Supporting Information

Malashkevich et al. 10.1073/pnas.0913660107

SI Text

SI Methods. *Structure determination.* Recombinant human S100A4 was purified as described previously (1). S100A4 in 20 mM Tris pH 7.5, 20 mM KCl, 10 mM DTT, 20 mM $CaCl_2$, and 0.02% NaN_3 was concentrated to 20–30 mg/mL (~0.85–1.28 mM dimer) and trifluoperazine (TFP) or prochlorperazine (PCP) was added to a concentration of 5 mM (final DMSO 2%) or 1 mM (final dioxane 4%), respectively. Diffraction quality crystals of Ca^{2+}-S100A4/ TFP were obtained by hanging drop vapor diffusion at 21 °C by mixing 1 μL of S100A4/TFP with 1 μL of reservoir solution containing 0.1 M Hepes, pH 7.0 and 30% v/v Jeffamine ED-2001 and equilibrating the samples against reservoir solution for 1–2 weeks. Diffraction from these crystals is consistent with the space group $P2_1$ with unit cell dimensions $a = 108.79$ Å, $b = 102.49$ Å, $c = 116.63$ Å, $\beta = 92.59°$ and twenty S100A4 chains in the asymmetric unit.

Diffraction quality crystals of Ca^{2+}-S100A4/PCP were obtained by hanging drop vapor diffusion at 21 °C by mixing 1 μL of S100A4/ PCP with 1 μL of reservoir solution containing 0.2 M ammonium sulfate, 0.1 M Tris pH 7.5, 20% w/v PEG-monomethyl ether (MME) 5000 and equilibrating the samples against reservoir solution for 23 weeks. Diffraction from these crystals is consistent with the space group $P2_1$ with unit cell dimensions $a = 54.58$ Å, $b = 102.28$ Å, $c = 117.37$ Å, $\beta = 92.6°$ and ten S100A4 chains in the asymmetric unit. The unit cells of the two S100A4 inhibitor complexes are very similar; however, in the S100A4/TFP complex the a axis is twice as long. The S100A4/TFP crystals demonstrate strong pseudo translational symmetry along the a axis and systematic near absences of odd reflections at low resolution, but at higher resolution the larger unit cell is apparent.

Crystals in mother liquor were flash-cooled in liquid nitrogen. For Ca^{2+}-S100A4/TFP crystals, diffraction data were collected at the LRL-CAT beamline (Advanced Photon Source, Argonne, USA) using an MAR-165 charge-coupled device (CCD) detector and 0.979 Å wavelength radiation. For Ca^{2+}-S100A4/PCP crystals, diffraction data were collected at the X-29 beamline (National Synchrotron Light Source in Brookhaven National Laboratory) using a Quantum-315 CCD detector and 1.073 Å wavelength radiation. Intensities were integrated using HKL2000 and reduced to amplitudes with TRUNCATE (2 and 3). The structures of the S100A4/TFP and S100A4/PCP complexes were determined by molecular replacement to 2.3 Å and 2.7 Å, respectively using the program PHASER (4) and the structure of the Ca^{2+}-S100A4 (PDB 2Q91, (5)) as the search model. Model building and refinement were performed using REFMAC and COOT (3 and 6). The quality of the final structure was verified with composite omit maps, and the stereochemistry was checked with WHATCHECK (7) and PROCHEK (8).

The final Ca^{2+}-S100A4/TFP model contained 20 subunit chains of 94 residues each (Ala2 to Pro95), 40 calcium ions, 40 TFP molecules, and 1,228 water molecules with an R_{cryst}/R_{free} of 0.206/0.259 (PDB entry 3KO0). The final Ca^{2+}-S100A4/PCP model contained 10 subunit chains of 94 residues each (Ala2 to Pro95), 20 calcium ions, 10 PCP molecules, and 11 water molecules with an R_{cryst}/R_{free} of 0.238/0.310 (PDB entry 3M0W). The LSQKAB and secondary-structure matching (SSM) algorithms were used for structural superimpositions (3 and 9). Quaternary structure and accessible surface areas were analyzed using the PISA server.

