Functional mapping of the anti-idiotypic antibody anti-TS1 scFv using site-directed mutagenesis and kinetic analysis

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Abbreviations: ACS, antigen combining site; CDR, complementary determining region; CK, cytokeratin; Fr, frame; ELISA, enzyme-linked immunosorbent assay; kₐ, association rate constant; k₆, dissociation rate constant; mAb, monoclonal antibody; scFv, single chain fragment variable

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Introduction

Numerous possible applications for monoclonal antibodies (mAb) and antibody fragments in biotechnology, diagnostics and therapy exist. As treatments for malignant tumors, administered radiolabeled mAbs may form complexes with selected tumor antigens and subsequently inhibit growth of the tumor cells.1 A major limitation with this approach is the remaining, non-targeting radiolabeled mAbs in the circulation and their uptake in normal tissues, which causes unintended exposure. Different methods to clear the primary, i.e., idiotypic (Id), mAb from the circulation have been presented and one is to use a secondary, i.e., anti-idiotypic (anti-Id) mAb.2–4 The mAbs included in this study are the Id TS1, which targets cytokeratin 8 (CK8) and the anti-Id, which targets TS1. The anti-Id anti-TS1 has been used successfully to clear the circulation5–6 of excess of the tumor localizing mAb TS1.7,8 It has also been shown that pre-formed complexes of the single-chain variable fragment (scFv) variants of both TS1 and anti-TS1 result in a higher tumor uptake of TS1 scFv both in vitro and in vivo.9

Many antibodies produced for therapy must be genetically engineered to achieve high expression levels, improved folding, high solubility, reduced HAMA, increased stability in vivo and optimal binding. A detailed description of the interactions between an Id mAb paratope, its antigen and an anti-Id mAb may enable more effective engineering since the effect of mutations intended to improve the properties and attributes of mAbs may be more reliably predicted.10–14

Many research groups have analyzed the structural basis of antibody-antigen recognition.11,15–17 It has been demonstrated that amino acid residues involved in establishing the complementary determining region (CDR) conformations are less frequently involved in antigenic interactions compared to residues in other positions in the CDR. Correlations between antigen type and the
length and amino acid composition of the CDR and between type of antigen and residue position involved in the binding have been demonstrated. The length of the CDR influences its shape and binding to antigen through formation of pockets, grooves or flat surfaces. In the antigen combining site (ACS) of antibodies, there are usually between 12–20 amino acid residues that contribute to the interaction between the antibody and its antigen and, if any of these interacting residues are altered, the effect is most likely a reduced affinity. The interaction between an antibody and its antigen, however, is mainly established by three to six very important and crucial residues.

Interaction kinetics between two proteins can be described by their association and dissociation rates, but it is difficult to predict the effects of particular mutations, especially on the dissociation rate, because a precise knowledge of the shape and chemistry of the binding interface would be required. The association rate of two proteins can be enhanced by favorable long-range electrostatic attractions that guide proteins towards each other by affecting the orientation and the time the two proteins are in close proximity. The association rate is therefore easier to predict and, if the desired result of a mutation is to change the association rate, residues affecting the electrostatic interactions outside the paratope should most likely be altered. In vivo, antibody diversity is created by a number of methods, including amino acid substitutions that are more frequent at certain positions, insertion and deletion of segments and variation in the association between the heavy and the light chain variable region. In order to regulate the function of therapeutic proteins, such as antibodies, control of both the association and dissociation rates is important.

Site-directed mutagenesis is frequently used both to regulate affinity, as well as other properties, and analyze the binding surfaces of antibodies and antigens. The effect of a certain mutation can be studied by methods such as enzyme-linked immunosorbent assay (ELISA) and optical biosensors based on surface plasmon resonance (SPR).

In this investigation, we functionally mapped amino acid residues important for the anti-TS1 interaction with TS1 by performing site-directed mutagenesis of a scFv construct of anti-TS1. Targets for mutagenesis were selected based on two previous studies that included amino acid residue interaction statistics, homology modeling and chemical modification of anti-TS1 and TS1. SPR-based Biacore systems were used to study the effects of the mutations on interaction kinetics and affinity.

Results

Mutagenesis, purification, quantification and SDS-PAGE. The sequences of the 17 anti-TS1 scFv mutants cloned in pET 26 were confirmed to be correct by sequencing of the insert in both directions. The expression level of the anti-TS1 scFv mutants varied and the concentration of scFv obtained from the media ranged from 2–1,800 nM as determined by quantitative ELISA. SDS-PAGE analyses of two of the ion exchange purified anti-TS1 mutants and the purified wild-type anti-TS1 scFv used as a standard in the quantitative ELISA are shown in Figure 1.

