times g in a GS-3 rotor using a RC2-B Superspeed centrifuge (Thermo, Asheville, NC) and the pellet stored at −80 °C until use. Bacterial pellets were suspended in 1 ml per gram with 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM DTT, 2 mM phenylmethylsulfonyl fluoride (PMSF) to which 0.08 ml per gram pellet of 10 mg/ml lysozyme was added for 15 min on ice. Then, 300 μl DNase solution (1 mg/ml) and 300 μl 4.9 M MgCl₂ were mixed for 15 min and lysis was completed by probe sonication. The sonicate was clarified by centrifugation at 4 °C for 45 min at 27,000 \times g in SS-34 rotor (Sorvall), the protein was captured on glutathione agarose beads (Novagen) using batch purification, and the beads were washed three times in 50 mM Tris, pH 7.4, 150 mM NaCl. GST-Hdm2 beads and control beads were then blocked with 5% BSA in the same buffer for 30 min at 37 °C, centrifuged briefly to pellet beads then washed with the same buffer without BSA with either 5 mM CaCl₂, no CaCl₂, or no CaCl₂ plus 5 mM EDTA and pelleted again before adding 10 μg S100B in the same buffer for 1 h at room temperature. The beads were then washed three times in the same buffer, without S100B, to remove unbound protein before the S100B was eluted with 25 μl EDTA (500 mM). Samples were run on an 18% SDS-PAGE, blotted, and detected using 5% BSA rabbit polyclonal antibody (Research Diagnostics Inc., Flanders, NJ) at 1:1000 dilution as done for the cellular p53 assays outlined below.

### 2.8. Cellular assays of p53 down-regulation by S100B

Human large cell lung carcinoma cells, H1299, were grown in Minimum Essential Medium (Invitrogen Inc.) containing 10% fetal bovine serum. The p53 expression vector (pCMV3-p53) was provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD). Using this wild-type p53 expression vector and the QuikChange site-directed mutagenesis kit (Stratagene Inc.), the phosphorylation site single and double mutant constructs were engineered including pCMV3.p53 S376A(TCT to GCC), pCMV3.p53 T377A (ACC to GCC) and pCMV3.p53 DM (S376A T377A). The pCMV3 empty vector, which was used as a control in several experiments, was purchased (Stratagene Inc.). The pCMV-Myc empty vector was purchased (Clontech Inc.), and the gene for rat S100B was cloned into the SalI/XhoI sites. To examine the effects that S100B has on p53 protein levels and function, H1299 cells were transiently transfected with the fixed amount of plasmid (12 μg) by the Fugene 6 transfection reagent (Roche Diagnostics) according to the manufacturer’s recommendation such that the S100B plasmid was in excess (9 μg) versus the p53 plasmid (3 μg). Next, western blot analyses were performed on 20 μg of H1299 protein extract after the cells were lysed in RIPA buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 5 mM DTT, 50 mM NaF, 5 mM EDTA). The proteins were analyzed on a 12% polyacrylamide gel, transferred on nitrocellulose, and reacted with either p53 mouse monoclonal antibody (DO-1, Oncogene Research Products, Boston, MA) at 1:1000 dilution, S100B rabbit polyclonal antibody (Research Diagnostics Inc., Flanders, NJ) at 1:1000 dilution, p21 rabbit monoclonal antibody (Zymed Laboratories Inc.) at 1:1000, or actin mouse monoclonal antibody (Oncogene Research Products) at 1:5000 dilution to control for even protein loading. The blots were then reacted with their respective secondary antibodies conjugated to horseradish peroxidase and reacted with a chemiluminescence substrate (ECL, GE Healthcare) as recommended by the manufacturer.

