Single-stranded telomere-binding protein employs a dual rheostat for binding affinity and specificity that drives function

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The proper management of ssDNA in the cell is required for numerous aspects of chromosome biology. In all kingdoms of life, ssDNA is formed transiently during the execution of many essential cellular processes including transcription, DNA replication, recombination, and repair. To coordinate these numerous activities, a diverse array of proteins has evolved to bind ssDNA, to facilitate normal events such as DNA replication, or to signal the appearance of inappropriate ssDNA and initiate repair (1).

Several of these ssDNA-binding proteins function in genome-wide maintenance (1, 2). Widely studied examples include the bacterial single-strand–binding protein (SSB) and its functional equivalent in eukaryotes, replication protein A (RPA) (3–5). SSB and RPA are both essential for DNA replication, binding nascent ssDNA that is generated when duplex DNA is unwound and thereby preventing reannealing and/or the formation of secondary structures that would impede progression of the replisome. Both proteins are also central to the cellular response to DNA lesions. Although RPA and SSB exhibit no sequence homology, they each employ an array of OB-folds for contacting ssDNA. Detailed analysis of RPA has revealed that it utilizes these OB-folds to contact ssDNA in distinct modes, engaging differing lengths of ssDNA with different subunits, presumably to orchestrate higher-order manipulations (6–8). Thus, to interact consistently throughout the genome, RPA also needs to bind ssDNA indiscriminately. Commensurate with this expectation, RPA displays little obvious sequence preference in vitro, binding ssDNA tenaciously with single-digit nanomolar affinities (6, 7).

In contrast to the genome-wide and the apparently sequence-nonspecific role performed by the canonical RPA complex (2), proteins that interact with ssDNA overhangs at telomeres exhibit sequence specificity tuned to the G-rich telomeric repeats (9–11). These telomere-dedicated proteins also show exceptional affinities for their ssDNA ligands, ranging from the tight nanomolar binding by human Pot1 to single-digit picomolar binding by the Saccharomyces cerevisiae Cdc13 protein (9, 11, 12). Remarkably, the Cdc13 protein performs its telomere-dedicated role as a subunit of a heterotrimERIC complex with a domain architecture that closely parallels that of RPA (13). In both the canonical and telomere-dedicated RPA, the large subunit is constitutively associated with two smaller proteins, Stn1/Ten1 with Cdc13 and Rpa32/Rpa14 with RPA70. In the CST complex, high affinity for ssDNA is conferred by the large subunit, whereas in RPA high

Significance

Proteins that bind nucleic acids are frequently categorized as being either specific or nonspecific, with interfaces to match that activity. In this study, we have found that a telomere-binding protein exhibits a degree of specificity for ssDNA that is finely tuned for its function, which includes specificity for G-rich sequences with some tolerance for substitution. Mutations of the protein that dramatically impact its affinity for single-stranded telomeric DNA are lethal, as expected; however, mutations that alter specificity also impact biological function. Unexpectedly, we found mutations that make the protein more specific are also deleterious, suggesting that specificity and nonspecificity in nucleic acid recognition may be achieved through more nuanced mechanisms than currently recognized.


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affinity is achieved through multivalency (2, 4, 8, 12–15). However, ssDNA binding by the telomere-dedicated RPA complex (t-RPA) is notably distinct from RPA, suggesting that these structurally similar domains have taken on distinct biochemical roles. Unlike RPA70, which uses two OB-fold domains for its core recognition of ssDNA, Cdc13 employs a single OB-fold augmented by an unusually long β2–3 loop (Fig. 1) (16) to contact DNA with exceptionally tight picomolar affinity. Furthermore, Cdc13 binds ssDNA with exquisite specificity for G-rich sequences (17, 18), which it achieves through recognition of a GxGT motif embedded in a larger oligonucleotide (10, 12). Nevertheless, Cdc13 presumably needs to show sequence flexibility to accommodate the heterogeneity of yeast telomeres (19), although the mechanism by which Cdc13 achieves this flexibility has not previously been elucidated.