Biacore® studies. Biacore studies were performed on the wild type and the 17 mutant scFvs. The kₐ and k₅ constants were determined using the BIAevaluation 3.2 (BIAcore, Uppsala, Sweden); Figure 2 presents a typical plot for one of the anti-TS1 scFv mutants. A plot of rate constants for the association, kₐ, versus dissociation, k₅, of the mutant anti-TS1 scFv, wild-type anti-TS1 scFv and the anti-TS1 IgG is presented in Figure 3. The negative control demonstrated that the remaining impurities in the samples after ion exchange chromatography purification did not affect the Biacore measurements. All dilutions with measurable concentration displayed the same kinetic rates. The anti-TS1 scFv wild type demonstrated a kₐ value of 1.15 x 10⁵ M⁻¹s⁻¹ and a k₅ value of 2.2 x 10⁻⁶ s⁻¹. Compared to the anti-TS1 IgG, with a kₐ value of 2.2 x 10⁶ M⁻¹s⁻¹ and a k₅ value of 2.6 x 10⁻⁶ s⁻¹, the affinity of the scFv was reduced 1.6 times, although, as the dissociation rate of the anti-TS1 scFv was above 10⁻⁵...
s\(^{-1}\), which corresponds to a complex half-life of more than 13 h, the dissociation time used in the experimental set up (10 min) was too short to accurately determine the dissociation rate constant.

All 17 scFv mutants displayed higher dissociation rates (60–1,300 times) and all except D101 and Y96 also demonstrated faster association rates (1.3–56 times) compared to the wild type. Three mutated residues, D100aA\(_h\) and D101A\(_h\), and Y96A\(_h\) had a more explicit negative effect on the binding and between 800–12,200 times lower affinities were calculated for these mutants (Fig. 3).

Seven additional mutations, Y32A\(_h\), K33A\(_h\), and Y52H\(_a\), as well as Y32A\(_h\), H34A\(_s\), K50A\(_s\), and V94A\(_s\), also demonstrated a high affinity reduction (between 48–164 times) and the nine other mutants displayed affinity reductions in the range of 1.6–17 times. Four of these nine mutants (Y32A\(_h\), D56A\(_h\), and H30aA\(_l\) and R54A\(_l\)) displayed increased association rates (36–56 times) and increased dissociation rates (78–1,790 times). As shown in Figure 3, the mutations of D56A\(_h\) and R54A\(_l\) demonstrated similar affinity as the wild type (iso-affinity).

Three-dimensional structures. The homology model of anti-TS1 ACS indicated that the molecule displays a surface that is mainly polar, with a centrally located region that is mainly hydrophobic. This hydrophobic region involves CDR3 of the heavy chain and also, to some extent, the CDR3 of the light chain. The model also showed that the anti-TS1 ACS displays several positively and a few negatively charged areas scattered over the surface. A front view of the three-dimensional model of anti-TS1 colored by polarity and charge is presented in Figure 4. None of the 17 mutations resulted in any visible structural changes in the model. The surface of the model was also colored by accessibility (data not shown) and if approximately 20% or more of a residue was visible, then we considered it as accessible. The accessibility of each mutated residue in the homology model and the frequency of involvement in antigen binding for the selected residue positions,\(^{22}\) as well as the affinity changes determined by BlAcore, are displayed in Table 1. No homologies between the epitope of CK8\(^{34}\) and the
distribution of functionally important residues in the three-dimensional structure of anti-TS1 ACS were seen.

**Discussion**

In antibody engineering, the ability to functionally map the interaction site on antibodies is very important. Detailed descriptions of interactions are necessary to facilitate understanding of the manner in which antibodies bind to antigenic surfaces and predict the effects of mutations.\(^{19-22,30,33,35}\) A system involving three molecular partners, e.g., an antibody that interacts with an antigen and an anti-Id antibody that binds competitively, is complex to study. Some anti-Id antibodies mimic the structure of the antigen,\(^{36,37}\) some demonstrate functional mimicry \(^{38,39}\) and some show no resemblance to the antigen.\(^{40}\) Anti-Id anti-TS1 antibodies that bind the Id anti-CK8 TS1 have shown potential in preclinical experiments as clearing agents. When administered in a scFv format simultaneously with TS1 scFv, anti-TS1 contributes to an increased accumulation of TS1 in the tumor.\(^{9}\)

We have previously studied interactions between the therapeutic mAbs TS1, its anti-Id antibody, anti-TS1 and its target antigen CK8. We identified the interacting surfaces of TS1 and CK8 and we showed mutual inhibition of CK8 and anti-TS1 to binding with TS1, although they do not show any obvious structural similarities.\(^{4,10,33,34}\) In this study, we used site-directed
alone or complexed with TS1.33 We also based the selection of residues and the effects of chemical modification of anti-TS1 based on a computer model that displayed accessibility of the Amino acid residues in anti-TS1 were selected for mutagenesis TS1 to further improve understanding of this complex system. were functionally important for the anti-TS1 interaction with mutagenesis of anti-TS1 scFv to identify amino acid residues that our results revealed that the complexes formed between TS1 and complexes between TS1 and anti-TS1 by electron microscopy and in the BIAcore. We previously studied the formation of com-
ficult for anti-TS1 to bind with both arms to immobilized TS1 hinge region of this anti-TS1 IgG antibody, which makes it dif-
reason for the lack of increased functional affinity of the IgG antibody as measured on the BIAcore were very similar. The abilities of certain amino acid residues in the CDRs of anti-TS1 to be involved in the interaction with TS1.