### 3. Results

#### 3.1. Binding of S100B to peptide and protein targets including p53, Hdm2, and Hdm4

Upon the addition of calcium, helix 3 of S100B rotates ∼90° and exposes a site that binds target proteins and peptides [61]. Using phage display, Ivanenkov et al. defined a S100B target...
sequence ([K/R]-[L/I]-x-W-x-[x-L]-I-L) and identified a 12 residue peptide, termed TRTK-12, which binds S100B and most closely resembles a sequence (residues 265–276) in the actin capping protein, CapZ [62] (Table 1). By expanding the search to include a larger number of protein sequences ([K/R]-[L/I]-[P/S/N/D]-[W/L/I]-[S/D/L]-x-[L/I]-[L/F]), additional S100B targets were identified. Of particular interest was the discovery that S100B associates rather tightly to peptides derived from Hdm2 ([K/D] = 3.1 ± 0.4 μM; Fig. 1A; Table 1) and Hdm4 (also termed MdmX; [K/D] = 0.4 ± 0.02 μM; Fig. 1B, Table 1) since these two proteins are involved in the regulation of p53. In addition to these two peptides, several other peptides were also found to bind S100B in the presence of calcium including new data reported here for peptides derived from p53, phosphorylated p53, human RAGE, neutrosin (nt), cathepsin L inhibitor (RKLLW), and a peptide derived from the regulatory domain of p53, human RAGE, neurotensin (nt), cathepsin L inhibitor

Although not yet supported by 3-dimensional structural data, it appears that the presence of positively charged arginine and lysine flanking both sides of the hydrophobic residue may tighten the interaction since the peptides that only have a positive charges on one side (i.e. RKLLW, XP-1, and Nt) bind more weakly; however, it should also be noted that these peptides are shorter as well, so the decreased affinity could also be due to lack of another interaction(s).

3.2. Chemical shift perturbations in S100B caused by target peptide binding

To further explore interaction of S100B with various target peptides, changes in the chemical shifts of S100B backbone 1H and 15N between Ca2+-bound to target peptide–Ca2+-bound were recorded (Fig. 3). The amino acids in S100B where chemical shift values are perturbed upon binding of different target peptides are very similar indicating that the target peptides as a whole interact with similar residues on S100B (Fig. 3); although, it should be noted that chemical shift changes in a protein may be the result of direct interaction of a ligand with amino acid residues and/or by changes in the overall protein structure caused by peptide binding. For the five S100B–peptide interactions examined here (Fig. 3), significant chemical shift perturbations and/or residues that are exchange broadened (i.e. resonances that disappear in peptide titrations) occur for residues in loop 2, termed the hinge region (S41, F43, L44, E45), helix 3 (V52, V53, V56, T59), the C-terminus of helix 4 (S78–F88), and in helix 1 (F14). With Phe-14 as an exception1, most of these

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1 Resonances of phenylalanine-14 in S100B likely shift during the titrations with the peptides because of a long-range structural perturbation rather than from a direct interaction with the S100B-binding peptides.
residues that show chemical shift perturbations in the five peptide complexes are also found to interact directly in the S100B–p53 peptide complex (via intermolecular NOE correlations) including residues in the hinge region of S100B (L40, H42, L44, I47), helix 3 (V52, V56), and in C-terminus of helix 4 (M79, V80, A83, F87) [48]. Interestingly, when the amino acid
sequences of human S100 proteins are compared (Fig. 4), the hydrophobic nature of the residues in S100B that interact with the target peptides are conserved throughout the family of 22 related S100 proteins including L40 (95.0%), L44 (85.0%), V52 (75.0%), V53 (100.0%), V56 (90.0%), V80 (100.0%), A83 (75.0%), F87 (60.0%) and F88 (65.0%). However, the negatively charged residues that interact with specific peptide targets are not well conserved. For example, the charged residues of S100B that interact with p53 are minimally conserved including E45 (15.0%) and E46 (25.0%) or E68 (50.0%), and in some cases, other S100 proteins have these analogous positions (i.e. E45, E46, and E86 in S100B) replaced with oppositely charged Lys or Arg residues, 35.0%, 15.0%, and 30.0% of the time, respectively. It is these variations in the charged residues that could perhaps give rise to binding specificity among various S100 proteins.

3.3. Do full-length Hdm2 and p53 interact with the well-defined peptide binding site on S100B?

Data in Table 1 and several previously published studies have demonstrated that S100B interacts with p53 including full-length p53 and peptides derived from the C-terminal region of p53. However, the binding site for full-length p53 on S100B has not yet been fully established. Nor has it been determined whether Hdm2 binds to this same site on Ca²⁺-bound S100B or to another yet to be discovered site. To answer this question, a newly developed method for examining protein–protein interactions was employed, which was developed by Dr. Sankar Adhya and colleagues at the National Cancer Institute, and termed the Lambda-2-hybrid method.