The sequence specificity and affinity displayed by Cdc13 provide a unique system for investigating how these two biochemical properties contribute to function in vivo. To do so, this study examined an extensive panel of mutations across the DNA-binding interface for their effects on both binding affinity and specificity and subsequently determined how perturbations in either property affected Cdc13 function in vivo. Not surprisingly, substantial reductions in Cdc13-binding affinity were lethal in vivo, whereas less severe declines in affinity were better tolerated. Unexpectedly, this approach also identified a second category of mutations that had little effect on affinity but large effects on specificity in vitro. These specificity mutations reduced the ability of Cdc13 to tolerate variations in telomere sequence, which substantially impaired Cdc13 function in vivo. Moreover, the magnitude of the in vivo defect closely correlated with the extent to which specificity was altered, thereby demonstrating that both affinity and sequence tolerance contribute to biological function. Thus, by conducting a systematic analysis of the Cdc13 DNA-binding domain (DBD) interface, we have uncovered a finely tuned binding rheostat of specificity and affinity that confers optimal biological function.

Results

Systematic Mutagenesis of the DNA-Binding Interface of Cdc13 Identifies a 35-Fold Span in Affinity. To address how the biochemical features of Cdc13 allow it to perform its biological roles, we introduced a set of eight alanine mutations into the DBD across the binding interface (16, 20), with an emphasis on the aromatic residues that play key roles in affinity and specificity, and measured the impact of these changes on binding characteristics (Fig. 1A). The change in binding affinity to the minimal Tel11 substrate (GTGTTGGGGTGG) exhibited by these mutant proteins was measured at the physiological salt conditions identified previously using an EMSA binding assay (SI Appendix, Fig. S1) (12). The DBD constructs exhibited a range of binding affinities, from slightly tighter than the very tight WT apparent K$_a$ of 2.1 pM to a reduced value of 71 pM (Fig. 1B and SI Appendix, Table S1). These defects in binding cannot be attributed to a change in protein structure or stability. Circular dichroic and NMR analysis suggest no alterations in secondary or tertiary structure (SI Appendix, Figs. S2 and S3). Furthermore, most of the mutations did not significantly alter the melting temperature of the protein, and the observed minor changes show no correlation with biochemical activity, presumably because they are all well above the temperature at which the binding and in vivo studies were conducted (SI Appendix, Fig. S2). Thus, the impact on the binding affinity to the Tel11 substrate exhibited by these mutant proteins spanned almost 35-fold, creating a set of proteins exhibiting a wide range, or rheostat, of binding affinities.

Large Defects in Binding Affinity Correlate with Substantial Impacts on in Vivo Viability. This range of binding affinities allowed us to ask whether the unusually tight affinity exhibited by Cdc13 was required and, indeed, what level of DNA binding was necessary, for function in vivo. To do so, the mutations described above were examined for their effects in vivo by integrating each mutation into the genome of a diploid strain of yeast in place of one copy of the WT $CDC13$. This panel of diploid strains was used to generate $cdc13$-DBD$^{-}$ haploid strains, which revealed a gradient of viability (Fig. 2). Changes in viability were not explained by changes in protein levels (SI Appendix, Fig. S4).

Not unexpectedly, the $cdc13$-Y522A and $cdc13$-K622A mutant strains, which exhibited greatly reduced binding, were capable of only two to five cell divisions (Fig. 2A), consistent with the 15- to 34-fold reduction in binding affinity for the minimal Tel11 substrate associated with these two mutations (Fig. 1B). For both strains, this severe growth defect was partially suppressed by exo1$^{-}$Δ and rad9$^{-}$Δ mutations (Fig. 2A); this recapitulates the behavior of previously characterized $cdc13$ muta-
tions (21–23), arguing that defects in Cdc13 DNA binding behaved in a manner comparable to other loss-of-function mutations in $CDC13$. Notably, the growth of the $cdc13$-K622A mutant strain (with a 15-fold reduction in binding affinity) was reproducibly less impaired than the $cdc13$-Y522A strain (with a 34-fold reduction in vitro binding), providing a strong correlation between the in vitro biochemical properties of these two mutations and their in vivo phenotypes.

