SARS Coronavirus Unique Domain: Three-Domain Molecular Architecture in Solution and RNA Binding

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Introduction

Severe acute respiratory syndrome (SARS) is an atypical pneumonia with flu-like symptoms that can lead to respiratory failure.1,2 SARS is caused by a coronavirus (CoV), SARS-CoV, which first became evident in 2003. CoVs are enveloped viruses with single-stranded positive-sense 30-kb RNA genomes.3,4 The SARS-CoV and ‘SARS-like’ viruses that have since been found in bats were phylogenet-
NMR Structure of the SARS-Unique Domain

This work describes new NMR structure determinations of SUD-C and of a two-domain construct SUD-MC with residues 527–720. Combined with the previous structure determination of SUD-M,13 these results are used for detailed comparisons of the SUD-M and SUD-C domains in isolated form and in the two-domain construct, and for investigations of the nature of the link between the two domains. We further present NMR data on SUD-N and SUD-NM in constructs comprising residues 387–524 and 387–651, respectively, which supplement the aforementioned crystal structure of SUD-NM with a description of the behavior of this protein in solution. Specifically, biochemical data and NMR experiments define the oligomerization state of SUD-NM and the interactions between the N- and M-domains in SUD-NM. The structure determinations are supplemented with investigations of the RNA binding properties of SUD-M, SUD-C, and SUD-MC based on biochemical data and NMR chemical shift perturbation experiments.

Results

Solution structure of SUD-C

The backbone assignment of SUD-C was essentially complete, with the only unassigned atoms being 15N and 1H of Ser655, 15N of Pro700, and 13C′ of Ser699. Table 1 summarizes the statistics of the structure calculation, which indicate a high-quality structure determination.

SUD-C adopts a fold consisting of seven β-strands arranged in an anti-parallel β-sheet, and two α-helices located at the N-terminus and C-terminus of the sequence, which are packed against the same side of the β-sheet (Fig. 1a and b). Helix α1 (residues 655–666) is followed by a small anti-parallel β-hairpin of strands β1 and β2 (residues 668–669 and 672–673), a short extended region, and another short strand, β3, of residues 678–679. There are two longer strands, β4 and β5, of residues 682–688 and 691–695, followed by a seven-residue loop, another hairpin of strands β6 and β7 (residues 703–705 and 708–710), and helix α2 (712–719). The seven extended strands form a twisted anti-parallel β-sheet (Fig. 1b), with the topology shown in Fig. 1c, where the two short strands β2 and β3 are both paired in anti-parallel fashion with β4. The fold is classified in the SCOP database as ‘N-terminal domain of CyaY-like.’

Solution structure of SUD-MC

The backbone assignment was essentially complete; the only unassigned atoms were 13C and 1H of Gly-4, 19N, 1HN, 13C′, and 1H of Ser-3, 18N and 1H of His-2 (these residues result from the vector-derived N-terminal expression tag); 15N of all proline residues, and 13C′ of the residues preceding prolines. These assignments were used as input for the analysis of the nuclear Overhauser enhancement
spectroscopy (NOESY) spectra with UNIO-ATNOS/ASCAN\textsuperscript{20,21} and UNIO-ATNOS/CANDID\textsuperscript{22} which yielded amino acid side-chain assignments and input for the structure calculation with CYANA\textsuperscript{23} (for details, see Materials and Methods). The resulting structure (Fig. 2a and b) was based on 3750 nuclear Overhauser enhancement (NOE) restraints (916 intraresidual, 954 short range, 767 medium range, and 1113 long range). While there were a small number of NOEs between domain hydrogen atoms and hydrogens in the linker peptide or in the short, chain-terminal tails, no NOEs that would connect hydrogen atoms in the two different domains were observed. In Fig. 2a and b, SUD-M and SUD-C are superimposed independently, and it is apparent that there is a large manifold of possible orientations for the domain that was not used for the superposition, showing that the two domains do not adopt a unique orientation relative to each other. In view of this result, we repeated the last cycle of the structure calculation separately for each domain, using NOE restraints within the two individual globular domains (residues 527–648 and 654–720) that had been measured in intact SUD-MC. The results of these two structure calculations (Table 1) show that the two domains are individually well defined in SUD-MC.

To further characterize the dynamics of the SUD-MC two-domain construct, we collected a steady-state \textsuperscript{15}N\textsuperscript{[1]H} NOE experiment, which is sensitive to the rapid motion of \textsuperscript{15}N–\textsuperscript{1}H moieties on the picosecond-to-nanosecond timescale (Fig. 2c). The linker residues 651–653 that connect the two domains exhibit \textsuperscript{15}N\textsuperscript{[1]H} NOE values ranging from 0.2 to 0.4, indicating significant segmental mobility, whereas the mobility of the residues within both globular domains is essentially limited to the overall molecular tumbling of the protein, with \textsuperscript{15}N\textsuperscript{[1]H} NOE values of 0.7–0.8. Overall, these data support the range of interdomain orientations observed in Fig. 2a and b is a realistic indication of the SUD-MC conformation in solution.

Additional evidence for a loose, flexible linkage of the two domains in SUD-MC comes from a com-

Table 1. Input for the structure calculations of the proteins SUD-C and SUD-MC and the statistics of the ensembles of 20 energy-minimized CYANA conformers used to represent the NMR structures