Specifically, competitive inhibition experiments using the Lambda-2-hybrid methodology (Bair et al., in preparation) confirmed that full-length p53 binds to a site on S100B that overlaps a small molecule inhibitor binding site (SBi1) and the TRTK-12 peptide site on S100B. In both of these experiments, recombinant lambda phage expressing S100B (Δλ-S100B) interacts with lambda phage expressing p53 (Δλ-p53) or a dimeric mutant of p53 (Δλ-p53MLQR; [60]) each of which are fused to the C-terminus of the phage major capsid protein D. Importantly, the S100B–p53 interactions detected by this method could be inhibited specifically by either the TRTK-12 peptide or by the S100B small molecule inhibitor, SBi1 (Fig 5). Similarly, a lambda phage expressing Hdm2 (Δλ-Hdm2) interacts with Δλ-S100B and this Hdm2–S100B protein–protein interaction was also inhibited by TRTK-12. It was also demonstrated that Hdm2 display phage, Δλ-Hdm2k, does interact with the Δλ-p53 and Δλ-p53MLQR phage indicating that this dimeric construct of p53 is folded correctly and, as expected, this interaction was not affected by the addition of up to 384 μM TRTK-12. SBi1 (80 μM), a small molecule that binds S100B [70], was also able to inhibit binding of Δλ-S100B to Δλ-p53MLQR (84%) and the binding of Δλ-S100B to Δλ-Hdm2 (46%). Together, these competition binding data illustrate that full-length Hdm2 and full-length p53 each bind a site on S100B that comprises the TRTK-12 peptide and the S100B small molecule inhibitor, SBi1 site(s) [64,65,70,71].

3.4. Covalent modifications in the C-terminal negative regulatory domain of p53 protects the tumor suppressor from binding to S100B

It is now established that p53 is protected from Hdm2-dependent degradation via phosphorylation in its N-terminal transactivation domain [72,73]. Based on the 3D structure of S100B bound to the C-terminus of p53, it was predicted that phosphorylated and perhaps acetylated p53 would not bind Ca²⁺–S100B as tightly as unmodified protein [48]. This indeed turned out to be the case since S100B was found to bind p53 peptides, phosphorylated at residues that were in the S100B–p53 peptide interface (S376, T377), more weakly than a corresponding full-length p53 peptide that was not covalently modified (Fig. 6; Table 1); whereas, phosphorylation of Ser-378, which is facing solution in the S100B–p53 peptide structure had little or no effect on binding (Table 1; Fig 6). Similarly, steric hindrance or loss of a salt bridge due to the acetylation of p53 Lys-383 could explain why the acetylated version of the p53 peptide also inhibits S100B binding by more than 10-fold (Fig 6A and B). Furthermore, it is likely that covalent modifications such as phosphorylation and perhaps acetylation stabilize the structure of the extreme C-terminus, as predicted by Arrowmith et al. [49], and as supported by data showing that phosphorylated p53 is more stable and binds DNA more tightly in vitro [74].

Further evidence for this “p53 protection model” is provided by our own earlier studies with the Δ30 p53 mutant (p531–363, ΔNRD), which has the C-terminus of p53 removed [25]. In this study, we showed that the Δ30 p53 mutant was down-regulated by S100B to a larger degree than that of full-length p53 in H1299 cells [25]. Here, it is thought that the inability of the Δ30 p53 mutant to get sufficiently phosphorylated leaves this construct open to S100B binding to the tetramer domain, as observed previously ([67]; Table 1), and subsequently to S100B-dependent p53 degradation. Based on this model, we anticipated that the S376A and the T377A p53 mutants (and perhaps others) will be more susceptible to S100B-dependent