Moderate Defects in Binding Affinity only Partially Correlate with in Vivo Viability. Surprisingly, the correlation between in vitro and in vivo behavior did not extend to other mutations introduced into the DBD interface of Cdc13. For example, two mutations,
cdc13-F539A and cdc13-Y626A, with only modest declines in affinity for the Tel11 substrate, nevertheless exhibited pronounced growth defects. Both these haploid mutant strains gave rise to barely visible colonies (Fig. 2A and SI Appendix, Fig. S5A) which were accompanied by a high percentage of inviable individual cells for both strains, resulting in a long delay in forming visible colonies. Thus, despite having only a 3.5-fold effect on in vitro binding affinity, the cdc13-F539A and cdc13-Y626A mutant strains exhibited a severe degree of in vivo telomere dysfunction.

Table 1. Apparent $K_d$ values for WT and mutant Cdc13 DBD proteins to Tel11 variants

<table>
<thead>
<tr>
<th>Protein Tel 11, pM</th>
<th>H1*, pM</th>
<th>H3*, pM</th>
<th>V4*, pM</th>
<th>H9*, pM</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>2.1 ± 0.2</td>
<td>31 ± 5</td>
<td>85 ± 20</td>
<td>180 ± 40</td>
</tr>
<tr>
<td>Y556A</td>
<td>0.6 ± 0.1</td>
<td>300 ± 30</td>
<td>310 ± 50</td>
<td>570 ± 200</td>
</tr>
<tr>
<td>I578A</td>
<td>1.0 ± 0.2</td>
<td>420 ± 20</td>
<td>130 ± 20</td>
<td>280 ± 70</td>
</tr>
<tr>
<td>Y561A</td>
<td>1.6 ± 0.2</td>
<td>15 ± 30</td>
<td>430 ± 80</td>
<td>380 ± 30</td>
</tr>
<tr>
<td>Y565A</td>
<td>4.7 ± 0.8</td>
<td>330 ± 50</td>
<td>260 ± 20</td>
<td>370 ± 70</td>
</tr>
<tr>
<td>Y626A</td>
<td>7.2 ± 0.9</td>
<td>1,230 ± 60</td>
<td>2,500 ± 60</td>
<td>290 ± 70</td>
</tr>
<tr>
<td>F539A</td>
<td>7.4 ± 0.2</td>
<td>1,200 ± 100</td>
<td>2,600 ± 400</td>
<td>1,300 ± 100</td>
</tr>
<tr>
<td>K622A</td>
<td>31 ± 3</td>
<td>2,400 ± 400</td>
<td>1,200 ± 200</td>
<td>4,500 ± 1,000</td>
</tr>
<tr>
<td>Y532A</td>
<td>71 ± 16</td>
<td>430 ± 50</td>
<td>770 ± 200</td>
<td>6,000 ± 1,000</td>
</tr>
</tbody>
</table>

*H refers to an equimolar pool of A, C, and T; V refers an equimolar pool of G, C, and A.

Mutations That Increase Cdc13-Binding Affinity Show Growth Defects in Strains Sensitized to Telomere Dysfunction. Equally striking was the behavior of strains expressing mutant proteins that slightly increased the affinity for the Tel11 substrate relative to the affinity of the WT protein for Tel11 (Fig. 1B and SI Appendix, Table S1). In an otherwise WT yeast background, strains bearing mutations in Y556, I578, or Y626 exhibited a growth phenotype that was indistinguishable from that of a WT strain (Fig. 2A and SI Appendix, Fig. S5B). However, when cdc13-Y561A or cdc13-I578A mutations were introduced into a strain background that is impaired for an additional aspect of telomere homeostasis (a telomerase deficiency), these mutant proteins were incapable of conferring the same level of function as the WT Cdc13 protein. Immediately following the loss of telomerase, the growth of a telomerase-defective strain is initially indistinguishable from that of a telomerase-proficient strain, although a decline in viability eventually becomes evident with continued propagation (24). In contrast, a newly generated telomerase-defective strain that also bore either a cdc13-Y561A or a cdc13-I578A mutation exhibited an immediate decline in viability (Fig. 2B). Similarly, these same mutations also exhibited a pronounced synthetic growth defect when combined with a mutation in the Ku heterodimer: the cdc13-Y556A, yku80-Δ, cdc13-I578A, yku80-Δ, and cdc13-Y561A, yku80-Δ double-mutant strains were close to inviable (SI Appendix, Fig. S5C). The synthetic lethality due to these mutations in the DBD interface were not readily explained by their small increase in affinity for the Tel11 substrate.