NMR spectroscopy. The Ca^{2+}-S100A4 NMR samples contained 0.35 mM S100A4 subunit, 0.34 mM NaN_3, 0–100 μM TFP, 15 mM NaCl, 10 mM $CaCl_2$, 10% D_2O, and 10 mM MES buffer,

pH 6.5. The chemical shift assignments for Ca^{2+}-S100A4 were used as the starting point during titrations with TFP to assign the two-dimensional ^1H-^{15}N HSQC spectra of S100A4 in the TFP-S100A4 complex. At the end of the two-dimensional HSQC titration, a three-dimensional ^{15}N-edited NOESY-HSQC experiment was performed to unambiguously confirm the assignments. Heteronuclear single quantum coherence NMR data were collected at 37 °C with a Bruker Avance III 600 (600.13 MHz for protons) or with an Avance 800 US2 (800.27 MHz for protons) NMR spectrometer both equipped with pulsed-field gradients, four frequency channels, and triple resonance, z-axis gradient cryogenic probes. Data were processed with NMRPipe (10), and proton chemical shifts were reported with respect to the H_2O or HDO signal taken as 4.658 ppm relative to external TSP (0.0 ppm). The ^{15}N chemical shifts were indirectly referenced as previously described using the following ratio of the zero-point frequency: 0.10132905 for ^{15}N to ^1H (11 and 12

Promotion of disassembly assays. Promotion of disassembly assays were performed with 3 μM S100A4 dimer, 3 μM myosin-IIA rod dimers, and 0–100 μM TFP in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.02% NaN_3, and 2% DMSO as described previously (1).

Chemical cross-linking of S100A4-inhibitor complexes. 3 μM S100A4 dimer was incubated for 1 h at room temperature in 20 mM Tris-HCl or 20 mM Hepes pH 7.5, 150 mM NaCl, 1 mM DTT, 2 mM $MgCl_2$, 0.3 mM $CaCl_2$, 0.02% NaN_3, and 2% DMSO. For experiments performed in the presence of EGTA the final concentration was 4 mM. The final phenothiazine concentration was approximately twofold higher than the IC50 for inhibition of the Ca^{2+}-induced fluorescence increase of the S100A4 biosensor (100 μM trifluoperazine, fluphenazine or prochlorperazine, 200 μM flupenthixol, or 500 μM chlorprothixene or perphenazine) (13). Disuccinimidylsuberate (DSS) (Pierce) was added to final concentration of 5 mM and the reactions were incubated for an additional hour at room temperature. The reaction was quenched by the addition of 5X sample buffer and heating.

Analytical ultracentrifugation. S100A4 was dialyzed into a buffer containing 20 mM Tris pH 7.5, 150 mM KCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP) , 0.02% NaN_3, and either 2 mM EDTA and 2 mM EGTA (Ca^{2+}-free) or 10 mM $CaCl_2$ (Ca^{2+}-bound). Sedimentation equilibrium experiments were performed using six channel centerpieces with 80 μM S100A4 subunit and 5–500 μM TFP (final DMSO 2%) at 25 °C. For centrifugation studies of crosslinked S100A4-TFP complexes, a 10 mL crosslinking reaction was performed as described above except that 1 mM TCEP was used instead of DTT and the final DSS concentration was 2.75 mM. The reaction was clarified by centrifugation for 15 min at $14,000 \times g$ and the crosslinked sample was concentrated to 80 μM S100A4 subunit. The sample was dialyzed against 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM TCEP, and 0.02% NaN_3 to remove free DSS. The experiments were performed with 13.3, 40, and 80 μM S100A4 subunit as described above.

Sedimentation profiles were monitored at 280 nanometers. Absorbance scans obtained following 24 h equilibrations at 15,000 and 22,000 rpm (TFP titrations) or 8,000 and 13,000 rpm (S100A4/TFP crosslinked complexes) in a Ti60 rotor (Beckman Coulter, Brea, CA) were globally analyzed using HeteroAnalysis v1.0.114 (Cole, J.L. and Lary, J.W., Analytical Ultracentrifugation Facility,

Biotechnology Services Center, University of Connecticut, Storrs, CT) to obtain the weight-average molecular weight of the S100A4 oligomers. The $\bar{\nu}$ value of 0.7346 was calculated from the amino acid composition of the wild-type S100A4. Density and viscosity values were provided by Sednterp v1.06 (Hayes, B., Laue T. and Philo, J. (2003), *Sedimentation Interpretation Program*, University of New Hampshire). The calculated mass of the wild-type S100A4 subunit is 11,597 Da. The titration data were plotted as a function of the TFP concentration and fit to the Hill equation to evaluate the midpoint and slope of the curve for S100A4 oligo-merization. The best-fit parameters and their 95% joint confidence intervals are reported.