<table>
<thead>
<tr>
<th>Quantity\textsuperscript{a}</th>
<th>SUD-M\textsuperscript{b}</th>
<th>SUD-C\textsuperscript{b}</th>
<th>SUD-C\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOE upper distance limits</td>
<td>2288</td>
<td>1399</td>
<td>2336</td>
</tr>
<tr>
<td>Intraresidual</td>
<td>555</td>
<td>339</td>
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<tr>
<td>Short range</td>
<td>586</td>
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<tr>
<td>Medium range</td>
<td>466</td>
<td>291</td>
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<tr>
<td>Long range</td>
<td>681</td>
<td>423</td>
<td>843</td>
</tr>
<tr>
<td>Restraints/residue</td>
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<td>35</td>
</tr>
<tr>
<td>Long-range restraints/residue</td>
<td>6</td>
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<td>13</td>
</tr>
<tr>
<td>Dihedral angle constraints</td>
<td>673</td>
<td>347</td>
<td>329</td>
</tr>
<tr>
<td>Residual target function value (Å\textsuperscript{2})</td>
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<td>1.20±0.24</td>
<td>6.27±0.18</td>
</tr>
<tr>
<td>Residual NOE violations Number &gt;0.1 Å</td>
<td>27±6</td>
<td>14±7</td>
<td>13±3</td>
</tr>
<tr>
<td>Maximum (Å)</td>
<td>0.21±0.17</td>
<td>0.13±0.01</td>
<td>0.13±0.01</td>
</tr>
<tr>
<td>Residual dihedral angle violations Number &gt;2.5°</td>
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<td>1±1</td>
<td>4±0</td>
</tr>
<tr>
<td>Maximum (°)</td>
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<td>2.06±0.89</td>
<td>28.88±0.31</td>
</tr>
<tr>
<td>Amber energies (kcal/mol)</td>
<td>Total</td>
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<td>−2474.23±83.15</td>
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<td>Van der Waals</td>
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<td>−198.23±9.28</td>
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<tr>
<td>Electrostatic</td>
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<td>−2811.92±73.72</td>
<td>−2865.62±46.61</td>
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<td>RMSD from ideal geometry</td>
<td>Bond lengths (Å)</td>
<td>0.0075±0.0002</td>
<td>0.0078±0.0005</td>
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<tr>
<td>Bond angles (°)</td>
<td>1.83±0.046</td>
<td>2.047±0.139</td>
<td>2.021±0.059</td>
</tr>
<tr>
<td>RMSD to the mean coordinates (Å)\textsuperscript{d}</td>
<td>BB</td>
<td>0.55±0.08 (527–648)</td>
<td>0.54±0.08 (655–720)</td>
</tr>
<tr>
<td>HA</td>
<td>0.95±0.08 (527–648)</td>
<td>0.91±0.06 (655–720)</td>
<td>0.69±0.06 (655–720)</td>
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<tr>
<td>Ramachandran plot statistics (%%%%)\textsuperscript{e}</td>
<td>Most favored regions</td>
<td>81.8</td>
<td>79.7</td>
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<tr>
<td>Additionally allowed regions</td>
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<td>15.8</td>
<td>10.9</td>
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<tr>
<td>Generously allowed regions</td>
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<td>5.0</td>
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<tr>
<td>Disallowed regions</td>
<td>0.4</td>
<td>0.6</td>
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</tr>
</tbody>
</table>

\textsuperscript{a} The top eight entries describe the input from NMR experiments. The other entries refer to a bundle of 20 CYANA conformers after energy minimization with OPA1P. The ranges indicate standard deviations.

\textsuperscript{b} SUD-M and SUD-C within the SUD-MC construct. Structure calculations were performed for the intact construct of SUD-MC and also for the two individual domains of residues 527–648 (SUD-M) and residues 655–720 (SUD-C) using the input measured with SUD-MC, since no medium-range or long-range distance constraints between the two domains were observed. In the table, we only list the statistics for calculations with the individual domains in SUD-MC (PDB codes 2KQV and 2KQW for SUD-M and SUD-C, respectively), since these coincide very closely with the result of a calculation for the intact SUD-MC, as presented by Fig. 2a and b, with backbone RMSDs of 0.23 and 0.21 Å for SUD-M and SUD-C, respectively.

\textsuperscript{c} Isolated SUD-C (PDB code 2KAF).

\textsuperscript{d} BB indicates the backbone atoms N, C\textsuperscript{\prime}, and C\textsuperscript{\"}; HA stands for all-heavy atoms.

\textsuperscript{e} As determined by PROCHECK.\textsuperscript{18}
Fig. 1. NMR solution structure of SUD-C. (a) Bundle of 20 energy-minimized NMR conformers representing the solution structure of SUD-C, superimposed for minimal RMSD of the N, C\(^\alpha\), and C\(^\prime\) atoms of residues 655-720. Residues in \(\alpha\)-helices are shown in red, those in \(\beta\)-strands are shown in green, and regions without regular secondary structure are shown in gray. Selected sequence positions are indicated by numerals. (b) Ribbon presentation of the conformer with the lowest RMSD to the mean coordinates of the ensemble shown in (a). Regular secondary structures are identified. (c) Topology diagram of SUD-C, where \(\alpha\)-helices are represented by rectangles and \(\beta\)-strands are represented by arrows. Dark gray, the plane closest to the viewer on which the \(\alpha\)-helices lie; light gray, the plane farthest from the viewer on which the \(\beta\)-strands lie.
parison with structures determined from data collected in solutions of separately expressed SUD-M and SUD-C. The root-mean-square deviation (RMSD) value between the mean coordinates of the backbone N, $C^\alpha$, and $C'$ atoms of SUD-C in SUD-MC and in the isolated form is 0.86 Å, and the corresponding value for the SUD-M domain in SUD-MC and in the previously reported structure of the isolated form is 0.97 Å. The 

structure superpositions in Fig. 3a and b, together with the $^{13}C^\alpha$ and $^{13}C'$ chemical shift data presented in Fig. 3c and d, further show that there are also no outstanding local differences between the SUD-M and the SUD-C polypeptide backbone folds determined either from data collected with individual domains or from data collected with SUD-MC.