Binding Specificity Is Profoundly Altered by Mutations in the DNA-Binding Interface. The above results strongly suggested that affinity was not the only important biochemical feature required for Cdc13 function in vivo. We therefore asked whether an additional biochemical property, binding specificity for telomeric substrates, was altered by these mutations. We have previously assessed Cdc13 specificity by measuring binding affinities for oligonucleotides with substitutions for the “pool” of the three other bases at specific positions within the minimal Tel11 oligonucleotide (10). This approach revealed a “specificity profile” defined by the relative loss of affinity when the identity of a base in the ligand is altered. The larger the loss in affinity for the pool relative to the cognate ligand, the more specifically the cognate base is recognized. This strategy revealed that bases at positions G1, G3, and T4 within the Tel11 (GTGGGGTGGT) substrate are the most specifically recognized by both the Cdc13 DBD and the full-length Cdc13 protein (10, 12). Substitutions at these three positions in the Tel11 sequence led to a significant loss of affinity (up to 87-fold) by the WT protein, whereas the change in affinity upon substitution at G9, a site which is less specifically recognized, was more modest (Table 1 and SI Appendix, Table S1).

To determine how specificity is impacted by mutations across the DBD interface, binding to these pools of oligonucleotides was performed with all mutant proteins (Fig. 3A, Table 1, and SI Appendix, Table S1). A wide range of effects was observed when the pool of bases was substituted at positions in the Tel 11 oligo, with the reductions in affinity ranging from 4.5-fold to nearly 3,000-fold.

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We noted above that in vivo phe-
86-fold decline in affinity, to 180 pM, for the WT protein. If
the most substantial decline in binding to the WT Tel11 ligand
previously designated as a hotspot for binding affinity, showed
the weakest binding mutant of Cdc13 DBD, Y522A, which has been
of several double-mutant pairs. For example, the
weakest binding mutant of Cdc13 DBD, Y522A, which has been
previously designated as a hotspot for binding affinity, showed
the most substantial decline in binding to the WT Tel11 ligand
(34-fold, to 71 pM). Similarly, substitutions at the most specifi-
cally recognized site on the oligonucleotide, T4, resulted in an
86-fold decline in affinity, to 180 pM, for the WT protein. If
these effects were simply additive, when assessing binding of the
mutant protein (Y522A) to the modified ligand the reduction in
affinity would be predicted to be 2,958-fold, which is quite simi-
lar to the observed value of 2,800-fold (SI Appendix, Table S1).
The fact that these sites behave independently is consistent with
their ~15-Å separation in the structure (30). Y522, however, is
physically proximal to G1. Here, the impact on binding in the
double substituted Y522A/H1 complex was less than additive,
with the observed net affinity down 200-fold relative to WT,
whereas the additive effect would be ~500-fold. This deviation
from a simply additive result supports the prediction from the
structure that Y522 specifically recognizes G1.

To visually identify protein/nucleic acid pairs whose combined
alteration deviates from thermodynamic additivity, we divided
the affinities for the binding of the doubly substituted pairs by
the binding affinity of each mutant protein for Tel11 (Fig. 3B
and SI Appendix, Table S1). Thermodynamic additivity would predict
that, with this normalization, the mutant proteins would show
the same specificity profiles as observed for WT Cdc13. This
scaled specificity profile indeed revealed that, in mutants for
which the effects were close to additive, such as Y522A, the
specificity profile mirrored that of WT. Exceptions included sites
of direct contact where the impact on binding was smaller than
expected, as discussed above for the Y522A/H1 pair.

Several mutant Cdc13 proteins unexpectedly exhibited a de-
viation from additivity in which the pairs led to a greater loss of
affinity than predicted by additivity. This was particularly evident
for Y626A and F539A and to a lesser extent for Y561A (Fig. 3B
and SI Appendix, Table S1). A case in point was the interaction
of F539A with H1, where the combined reduction in affinity
(600-fold) was about 11-fold greater than the product of the
F539 vs. Tel 11 (3.5-fold) and WT vs. H1 (15-fold) differences
between WT binding with Tel 11. This was also the case for the interaction between F539A and position H3 in the DNA: F539A
exhibited a 1,300-fold loss in binding affinity at this site even
though F539A exhibited only a 3.5-fold reduction in Tel11
binding. Again, the net impact of the combination of amino acid
change and oligonucleotide substitution was highly nonadditive.