Fig. 3. Changes in chemical shifts for 1H–15N correlations in Ca²⁺-bound S100B upon binding several target peptides. Similar residues in S100B undergo chemical shift perturbations upon binding (A) p53367–388, (B) p53321–346, (C) TRTK-12, (D) PKC19–36, or (E) the RKKLW peptides. The bar graphs on the left show the combined backbone 1H and 15N chemical shift perturbations upon peptide binding to Ca²⁺–S100B except for residues G22, D23, K24, I47, and Q50, which are severely exchanged broadened. Correlations for H42, L44, E45, and E86 for the p53321–346 peptide, F43 for TRTK-12, and M79 and V80 for the PKC19–36 peptide broadened upon binding, so Δδ values could not be determined for these residues. To the right of each bar graph are ribbon diagrams of the 3D solution structure for p53367–388 bound S100B (PDB ID T17) with the p53367–388 peptide in each subunit removed; these ribbon diagrams show (in yellow) the amino acid residues having significant chemical shift perturbations (i.e. Δδ values that are greater than that observed for F14) and/or residues for which extreme exchange broadening effects was observed during the peptide titration. The residues that were perturbed (in yellow) are shown for titrations with each peptide, respectively, on only one S100B subunit (red), the other S100B subunit (blue) and the Ca²⁺ ions (gray spheres) are also illustrated.
degradation than wild-type and S376A p53. In H1299 cells, this indeed turned out to be the case for the T377A mutant (Fig 6C). However, no additional effect on p53 degradation was observed when the T377A p53 and S376A +T377A double mutant were compared, which is evidence that the mutation at Thr-377 is more deleterious for S100B-dependent degradation of p53 in cells, and are consistent with the hypothesis that phosphorylation of Thr-377 in p53 is an important phosphorylation site for protecting p53 from S100B. This idea is also in agreement with cellular reporter gene assays from Youmell et al., done with full-length p53, which show that mutating T377 is the most deleterious PKC site mutant when p53 transcription activation activity is measured; although, a small effect was also observed for the S376 mutant[75]. The differences observed in comparisons of the peptide-binding data (Table 1) to the cellular assays done with full-length p53 (Fig. 6) are suggestive that perhaps full-length p53 binds S100B slightly differently than found in the S100B–p53 peptide complexes, although this remains to be determined more rigorously. Nonetheless, it is important to note that the absence of the extreme C-terminus (Δ30 p53;[25]) and/or the lack of a phosphorylation site in the C-terminus of p53 (at Thr-377) does reduce the stability of p53 in treatments with S100B (Fig. 6C) and is consistent with previously measured transcription activation assays done with the same phosphorylation mutants performed by Youmell et al. [75].

4. Summary

A schematic is presented (Fig. 7) that summarizes how calcium-loaded S100B and Hdm2 participate in the down-regulation of the tumor suppressor protein, p53. Furthermore, feedback loops are in place in such that the tumor suppressor protein levels are kept under tight regulatory control (Fig. 7). Evidence of an S100-p53 interaction in vivo was provided by co-immunoprecipitation (co-IP) experiments with S100B done in human primary malignant melanoma cancer cells [76]. Similar co-IP experiments were also demonstrated for the S100A4–p53 interaction [24]. While most proteins that bind or modify the C-terminus of p53 activate the tumor suppressor, the opposite effect was observed for both S100A4 and S100B; in both cases, DNA binding to p53 in gel shift assays is decreased, and correspondingly, p53 function as a transcription activator is decreased when bound to S100 protein [24,25]. In the case of S100A4 (mts1), the S100-dependent affect on p53 transcription activation varied for some genes and showed a time and cell-density dependence [24,77]. For S100B, p53 protein levels and downstream effector genes, Hdm2 and p21 were also measured after human large-cell lung carcinoma cells (H1299; p53−/−) were transiently co-transfected with p53 and S100B expression vectors [25,78]. As expected, expression of p53 triggered expression of Mdm2 and p21, but co-expression with the S100B protein markedly reduced the accumulation of p53,
Hdm2 and p21 protein levels [25]. Similarly, endogenous wild-type p53 in human breast cancer cell line MCF-7 (p53+/+) [79] was inhibited when transiently transfected with S100B [25]. These earlier data indicate that the basal levels of p53, Hdm2 and p21 can be induced by exposure to bleomycin [25], while over-expression of S100B protein reduces p53 levels and its downstream effector genes. Additional evidence that S100B down-regulates p53 protein levels is provided by siRNA data since when S100B levels are reduced using siRNA directed against S100B (siRNAS100B), functional wild-type p53 protein is restored in primary malignant melanoma cells [76].