Fig. 2. NMR solution structure of SUD-MC. (a) Bundle of 20 energy-minimized NMR conformers superimposed for minimal RMSD of the backbone N, $C^\alpha$, and $C'$ atoms of SUD-M (residues 527–648). In SUD-M, residues in $\alpha$-helices are shown in red, those in $\beta$-strands are shown in green, and regions without regular secondary structure are shown in gray. SUD-C is shown in gray. The N-terminus of SUD-M is labeled, and the backbone of the C-terminal residue of SUD-C is shown in blue. (b) The same bundle of conformers as in (a) superimposed for minimal RMSD of the backbone N, $C^\alpha$, and $C'$ atoms of SUD-C (residues 655–720). In SUD-C, residues in $\alpha$-helices are shown in red, those in $\beta$-strands are shown in green, and regions without regular secondary structure are shown in gray. SUD-M is shown in gray. The C-terminus of SUD-C is labeled, and the backbone of the N-terminal residue of SUD-M is shown in blue. (c) $^{15}N{^{1}H}$ NOE values ($I_{\text{rel}}$) plotted versus the sequence of SUD-MC. The sequence positions of regular secondary structures are indicated at the top of the panel.
Fig. 3 (legend on next page)
The isolated SUD-N formed by the polypeptide of nsp3 residues 387–524 is monomeric in solution, as judged by the two-dimensional (2D) $^{15}\text{N},^{1}\text{H}$ heteronuclear single quantum coherence (HSQC) spectrum (Fig. 4a and b, blue peaks), which contains 130 of the 133 expected backbone $^{15}\text{N}--^{1}\text{H}$ correlation peaks and shows line shapes that are typical of a small globular protein. This conclusion is supported by size-exclusion chromatography (Fig. 5a), where SUD-N elutes at an apparent molecular mass of 19 kDa, which is close to the actual molecular mass of 15.2 kDa and would be inconsistent with a 30.4-kDa dimer.
NMR Structure of the SARS-Unique Domain

SUD-NM was reported to form a dimer in the single crystals used for X-ray structure determination, with a disulfide bond joining the SUD-N and SUD-M domains within each subunit, in addition to the backbone link between these domains. In contrast, we found that SUD-NM is monomeric in solution. The monomeric state is clearly apparent from the NMR data, as the 2D $^{15}$N,$^1$H HSQC spectrum (Fig. 4, red peaks) would be inconsistent with a molecular size of 60 kDa. Independently, size-exclusion chromatography showed that the protein eluted at an apparent molecular mass of 35 kDa, which fits closely with its monomeric size (Fig. 5b). Based on the similarity of the correlation peaks (Fig. 4), we conclude that the globular structures of SUD-N and SUD-M are conserved in the SUD-NM construct. Furthermore, SUD-N and SUD-M are flexibly connected with each other, as evidenced by the close similarity of the peak positions and peak shapes of the isolated domains and the SUD-NM construct (Fig. 4). Since the resonances of SUD-N have been identified as a group, but not individually assigned, the peaks of the $^{15}$N,$^1$H moieties of residues 525–526, which form the interdomain linker, had to be assigned by exclusion. There is one $^{15}$N,$^1$H correlation peak (identified in Fig. 4b and d) that has no counterpart in the spectra of the individual domains and was therefore tentatively assigned to the linker peptide segment. This is one of about 20 peaks in SUD-NM that exhibit $^{15}$N,$^1$H NOE values smaller than 0.6 (Fig. S1), thus indicating that there is increased mobility in parts of SUD-N and in the linker peptide when compared to the bulk of either of the two globular domains.

RNA binding to SUD-C and SUD-MC

We collected new data on the RNA affinity of SUD-MC, SUD-M, and SUD-C, and then included earlier data on SUD-M in comparative studies. Initially, gel-shift experiments [electrophoretic mobility shift assay (EMSA)] were used to screen a wider selection of RNA ligands, and evidence for binding was then followed up with chemical shift perturbation NMR experiments.

EMSA experiments showed weak binding of SUD-MC to $A_{10}$, $U_{10}$, “TRS(+)” (5′-CUAAACGACAC-3′), “TRS(−)” (5′-GUUCGUUAGC-3′), and “GAUA” (5′-CCGGAUAACCAC-3′) (Fig. 6a and b). Binding was observable at RNA/protein ratios of 1.8:1 or higher, except that binding to $C_{10}$ could not be determined in the experiment of Fig. 6a due to low staining efficiency. There was no observable binding of SUD-C to the same set of RNA ligands (data not shown).

Both SUD-M and SUD-MC bind to a 20-base RNA containing only purine bases, (GGGA)$_3$. The SUD-MC/(GGGA)$_3$ complex is seen as a discrete band in EMSA (Fig. 6b and e). SUD-M does not enter the polyacrylamide gel due to its basic pI (calculated pI=6.7) and does not enter the gel, but its (GGGA)$_3$ complex is sufficiently stable and has enough negative charge to enter the gel and to be observed as a discrete band (Fig. 6b and e). In contrast, the acidic SUD-C (calculated pI=5.0) does enter the gel, as shown by the protein staining in Fig. 6b, but we do not observe significant RNA staining at the corresponding position (Fig. 6g) and thus conclude that SUD-C does not bind to (GGGA)$_3$ under the assay conditions used. From Fig. 6a and g, it is also seen that, in contrast to SUD-M (Fig. 6c and previous work), SUD-MC and SUD-C do not bind to (ACUG)$_5$ under these assay conditions. Finally, both SUD-M and SUD-MC bind to a mixture of random 20-base RNA sequences, while there was no evidence of binding to a mixture of random 20-base DNA sequences (Fig. 6c, e, and g).

Binding to purine-containing RNAs was further investigated with six 10-base oligonucleotides containing variable combinations of G and A (Fig. 7). SUD-M was found to bind to all six of these sequences and also to the octamer GGGAGGGGA (Fig. 7a, c, and e), showing that neither the number of consecutive guanosines nor their positions in the sequence had significant effects on binding. In contrast, SUD-MC bound only to GGGAGGGAG (Fig. 7d and f). The different affinities of SUD-MC for GGGAGGGA (low affinity), GGGAGGGAG (high affinity), and SUD-M were reported to form a dimer in the single crystals used for X-ray structure determination, with a disulfide bond joining the SUD-N and SUD-M domains within each subunit, in addition to the backbone link between these domains. In contrast, we found that SUD-NM is monomeric in solution. The monomeric state is clearly apparent from the NMR data, as the 2D $^{15}$N,$^1$H HSQC spectrum (Fig. 4, red peaks) would be inconsistent with a molecular size of 60 kDa. Independently, size-exclusion chromatography showed that the protein eluted at an apparent molecular mass of 35 kDa, which fits closely with its monomeric size (Fig. 5b). Based on the similarity of the correlation peaks (Fig. 4), we conclude that the globular structures of SUD-N and SUD-M are conserved in the SUD-NM construct. Furthermore, SUD-N and SUD-M are flexibly connected with each other, as evidenced by the close similarity of the peak positions and peak shapes of the isolated domains and the SUD-NM construct (Fig. 4). Since the resonances of SUD-N have been identified as a group, but not individually assigned, the peaks of the $^{15}$N,$^1$H moieties of residues 525–526, which form the interdomain linker, had to be assigned by exclusion. There is one $^{15}$N,$^1$H correlation peak (identified in Fig. 4b and d) that has no counterpart in the spectra of the individual domains and was therefore tentatively assigned to the linker peptide segment. This is one of about 20 peaks in SUD-NM that exhibit $^{15}$N,$^1$H NOE values smaller than 0.6 (Fig. S1), thus indicating that there is increased mobility in parts of SUD-N and in the linker peptide when compared to the bulk of either of the two globular domains.