A standard double-mutant thermodynamic cycle can be used
to assess the effects of independently mutating the protein or the
nucleic acid substrate and then combining these two different
alterations to the protein/nucleic acid interface (25–27). If these
are independent changes, the effects of implementing them si-
multaneously on binding free energy will simply be additive,
with the net observed $K_d$s being the product of the $K_d$s for the
individual changes. However, if the combination has a nonadditive
effect on affinity, it suggests that the two alterations are ther-
nodynamically coupled in some way. A net effect that is less
than simply additive could be due to the sites being physically
proximal, so that the loss represents both sides of a direct amino
acid/base contact and removing either side of the interaction is
sufficient to abrogate it. For this reason, strong couplings are
most commonly observed for physically proximal residues (25–
27). Moreover, it could also suggest that a mechanism of ac-
commodation is in place whereby the loss of a favorable inter-
action is compensated by the gain of a new favorable interaction,
as seen previously in other ssDNA/protein complexes (28, 29).
Conversely, a net effect that is greater than the sum of the free
energy changes of the individual alterations could suggest a loss
of cooperativity or a structural change at the interface. This classic
analysis allows us to identify the amino acids that perform un-
expected roles in determining binding specificity.

Simple thermodynamic additivity explained the binding affini-
ties observed for several double-mutant pairs. For example, the
weakest binding mutant of Cdc13 DBD, Y522A, which has been
previously designated as a hotspot for binding affinity, showed
the most substantial decline in binding to the WT Tel11 ligand
(34-fold, to 71 pM). Similarly, substitutions at the most specifi-
cally recognized site on the oligonucleotide, T4, resulted in an
86-fold decline in affinity, to 180 pM, for the WT protein. If

Fig. 3. Creation of a rheostat of binding affinities in Cdc13. (A) Skyscraper
plot of $K_d$s for each alanine mutant of Cdc13 DBD mutant protein with
various substituted oligonucleotides. (B) Specificity data for select mutant
proteins scaled to the loss of affinity observed for that mutant to Tel11. Data
used to construct this plot are in SI Appendix, Table S1.

Binding Specificity in Conjunction with Affinity Predicts in Vivo
Phenotypes More Accurately. We noted above that in vivo pheno-
types correlated roughly with severe losses of binding affinity
but that more moderate changes in binding affinity did not fully
explain the phenotypes. The reduced tolerance by F539A and
Y626A for deviations from the Tel11 sequence (i.e., increased
specificity) provides a biological explanation for their in vivo
phenotypes, which were significantly functionally impaired,
particularly when contrasted with the similarly affinity-impaired
Y561A. The severe growth defects associated with the cdc13-
F539A and cdc13-Y626A strains, as well as the more subtle
growth defects in the cdc13-I578A, cdc13-Y561A, and cdc13-
Y556A yeast strains (with mutations that conferred increased
affinity but reduced specificity) demonstrate that binding speci-
ficity contributes substantially to Cdc13 function.
Discussion

In this study, we have performed a systematic analysis of the ssDNA-binding surface of Cdc13 by generating a panel of alanine mutations that span the interface and then probing the impact of these mutations on binding affinity and specificity. This detailed biochemical analysis was combined with an in vivo phenotypic readout that was sensitive enough to detect even minor differences in function, revealing a gradient, or rheostat, of functionality. As expected, strains expressing mutant proteins with a reduction in binding affinity of more than 15-fold were inviable, demonstrating that high-affinity DNA binding is an essential function of the yeast t-RPA complex which contains the Cdc13 protein. Surprisingly, this systematic analysis identified a second category of mutations that did not confer substantial changes in ssDNA-binding affinity but altered the ssDNA-binding specificity of the Cdc13-binding interface, such that the surface was less tolerant of changes in the ssDNA. This second category of cdc13+ mutations also had a substantial impact on viability, thereby revealing that sequence tolerance is as important as binding affinity for biological function in vivo.