S100A4/myosin-IIA model. The S100A6/SIAH-1 interacting protein peptide complex (PDB 2JTT) was used to build the S100A4/myo-sin-IIA model. The model structure of the myosin-IIA coiled-coil (residues 1,730–1,928) (14) was unwound at the C terminus to allow single peptide interactions with each S100A4 subunit. The S100A4 binding domain on myosin-IIA (residues 1,908–1,923) (1 and 5) was aligned with the bound peptide in the 2JTT structure so that the hydrophobic residues of the myosin-IIA coiled-coil (i.e., heptad positions *a* and *d*) interacted with the hydrophobic cleft formed by helices 3 and 4 on S100A4. The myosin-IIA heavy chain was bent similarly to the SIAH-1 peptide to maximize interactions with residues exhibiting chemi-cal shift perturbation upon myosin-IIA peptide binding (5).

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Fig. S1. Stereo image of TFP1(A) superimposed on TFP2(A) (yellow). The piperazine rings have opposite orientations relative to the superimposed phenothiazine moieties.

Fig. S2. Ca^{2+}-S100A4/PCP structure. (*A*) Ribbon diagram showing the crystal packing of the Ca^{2+}-S100A4/PCP molecules. The protein dimers are arranged in a pentameric ring facilitated by PCP molecules (dark gray). (*B*) Ribbon diagram of the AB (light and dark blue) and CD (dark and light green) S100A4 dimers showing the Ca^{2+}-S100A4/PCP dimer-to-dimer crystal interface. The Ca^{2+} atoms are shown as light gray spheres. The interhelical loops connecting helices 1 and 2 (1–2) and helices 2 and 3 (hinge), which are involved in crystal contacts, are indicated. (*C*) PCP interactions with the symmetry-related molecule. View from subunit A (light blue) towards CD dimer. The single PCP molecule (PCP(A)) bound to subunit A is shown in yellow. Helix 4 of subunit C is dark green and helix 1 of subunit D is light green. Hydrogen bonds are shown as dashed pink lines. (*D*) Overlay of Ca^{2+}-S100A4/TFP and Ca^{2+}-S100A4/PCP complexes. A single PCP(A) molecule (red) occupies the same position as the TFP2(A) (yellow) molecule.

Fig. S3. Chemical shift perturbations following addition of TFP to Ca^{2+}-S100A4. (*A*) Bar graph showing the cumulative ^1H and ^{15}N chemical shift perturbations observed per residue upon the addition of 100 μM TFP. The inset shows an expanded region of the HSQC spectrum illustrating the perturbations to the ^1H-^{15}N correlation for F55 in the absence (black contours) and presence of 10 (red contours), 20 (blue contours), 40 (green contours), 50 (orange contours), 80 (violet contours), and 100 μM (light blue contours) TFP. (*B*) Ribbon diagram of Ca^{2+}-S100A4 showing the subunits in light and dark blue. Residues highlighted in dark pink exhibited the largest perturbations (as per the red line at 25 Hz in A).

Fig. S4. Chemical cross-linking of Ca^{2+}-S100A4 in the presence of phenothiazines. (A) 6% SDS-PAGE of S100A4/TFP/DSS complex. In the presence of 100 μM TFP and DSS, a complex with an approximate mass of 120 kDa is observed. Larger oligomers are not detected by SDS-PAGE. (B) Chemical cross-linking of Ca^{2+}-S100A4 in the presence of various phenothiazines. Monomeric and dimeric S100A4 have apparent molecular weights of approximately 11.5 kDa and 23 kDa, respectively. The addition of DSS in the presence of five different phenothiazines resulted in the appearance of S100A4 oligomers with apparent masses ranging from 32 to 120 kDa.