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(GGGA)$_5$ (low affinity) observed in buffer containing potassium chloride (Fig. 7f) further suggests that the presence of 3′-terminal G residues is an important determinant of SUD-MC binding. Two oligonucleotides, GGGAGGGAGG and (GGGA)$_5$, were tested both in saline buffer (Fig. 6a–f) and in KCl buffer (Fig. 7e and f), whereby the binding of both SUD-M and SUD-MC to (GGGA)$_5$ was found to be weaker in KCl buffer. Binding of G-rich sequences to SUD-M and SUD-MC was also inferred by smearing of the bands.
indicating that there was a range of RNA electrophoretic mobilities rather than a single complex with a unique mobility; we concluded that this probably manifests reversible dissociation of the complexes during electrophoresis.

NMR spectra of SUD-MC in the presence and in the absence of the single-stranded RNA (ssRNA) A10 (Fig. 8a and b) revealed highly specific changes in a small number of peaks, whereas the positions of the other peaks were unchanged. The magnitudes of the chemical shift changes ($\Delta \delta$) for each residue are plotted in Fig. 8c. The molecular surface area of SUD-M that is affected by A10 binding to SUD-MC was found to localize in a positively charged surface cavity (Fig. 9b). In contrast, the molecular surface of SUD-C that is affected by A10 binding to SUD-MC (Fig. 8a–c) shows an excess of negative charge (Fig. 9c and d).

NMR chemical shift perturbation measurements revealed that the isolated SUD-C also binds to A10 and, additionally, to G10 (Fig. 8d). Both homooligonucleotides caused similar patterns of chemical shift perturbations, as described in detail in the Discussion. Much smaller perturbations were induced by U10 (Fig. 8d). SUD-C thus shows similarity of RNA binding to SUD-M in the sense that stronger binding is observed for RNA sequences containing purine bases. No binding was apparent in EMSAs at RNA/protein ratios between 0.5:1 and 2.25:1 (data not shown), which is likely due to the fact that RNA binding to the
isolated SUD-C is weaker than RNA binding to SUD-MC; a 5:1 excess of A₁₀ over SUD-C was required to induce chemical shift perturbations comparable to those observed for SUD-MC at a 1:1 ratio.

Discussion

SUD-C completes the structural coverage of nsp3(1–1203)

With the SUD-C structure determination, there is now continuous structural coverage for the nsp3 polypeptide segment of residues 1–1203, which comprises 63% of the protein. This includes high-resolution structures of all globular domains and characterization of flexibly disordered polypeptide segments linking these domains (Fig. 10). The data presented in Figs. 2 and 4 provide additional information on the plasticity in solution of two two-domain constructs (see the following section, which extends previous NMR characterizations of flexibly extended interdomain regions of nsp3 (Fig. 10) in constructs where they were linked to a single globular domain.

With the exception of nsp3e, which is an RNA-binding domain and putative nucleic acid chaperone, all nsp3 domains in Fig. 10 adopt known protein folds, although some of the domains have very low levels of sequence identity to other proteins. This is also the case for the new domain structure presented in this work, SUD-C, which has a fold that is named after...
the N-terminal domain of CyaY, a member of the frataxin family of metal-binding proteins involved in iron homeostasis.26,27 Despite the structural similarity, there is only a 10% sequence identity between SUD-C and CyaY, including that only one of the eight residues believed to be involved in iron binding by CyaY is conserved in SUD-C. This makes it unlikely that SUD-C performs the same function. A search of the

Fig. 9. Visualization of the RNA-binding protein surface areas implicated by the NMR chemical shift perturbation experiments of Fig. 8. (a) Perturbations induced on the surface of SUD-M by the addition of 1 Eq of A10 to SUD-MC. The residues with Δδ ≥ 0.03 (data plotted in green in Fig. 8c) are shown in green and labeled. (b) Electrostatic potential surface of SUD-M. The molecule is shown in the same orientation as in (a). Red, negatively charged areas; blue, positively charged areas; white, neutral areas. Selected surface residues are labeled. (c) Perturbations induced on the surface of SUD-C by the addition of 1 Eq of A10 to SUD-MC. The residues with Δδ ≥ 0.03 (data plotted in green in Fig. 8c) are shown in green and labeled. (d) Electrostatic potential surface of SUD-C. The molecule is shown in the same orientation as in (c), with the same color scheme as in (b). (e) Perturbations induced on the surface of SUD-C by the addition of a 5-fold excess of A10 to SUD-C. The residues with Δδ ≥ 0.03 (data plotted in magenta in Fig. 8d) are shown in magenta and labeled. In (c) to (e), the orientation of SUD-C is related to that of Fig. 1 by a rotation of approximately 90° about the horizontal axis in the projection plane.