It is well established that p53 activates the transcription of Hdm2 (Fig. 7), a protein that is involved in ubiquitin-dependent degradation of p53 itself as part of a feedback loop [33,45,80]. In an analogous situation to Hdm2, p53 also activates the transcription of S100 proteins, including S100B [76,81]. The promoter for S100B has six relatively equally spaced sequences that correspond to the consensus sequence for p53 binding (>16/20 nucleotide match), and one region has a nucleotide sequence that matches the p53-binding consensus sequence perfectly (20/20 nucleotide match). In DNA band shift binding assays, regions of the S100B promoter bind p53 and, not surprisingly, the constructs containing the 20/20 matching sequence have the highest DNA binding affinity [25]. Furthermore, p53 activates transcription in reporter gene assays (i.e. CAT assays) containing various constructs of the S100B promoter [76].

It is now clear that the p53-dependent activation of the S100B promoter is also itself negatively regulated. This conclusion was first suspected from a comparison of the transcription activity of the full-length S100B promoter to Hdm2 and p21 protein levels [25]. Similarly, endogenous wild-type p53 in human breast cancer cell line MCF-7 (p53+/+) [79] was inhibited when transiently transfected with S100B [25]. These earlier data indicate that the basal levels of p53, Hdm2 and p21 can be induced by exposure to bleomycin [25], while over-expression of S100B protein reduces p53 levels and its downstream effector genes. Additional evidence that S100B down-regulates p53 protein levels is provided by siRNA data since when S100B levels are reduced using siRNA directed against S100B (siRNAS100B), functional wild-type p53 protein is restored in primary malignant melanoma cells [76].

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promoter constructs from a gene involved in cell-cycle control are both activated by elevated levels of p53 [76]. An interaction between S100B and p53 requires that S100B binds calcium and undergoes a conformational change (green circle with p53) to activate transcription. Subsequent down-regulation of p53 protein levels is mediated by (i) S100B and (ii) Hdm2 that down regulate p53, S100B is also (i) activated at the transcriptional level by p53 and (ii) then subsequently inhibits p53 function via feedback control. The interaction described here between S100B and Hdm2 further implies that these two proteins may work together to downregulate p53; however, the relevance of this interaction needs to be explored in more detail in vivo. This interaction between S100B and the E3 ligase for p53 ubiquitination, Hdm2, is particularly intriguing because of precedence for an S100 protein, S100A6, interacting with an E3 ubiquitin ligase, now called CacyBP/SIP; this E3 ubiquitin ligase, in turn, interacts with Siah-1 and Skp1 and contributes to the ubiquitination and degradation of beta-catenin [84,85].

The therapeutic relevance of inhibiting S100B is now established because (i) S100B contributes to the depletion of wild-type p53 protein in malignant melanoma and (ii) because S100B protein levels are elevated in this and other cancers. Therefore, such an effort to restore wild-type p53 tumor suppressor function, as is typically found in melanoma, is now underway using a rational drug design approach to find small molecule inhibitors of S100B [70] that mimic, in some respects, the activities observed previously with siRNA directed against S100B [76]. The next step in this process is to fully understand the target-binding site on S100B and how this site is different from other S100 proteins. In this paper, the p53 binding site on S100B is explored in detail, so that inhibitors can more readily be prepared that bind S100B specifically. Furthermore, these studies led to the discovery of a new and likely an important protein–protein interaction between S100B and Hdm2 and possibly with another regulator of p53, Hdm4. Lastly, data were presented that show how cells may prevent the S100B–p53 interaction, via phosphorylation and acetylation. These data suggest that the mechanism for the protection of the p53 tumor suppressor from two of its known negative-regulatory proteins, S100B and Hdm2, in vivo, is likely to be a very similar one (i.e. via covalent modification of the tumor suppressor).

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