Typically, mutating a contacting amino acid increases sequence tolerance by removing the H-bond donors and acceptors and steric interactions that enforce specific recognition. In contrast, mutations in a subset of residues of the Cdc13-binding interface confer a decrease in sequence tolerance. Notably, mutating these amino acids impacts recognition of bases 10 Å away (Fig. 1L), indicating that long-range effects across this interface contribute to specificity. Furthermore, removal of these aromatics from the interface does not make binding more promiscuous, suggesting that these side chains are not driving local specificity and instead are accommodating sequence diversity. We therefore propose a model in which DNA binding by Cdc13 employs a highly cooperative interface with sequence diversity accommodated through plastic binding modes. This argues that ssDNA binding employs localized contacts between a subset of amino acids and adjacent bases that are important for binding affinity as well as long-range effects across the interface that are critical for sequence tolerance.

Analysis of the biochemical data in the context of the Cdc13 DBD/Tel111 structure points to three distinct functional parts of the interface (30). The first region is the segment of the OB-fold barrel that interacts with the 5' end identified in previous mutagenesis studies as driving both affinity and specificity of interaction. This region includes Y522 and K622, the residues whose substitution has the largest impact on affinity without significant changes in specificity. The second is the long β3 loop (highlighted in blue in Fig. 1L), encompassing mutations spanning residues Y556A to Y565A, that interacts with the 3' end of the ligand. Mutations in this loop have more moderate impacts on affinity and specificity, consistent with a "Velcro-like function," that is, a sticky surface suited to binding any sequence. The final structural region bridges these two, spanning the middle part of the barrel. Here substitution of two key aromatic residues, F539A and Y626A, results in a modest loss in affinity as well as long-range effects across the interface that are critical for sequence tolerance.

Specificity in nucleic acid recognition by proteins has been studied from both a biochemical and structural perspective (reviewed in ref. 35), which has suggested that the nucleic acid-binding interface is malleable. For example, single-stranded recognition interfaces can be remodeled to match different substrates to achieve specific recognition for both DNA and RNA, as exemplified by the Oxytricha nova telomere end-binding protein (36), the S. pombe Pot1 protein (28, 37), and, in RNA recognition, the PUF protein (38) and the MS2 coat protein (39). This malleability could be dynamic in origin (40, 41). Lacking in all these prior studies, however, has been a demonstrated link between the requirement for recognition malleability and function.

The observation that mutations in the DNA-binding interface of Cdc13 render the protein more specific and less functional was unexpected. While it is common to observe loss of function upon loss of a biochemical activity, the enhancement of specificity leading to a substantial reduction in biological function has not been reported previously in nucleic acid recognition, to the best of our knowledge. As a systematic evaluation of the binding specificity of mutant proteins is not commonly undertaken, this disruption of multiple biochemical behaviors may be a broader phenomenon than previously appreciated. A case in point is the human CST complex, a heterotrimer with a domain organization very similar to that of the yeast t-RPA complex (42–44). Unlike t-RPA, the CST complex is not a telomere-dedicated protein; although it displays a preference for G-rich sequences, the arrangement of guanosine nucleotides needed for high-affinity binding does not correspond to the repeat characteristic of...
telomeres (45–48). This allows the complex to function as a replication accessory factor genome-wide as well as facilitating proper maintenance of G-rich sequence at telomeres. Although the complete DNA-binding interface of CST has not yet been identified, we suggest that the results reported here for the yeast Cdc13 protein may extrapolate to other moderately specific ssDNA-binding complexes such as CST. Moreover, perhaps ssDNA-binding proteins, such as RPA and SSB, which are largely non-specific, achieve non-specific binding through a similar mechanism, as suggested by dynamic analysis of the RPA-binding domain (49). As the alteration in the specificity of Cdc13 was discovered only through the comprehensive mutagenesis of the protein surface that contacts DNA, it suggests that the systematic mutation and characterization of an entire interaction surface is essential to understand the full complexity underlying nucleic acid binding and in vivo function.

Methods
Protein expression, purification, binding and specificity studies, and in vivo analysis are described in detail in SI Appendix, Supplemental Materials and Methods.

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