Fig. 10. Globular domains and nonglobular linker peptide segments formed by residues 1–1203 of nsp3. The globular domains are shown as ribbon presentations, flexibly disordered linker segments characterized by NMR spectroscopy are shown as blue lines, and disordered segments implicated by the absence of X-ray diffractions in crystallographic studies are represented by green lines. The black line at the top indicates the initial domain annotation based on bioinformatics and phylogenetic analyses.15 Abbreviations for the common names of the globular domains are shown below the structures: UB1, first ubiquitin-like domain; AC, acidic domain; ADRP, ADP-ribose-1″-phosphatase; SUD-N, N-terminal region of SUD; SUD-M, middle region of SUD; SUD-C, C-terminal region of SUD; UB2, second ubiquitin-like domain; PLpro, papain-like protease; NAB, nucleic-acid-binding domain.
Protein Data Bank (PDB) with the program Dali\textsuperscript{28,29} identified several additional structural homologues with Z-scores of $\leq 4.4$ (Table S1). These include anthrax edema factor,\textsuperscript{30} which has adenylate cyclase activity, and AcsD, which is an adenylating enzyme of which SUD-C resembles the N-terminal dimerization domain.\textsuperscript{31} Similar to the comparison with CyaY, SUD-C lacks any apparent conservation of the active-site residues of these proteins; therefore, the observation that two different adenylate-binding enzymes have SUD-C-like folds does not appear to provide a new lead toward the as yet unknown SUD-C function. Among the structural homologues of SUD-C (Table S1), there are also small protein domains that perform regulatory functions for DNA-modifying enzymes. Examples are the N-terminal domain of DNA primase, which is a zinc-binding domain that is responsible for DNA binding and also regulates the activity of the catalytic domain of the enzyme,\textsuperscript{32} and responsible for DNA binding and also regulates the primase, which is a zinc-binding domain that is responsible for DNA binding and also regulates the activity of the catalytic domain of the enzyme,\textsuperscript{32} and double-wing motif DNA-binding proteins such as YjbR and MotA.\textsuperscript{33,34} Although the key DNA-binding residues are in no case conserved in SUD-C, it is interesting that other proteins with SUD-C-like folds bind nucleic acids, especially when considering that nucleic acids were observed to copurify with recombinant SUD-C expressed in \textit{Escherichia coli} (see Materials and Methods).

In summary, data bank searches for structural homologues of SUD-C show that metal binding, adenylate binding, and nucleic acid binding may be key functions associated with its fold. Due to lack of active-site conservation, however, the present homology search alone did not yield straightforward leads to possible SUD-C functions, although its results are of considerable interest in the context of the experiments in Figs. 6–8 (see the text below).

**Solution behavior of the SUD inferred from investigations of SUD-NM and SUD-MC**

Previously reported NMR solution and X-ray crystal structures\textsuperscript{16,17} and the data reported in this work show that the SUD forms three globular domains. The NMR structure determination of SUD-MC in solution further reveals that the short linker peptide segment of residues 652–654 between SUD-M and SUD-C allows nearly unrestricted freedom of movement of the two domains relative to each other (Fig. 2), with no evidence for a preferred interdomain orientation detected by the methods used. Similarly, solution studies of the construct SUD-NM, for which crystal structure determination has recently been presented,\textsuperscript{17} indicate extensive freedom of motion of SUD-N and SUD-M relative to each other. SUD-NM is monomeric in solution (Fig. 5), contrasting with the observation of a dimeric form in the crystal structure.\textsuperscript{17} The observed flexible linkage of the two domains further indicates that an interdomain disulfide bond that has been reported to link SUD-N and SUD-M in the crystal structure is not present in the reducing solvent milieu used for the NMR studies. Since the eukaryotic cell cytoplasm, where the SUD is expected to be located,\textsuperscript{10,25} also presents a reducing milieu,\textsuperscript{35} it would appear that the monomeric sulfhydryl form observed in solution, with flexibly linked SUD-N and SUD-M domains, might be closely related to the physiologically relevant form of the protein. There are no significant chemical shift perturbations that would indicate tight transient or static contacts between the two domains, and the SUD-NM \textsuperscript{15}N–\textsuperscript{1}H correlation NMR signals have linewidths narrower than one expects for a compact globular domain with a molecular mass of 29.6 kDa. In view of these observations, the following discussion on nucleic acid binding and on the possible physiological roles of the SUD will be based on an SUD-NM molecular model containing three flexibly linked globular domains.

**RNA binding properties of the SUD**

Previous NMR studies\textsuperscript{16} had shown that SUD-M binds $A_{10}$ with higher affinity than $U_{10}$, and EMSAs further revealed weak binding to $A_{15}$ (ACUG)$_5$, and the reverse complement of the SARS-CoV transcription regulatory sequence (TRS), TRS(–). No affinity was observed by EMSA either for TRS(+) or for an RNA sequence ‘GAUA’, which had previously been found to be recognized by the N-terminal domain of nsp3, nsp3a.\textsuperscript{14} The binding studies of Figs. 6–8 now add the information that SUD-C also binds preferentially to purine-containing RNAs and further provide a basis for a comparison of nucleic acid binding by SUD-M and SUD-C in isolated form and in SUD-MC.

As a first step toward gaining insight into the mode of nucleic acid binding by SUD-C, we evaluated the locations of residues that experience chemical shift perturbations upon addition of purine-containing ssRNAs (Fig. 8), in context with electrostatic potential distribution on the molecular surface (Fig. 9d). This shows that the binding surface of SUD-C does not have the characteristics of a typical RNA binding site, since besides two positively charged residues, of which the histidine in position 658 has the largest perturbation (Fig. 8d), there are also several negatively charged amino acid side chains (Fig. 9e). In SUD-MC, the $\Delta \delta$ values of SUD-C residues at a 1:1 $A_{10}$/protein ratio (Fig. 8c) are significantly increased relative to those of the isolated SUD-C. The presence of SUD-M in SUD-MC thus stabilizes SUD-C–RNA interactions, indicating that the coordinated binding of the linked domains might reduce the repulsion of RNA due to the negative surface charge of SUD-C (Fig. 9d). A putative binding site on SUD-C in SUD-MC is indicated by the fact that the largest $\Delta \delta$ values were seen for H658, H695, T696, E702, G707, and V709 (Fig. 9c). Except for H658 in helix $\alpha_1$, these residues are all in strands $\beta_5$, $\beta_6$, and $\beta_7$ of the $\beta$-sheet (Fig. 1), or in loops joining these strands, and the set of residues with large $\Delta \delta$ values is somewhat smaller but nonetheless clearly similar to that seen for RNA binding to the isolated SUD-C (Fig. 9e).