Fig. S5. Ligand binding to S100 proteins. (A) Surface diagrams of Ca^{2+}-S100A4 showing the two subunits in light and dark blue. Residues colored dark pink or orange demonstrate significant chemical shift perturbations and exchange broadening effects upon binding TFP or the MIIA$^{1908-1923}$ peptide, respectively. (B) Target peptide (yellow) binding to different S100 proteins (blue). Peptide orientations in the binding clefts are indicated. 2JTT: S100A6 bound to the C terminus of SIAH-1 interacting protein; 2KBM: S100A1 bound to TRTK12; 2K2F: S100A1 bound to the ryanodine receptor; 1BT6: S100A10 bound to the N terminus of annexin II, and 1PSB: and S100B bound to the N-terminal domain of NDR kinase.

Fig. S6. Hypothetical model of the S100A4/myosin-IIA pentamer. (*A, B*) The ten S100A4 protein chains are shown as light blue ribbons with residues exhibiting chemical shift perturbation in titrations with the MIIA[1908–1923] peptide shown in green. The bound myosin-IIA heavy chains (coiled-coil region) are shown in red and blue. The myosin-IIA N termini face outside the pentameric ring with the C termini inside the rings. The polypeptide chains from neighboring S100A4 subunits would overlap in the pentameric assembly.

Table S1. Crystallographic data and refinement statistics.

Data collection

	Ca^{2+}-S100A4/TFP	Ca^{2+}-S100A4/PCP
Beamline	LRL-CAT	X29A
Wavelength (Å)	0.9793	1.073
Resolution limits (Å)	20-2.3	25-2.8
Observed reflections	267,996	61,500
Unique reflections	110,771	31,659
Completeness (%)	96.8 (89.2) *	99.6 (100.0) *
R_{merge} [†]	0.077 (0.744)	0.065 (0.480)
Refinement statistics		
Protein nonhydrogen atoms	15,930	7,351
Water molecules	1,228	11
R_{cryst} [‡]	0.206 (0.333) *	0.252 (0.315) *
R_{free} [‡]	0.259 (0.378) *	0.302 (0.389) *
Average B-factor (Å²)	28.8	47.6
RMSD from ideality		
Bond length, Å	0.015	0.01
Bond angles (°)	1.75	1.212
Torsion angles (°)	19.3	23.9
Ramachandran plot		
Core (%)	95.8	93.2
Allowed (%)	3.5	6.8
Generous (%)	0.7	0.0

*Values in parentheses indicate statistics for the high resolution bin.
[†]$R_{merge} = \Sigma\Sigma j |Ij(hkl)- < I(hkl) > | / \Sigma\Sigma j | < I(hkl) > |$, where Ij is the intensity measurement for reflection j and $\langle I \rangle$ is the mean intensity over j reflections.
[‡]$R_{cryst}/(R_{free}) = \Sigma||F_o(hkl)| - |F_c(hkl)||/\Sigma|F_o(hkl)|$, where F_o and F_c are observed and calculated structure factors, respectively. No σ-cutoff was applied. 5% of the reflections were excluded from refinement and used to calculate R_{free}.

Table S2. Molecular contacts of bound TFP molecules *

Primary interactions with the target S100A4 molecule

	Subunit A	Subunit B
TFP1(A)	Ser44(A), Phe45(A), Leu46(A), Gly47(A), Ile82(A), Met85(A), Cys86(A), Phe89(A), TFP2(A), 3 water molecules	none
TFP2(A)	Leu42(A), Ser44(A), Phe45(A), Ile82(A), Cys86(A), Phe89(A), TFP1(A), 8 water molecules	Glu6(B), Leu9(B), Asp10(B)

Secondary interactions with
symmetry-related molecules

	Subunit C	Subunit D
TFP1(A)	Phe89(C), Phe90(C), Gly92(C), Phe93(C), TFP1(C)	none
TFP2(A)	Cys86(C), Phe89(C), TFP2(C)	Asp10(D), Ser14(D)

*Calculated with the program CONTACTS using a 4 Å cutoff.

Table S3. Contact area of bound TFP molecules

	TFP1(A)	TFP2(A)
Surface accessible area ($Å^2$) *	578	574
Change due to binding to:		
Ca^{2+}-S100A4	−299	−307
Ca^{2+}-S100A4/TFP1(A)	-	−337
Ca^{2+}-S100A4/TFP2(A)	−335	-
Symmetry-related molecules	−170	−228
Whole pentamer-of-dimers	−467	−502

*Calculated with the program AREAIMOL.