Compared to SUD-MC, SUD-M binds to a larger range of purine-containing RNA sequences, including
that it binds to (ACUG)₅ and interacts more strongly with a mixture of random RNA 20-mers than does SUD-MC. It thus appears that coordinated action of the two domains in SUD-MC results in greater specificity of RNA binding by SUD-MC than by SUD-M, and may thus lead to binding interactions that are unique to SUD-MC. For example, SUD-MC binds to SARS-CoV TRS(+) (5′-CUAAACGAAC-3′) and to its reverse complement in the anti-genome, TRS(−) (5′-GUCCGUUUAAG-3′) (Fig. 6b). NMR chemical shift perturbations (data not shown) were larger in the presence of TRS(+) than in the presence of TRS(−), and localized to the same site of SUD-M in SUD-MC that was previously identified for A₁₀ binding (Fig. 9a). However, in contrast to A₁₀ binding (Fig. 8a–c), there was minimal contact of SUD-C with TRS. Since the isolated SUD-M did not bind either to TRS(+) or to TRS(−), this shows that the SUD-C in SUD-MC modifies binding without being directly involved in the ligand interaction. Since TRS(+) and TRS(−) contain six and four purine residues, respectively, the aforementioned larger shift perturbations by TRS(+) again seem to reflect the preference of SUD for purine binding. Poly-A is found at the 3′-end of the viral RNA genome, and these interactions could thus indicate a role for SUD-MC in viral genome or subgenomic RNA transcription.

We further used NMR spectroscopy to explore the binding of SUD-M and SUD-MC to other purine RNAs, which yielded results that could only tentatively be interpreted due to line broadening and/or precipitation. Addition of [GGA]₄ resulted in broadening of SUD-M resonance linewidths in SUD-MC with increasing RNA/protein ratio. Thus, at a [GGA]₄/protein ratio of 0.5:1, most peaks of SUD-M were broadened beyond detection at the contour levels shown in Fig. S2, whereas those of SUD-C were nearly unaffected. For GGGAGGGAGG binding with SUD-MC, a similar pattern of line broadening (Fig. 6b) was observed at a 1:1 RNA/protein ratio, and both [GGA]₄ and GGGAGGGAGG caused precipitation when added to SUD-M. Eventually, chemical shift perturbations in SUD-MC and SUD-M were measurable only for [GGA]₄ at a 0.2:1 RNA/protein ratio, where the binding of [GGA]₄ involves the same surface residues (Fig. S2a–d) as previously observed for A₁₀ binding (Fig. 9a). In all of these experiments, SUD-C NMR signals showed neither sizeable chemical shift perturbations nor line broadening (Fig. S2a–c and e). We speculate that this indicates the formation of high-molecular-mass aggregates characterized by binding of SUD-M to RNA, with the NMR signals of the flexibly linked SUD-C only minimally affected by this process. These aggregates would involve multiple SUD-MC proteins binding via their SUD-M to the same RNA. Similar to conclusions from the data in Figs. 6–8, in this interpretation, SUD-C would again appear to regulate the specificity of SUD-MC for RNA binding without direct contacts with the ligand, which may be physiologically important in directing the protein only to certain viral or host cell RNA sequences. Alternatively, chemical exchange between free SUD-MC and a 1:1 complex with RNA in the intermediate rate regime on the chemical shift timescale could also lead to line broadening; however, in this situation, one would expect the line broadening to be limited to binding site residues rather than affecting the entire SUD-M.

**Comparison with RNA binding by other macrodomains**

ADRP-type macrodomains have been shown to have poly-ADP-ribose binding activity, with so far unknown binding mechanisms.⁵³ Human macrodomains have also been found to have different specificities for monomeric and poly-ADP-ribose and for RNA. For example, the human macrodomain ganglioside-induced differentiation-associated protein 2 (GDAP2), which is implicated in neuronal differentiation and expressed during brain development, does not bind to poly-ADP-ribose or hydrolyze ADPR-1′-phosphate, but does recognize poly-A RNA.³⁶ Since we and others have shown that neither SUD-M¹⁶ nor SUD-NM⁷ binds to monomeric ADP-ribose, the substrate specificity of SUD appears to be similar to that of GDAP2. No sequence similarity between GDAP2 and SUD-M was detected by BLAST, but a pairwise alignment with FFAS²⁷ identified about a 15% sequence identity. While cellular and viral macrodomains occur in combination with a variety of C-terminal domains, SUD-M appears to be a first example of a macrodomain occurring together with a C-terminal domain that has similar RNA binding specificity and regulates the RNA binding behavior of the macrodomain. Since the fifth globular domain of nsp3, nsp5e, which is separated from SUD-MC in the nsp3 sequence by a papain-like protease (Fig. 10), also binds to G-containing and A-containing RNAs,²⁵ there is also the possibility of a longer-range concerted action with SUD-MC in RNA recognition. Physiologically, this increased specificity is likely to be important in directing nsp3 activity to the desired RNA sequences; binding to a broader range of RNA sequences, as observed for the isolated SUD-M, might affect host cell viability and thus reduce the ability of the virus to replicate.

**Implications for possible functions of the SUD**

Several lines of evidence,¹¹,¹⁴,¹⁶,¹⁷,²⁵,³⁸ have shown links between nsp3 and RNA, and here we characterize SUD-MC as a purine-binding protein. SUD-M was previously shown to bind to poly-A RNA, which might relate to a possible function in initiating negative strand synthesis by binding to poly-A tails of the viral genome.¹⁴ It has also been proposed that due to its affinity for G-quadruplexes, SUD-NM could bind to G-quadruplex-containing host cell mRNAs encoding proteins involved in cellular signaling pathways, which could lead to disruption of the host cell environment and could favor viral replication.¹⁷ When we tested (GGGA)₅ binding to
SUD-MC in KCl buffer, which favors the formation of G-quadruplexes, the binding was much weaker than that in saline buffer (Fig. 7), which would argue against a G-quadruplex being the actual molecular structure recognized by SUD-MC. We note, however, that several of the G-rich RNA sequences displayed multiple bands on native gels (Figs. 6 and 7), which may be due to the presence of different secondary structures, suggesting that variable types of folded RNA structure could indeed be recognized or induced by the interaction with the protein. In the following, we investigate the occurrence of alternative oligopurine sequences in SARS-CoV genomes that could function as additional SUD binding sites.

In the SARS-CoV Tor2 strain, there are three G\(_6\) stretches and three G\(_3\) stretches, but none of these is conserved in all SARS-CoV strains. Of 25 G\(_4\) sequences (on both (+) and (−) strands), eight are conserved in all known SARS-CoV strains; seven of these are in protein-coding regions, and one is in the 3′-untranslated region. Of these eight conserved G\(_4\) sequences, all but one are unique to SARS-CoV, and if these sequences were indeed physiologically relevant substrates for SUD-MC, this would be consistent with the finding that SUD is unique to SARS-CoV. An additional search for the GGGAGG-GAGG sequence in the SARS-CoV Tor2 genome did not result in an exact hit, but we found three GAGG sequence in the SARS-CoV Tor2 genome

Within nsp3 and possibly in concerted action with other nsp3 domains (see the text above), the SUD may have multiple roles, perhaps interacting with viral RNA to initiate transcription and interfering with mRNAs of the host cell. There is a precedent for a remarkable variety of biological functions carried out by single viral proteins, where nsp1, which is a key virulence factor of the influenza A virus, provides a particularly striking illustration.

### Materials and Methods

#### Preparation of SUD-N, SUD-NM, SUD-MC, and SUD-C

The DNA sequence encoding nsp3 residues 1–723 obtained as a synthetic gene from DNA 2.0 (Menlo Park, CA) was codon optimized for expression in E. coli. All four constructs (Fig. 55) were cloned from this starting sequence into pET-28b. SUD-N and SUD-NM consisting of nsp3 residues 387–524 and 387–651, respectively, were cloned with a tobacco etch virus (TEV) protease cleavage site, and SUD-MC consisting of residues 527–720 was cloned with a thrombin cleavage site after an N-terminal 6× His tag. For SUD-C, the highest expression levels were obtained for a construct with a long linker to the 6× His tag, containing both a thrombin and a TEV protease cleavage site.

The four constructs were expressed in E. coli strains BL21(DE3)-RIL (SUD-N and SUD-NM), Rosetta(DE3) (SUD-MC), and BL21(DE3) (SUD-C). To produce uniformly \(^{15}\)N-labeled or \(^{13}\)C,\(^{15}\)N-labeled protein, we grew cultures in M9 minimal medium containing 1 g/L \(^{15}\)NH\(_4\)Cl as the sole nitrogen source and 4 g/L of either unlabeled glucose or \(^{13}\)C\(_6\)-glucose as the sole carbon source. For SUD-N, SUD-NM, and SUD-MC, the cell cultures were grown at 37 °C with shaking to an optical density at 600 nm of 0.8. The temperature was then lowered to 18 °C, expression was induced with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside, and the cultures were grown for a further 18 h. For SUD-C, the cultures were grown for 28 h after induction with 1 mM isopropyl-\(\beta\)-D-thiogalactopyranoside at 18 °C.

The proteins were extracted from frozen cell pellets by sonication in lysis buffer (Table 2). After centrifugation to remove cell debris, the supernatants were applied to a 5-mL HisTrap Crude column (GE) equilibrated with buffer A.

<p>| Table 2. Buffers used in the purification of the four proteins |
|----------------|----------------|----------------|----------------|</p>
<table>
<thead>
<tr>
<th>Lysis(^a)</th>
<th>Buffer A</th>
<th>Cleavage</th>
<th>Final NMR sample</th>
</tr>
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<tbody>
<tr>
<td><strong>SUD-N</strong></td>
<td>25 mM Na(_{2})PO(_4) (pH 8.0), 200 mM NaCl, 2 mM DTT, 1% TX-100</td>
<td>25 mM Na(_{2})PO(_4) (pH 8.0), 200 mM NaCl, 2 mM DTT, 20 mM imidazole</td>
<td>25 mM Na(_{2})PO(_4) (pH 8.0), 200 mM NaCl, 2 mM DTT, 20 mM imidazole, 2 mM DTT</td>
</tr>
<tr>
<td><strong>SUD-NM</strong></td>
<td>25 mM Na(_{2})PO(_4) (pH 6.6), 200 mM NaCl, 2 mM DTT, 1% TX-100</td>
<td>25 mM Na(_{2})PO(_4) (pH 6.6), 200 mM NaCl, 2 mM DTT, 20 mM imidazole</td>
<td>25 mM Na(_{2})PO(_4) (pH 6.6), 200 mM NaCl, 20 mM imidazole, 2 mM DTT</td>
</tr>
<tr>
<td><strong>SUD-MC</strong></td>
<td>25 mM Na(_{2})PO(_4) (pH 8.0), 200 mM NaCl, 2 mM DTT, 1% TX-100</td>
<td>25 mM Na(_{2})PO(_4) (pH 8.0), 200 mM NaCl, 20 mM imidazole</td>
<td>50 mM Tris–HCl (pH 8.0), 200 mM NaCl, 5 mM CaCl(_2), 20 mM imidazole</td>
</tr>
<tr>
<td><strong>SUD-C</strong></td>
<td>20 mM Na(_{2})PO(_4) (pH 7.3), 500 mM NaCl, 10 mM imidazole</td>
<td>20 mM Na(_{2})PO(_4) (pH 7.3), 500 mM NaCl, 10 mM imidazole</td>
<td>20 mM Na(_{2})PO(_4) (pH 7.0), 50 mM NaCl</td>
</tr>
</tbody>
</table>

\(^a\) All lysis buffers also contained Complete EDTA-Free protease inhibitors (Roche).
The backbone assignment of SUD-C was carried out based on four-dimensional (4D) automated projection spectroscopy (APSY) HACANH, five-dimensional (5D) APSY-CBCACONH, and 5D APSY-HACACONH experiments, which were recorded in a total measurement time of 16.5 h on a Bruker Avance 600 spectrometer with a TXI z-gradient probe. The 5D APSY-HACACONH experiments were measured using experiments based on transverse relaxation optimized spectroscopy (TROSY) z-gradient probe. APSY data were analyzed with the software GAPRO, and GAPRO peak lists were used as input for automated backbone assignment with the program MATCH. The peak lists produced by MATCH were checked and completed interactively. A three-dimensional (3D) 15N-resolved 1H, 1H NOESY spectrum and two 3D 13C-resolved 1H, 1H NOESY spectra with the carrier frequency centered on the aliphatic and aromatic carbon regions, respectively, were recorded with a mixing time of 150 ms on a Bruker Avance 800 spectrometer with a TXI z-gradient probe. Automated si-chain assignment was performed with the program ASCAN, using backbone chemical shifts and the three NOESY data sets as input. During the input preparation, the backbone chemical shifts in the MATCH output were adjusted for optimal fit with the NOESY spectra, with adjustments of 0.01 ppm for 1H, 0.02 ppm for all other 1H, 0.09 ppm for 13N, 0.1 ppm for Gly 15C, and 0.3 ppm for all other 13C positions and all 15N positions.

The chemical shift lists from the ASCAN resonance assignments were used for an initial structure calculation with the stand-alone program suite ATNOS/CANDID 2.2 and the torsion angle molecular dynamics program CYANA 3.0. The ASCAN chemical shift lists were then interactively corrected and extended for the final structure calculation. Backbone ϕ and ψ dihedral angle constraints derived from the 13Cα chemical shifts were used as supplementary data in the input for the structure calculation. The 20 conformers with the lowest residual CYANA target function values obtained from the seventh ATNOS/CANDID/CYANA cycle were energy minimized in a water shell with the program OMP@, using the AMBER force field. The program MOLMOL was used to analyze the ensemble of 20 energy-minimized conformers. The stereochemical quality of the models was analyzed using the PDB validation server.

SUD-MC structure determination was performed with the same strategy and the same NMR equipment, using the following experimental data: 4D APSY-HACANH, 4D APSY-HNC(O)CA, 5D APSY-CBCACONH, and 5D APSY-HACACONH experiments recorded in a total of 120 h. A 3D 15N-resolved 1H, 1H NOESY spectrum and two 3D 13C-resolved 1H, 1H NOESY spectra were recorded with a mixing time of 60 ms. The adjustments of backbone chemical shifts to fit the NOESY data sets were 0.016 ppm for 1H, 0.01 ppm for all other 1H, 0.12 ppm for 13N, and 0.31 ppm for all 15C and 13C. The software versions employed were UNIO 1.0.4 (which includes MATCH, ATNOS, ATNOS, and CANDID) and CYANA 3.0. In the seventh ATNOS/CANDID/CYANA cycle, 20 conformers were generated and subjected to energy minimization in a water shell with OMP@ and the 20 best energy-minimized conformers were selected to represent the solution structure.

NMR experiments with SUD-N and SUD-NM

One-dimensional 1H NMR and 2D 15N, 1H HSQC spectra of the uniformly 15N-labeled SUD-N (0.8 mM) and SUD-NM (1.0 mM) were recorded on a Bruker DRX 700 spectrometer with a 1.7-mm TXI z-gradient probe. 1H NOE experiments were measured using experiments based on transverse relaxation optimized spectroscopy, with a saturation period of 3.0 s and a total interscan delay of 5.0 s. The peaks in the SUD-NM 2D 15N, 1H HSQC spectrum arising from SUD-M were identified by comparison with the previously obtained assignments for the single-domain construct SUD-M.

The residual peaks in the 2D 15N, 1H HSQC spectrum of SUD-NM were assigned as a group to SUD-N. One peak

‡ http://deposit.pdb.org/validate
was tentatively assigned to the interdomain linker based on the lack of any apparent corresponding peak in either the SUD-N spectrum or the SUD-M spectrum.

**NMR studies of RNA binding**

The interactions of SUD-C and SUD-MC with RNA were evaluated by comparing the 2D $^{15}$N,$^1$H HSQC spectra in the presence and in the absence of ssRNA. For the experiments with SUD-C, ssRNA solutions in water were added to an empty tube and frozen, and water was removed by speed vacuum. Ten microliters of 0.68 mM uniformly $^{15}$N-labeled protein were added, and the RNA was gently resuspended. The $^{15}$N,$^1$H HSQC spectra were recorded on a Bruker DRX 700 spectrometer with a 1-mm TXI z-gradient probe. The combined changes in $^1$H and $^{15}$N chemical shifts in the presence versus the absence of the RNA were calculated as $\Delta \delta = \left[ \Delta \delta(^{1}H) + \Delta \delta(^{15}N)/5 \right]^{1/2}$.

For RNA binding experiments with SUD-M and SUD-MC, a similar procedure was applied, except that for most experiments, the ssRNA solutions in buffer were added directly to the protein solution. Exceptions are the experiments with a 5:1 (GGA)$_5$/SUD-MC ratio and with equimolar mixtures of both proteins and (GGGA)$_5$ or GGGAGGGAGG, where the procedure described for SUD-C was used. Uniformly $^{15}$N-labeled SUD-MC at 0.38 mM concentration and uniformly $^{15}$N,$^{13}$C-labeled SUD-M at 0.5 mM concentration, both in 25 mM phosphate buffer (pH 6.8) containing 150 mM NaCl, were used for these experiments. For these proteins, 40-μl samples were measured on a Bruker DRX 700 spectrometer with a 1.7-mm TXI z-gradient probe.

**Electrophoretic mobility shift assays**

Purified SUD-M, SUD-MC, or SUD-C was incubated with ssRNA or single-stranded DNA oligomers either in “saline buffer” containing 150 mM NaCl, 7% glycerol, 4 mM MgCl$_2$, and 50 mM NaPi (pH 6.5), or in “KCl buffer” containing 100 mM KCl, 50 mM NaCl, and 50 mM NaPi (pH 6.5). The protein–nucleic acid mixtures were incubated at 37 °C for 60–90 min and analyzed by native electrophoresis on precast 6% acrylamide DNA retardation gels (Invitrogen). Nucleic acid bands were detected by SYBR-Gold (Invitrogen) staining and photographed using a UV light source equipped with a digital camera. SYBR-Gold was rinsed out, and the protein was subsequently detected by SYPRO-Ruby staining (Invitrogen).

**Data bank depositions**

The chemical shifts of SUD-C and SUD-MC were tentatively assigned to the interdomain linker based on the lack of any apparent corresponding peak in either the SUD-N spectrum or the SUD-M spectrum.

**Acknowledgements**

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**Supplementary Data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.05.027

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§ http://www.bmrb.wisc.edu
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