Figure 4. A three-dimensional model of anti-TS1 viewing the complementary determining regions (CDR) and antigen binding surface from the front. The upper part of the model represents the CDR of the heavy chain and the lower part the light chain. Grey color represents hydrophobic areas, yellow color represents polar parts, blue represent positive charges and red represent negative charges. Accessible residues are depicted with circles. Amino acids within thick black circles resulted in a substantial reduction (>2.5 kcal/mol) in binding energy (2.6–3.9 kcal/mol). Amino acids within thin black circles resulted in a moderate reduction in binding energy (1.6–2.3 kcal/mol). Amino acids with dashed circles resulted in a low reduction in binding energy (0.2–0.3 kcal/mol). One letter abbreviation of amino acids is used.

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mutagenesis of anti-TS1 scFv to identify amino acid residues that were functionally important for the anti-TS1 interaction with TS1 to further improve understanding of this complex system. Amino acid residues in anti-TS1 were selected for mutagenesis based on a computer model that displayed accessibility of the residues and the effects of chemical modification of anti-TS1 alone or complexed with TS1.33 We also based the selection of residues to be mutated on statistical calculations21 of the probability of certain amino acid residues in the CDRs of anti-TS1 to be involved in the interaction with TS1.

The properties of the IgG and scFv versions of the wild-type antibody as measured on the BIACore were very similar. The reason for the lack of increased functional affinity of the IgG form based on its bivalent binding is most likely due to the stiff hinge region of this anti-TS1 IgG antibody, which makes it difficult for anti-TS1 to bind with both arms to immobilized TS1 in the BIACore. We previously studied the formation of complexes between TS1 and anti-TS1 by electron microscopy and our results revealed that the complexes formed between TS1 and anti-TS1 were mainly ring shaped structures comprising four or more antibodies.5

All 17 mutants demonstrated an increase in their dissociation rate constant (k d) of between 60–1,300 times, which probably occurred because we selected residues that were expected to have a negative effect on the binding. Fifteen mutants demonstrated an increase in their association rate constant (k a) of between 1.3–56 times, indicating that a truncation of these residues in the ACS to the less polar amino acid alanine had some advantage on the association. Due to this effect on binding, all analyzed residues should be considered important for the interaction with TS1 or stabilization of the structure of anti-TS1 or both.

Based on the observations in this and other studies, three residues, D101 H, R24 L, and H34 L, were considered structurally important. D101 H and H34 L had low accessibility in the homology model (Fig. 4) and, according to Almagro,22 these positions have low antigen binding frequency (Table 1). D101 H is structurally important because of its interaction with the arginine at position 94 in the heavy chain, which establishes the structure required for antigen binding.24 The arginine at position 24 probably forms an intramolecular salt bridge with D70, both of which are found in the light chain.52 Vargas-Madraso and Paz-Garcia demonstrated that position 34 in the light chain frequently participates in the interface by contacting residues in the heavy chain.29 We therefore consider the role of H34 L, which is not visible from the surface of anti-TS1, as structural (Fig. 4).

For the mutants D100aA H and Y96A L, the association rate constants were increased only slightly while the dissociation rate constant increased more than 1,000 times (Fig. 3). D100aA H is accessible (Fig. 4) and positioned close to the center of CDR3 with its side chain oriented towards the center of the combining site (antigen binding frequency 33%)25. The side chain of Y96 L is also oriented towards the antigen binding site, but is less accessible and has an antigen binding frequency of 42%.22 It has been demonstrated that both position 100aH and position 96 L are involved in stabilization of the V H/V L interface.29 The roles for D100aH and Y96 L were therefore considered as probably both structurally important and important for the interaction with TS1.

The additional 12 mutated residues were considered important for the interaction since all but two residues (D56A H and R54A L, which displayed nearly iso-affinity with the wild type) contributed >1 kcal/mol to the interaction (Fig. 3 and Table 1). Four of these 12 residues, Y32 L, K50 L, Y52 H and K33 H, were considered very important for the interaction because they contributed extensively to the interaction energy (≥2.5 kcal/mol) reducing the affinity between 48–164 times (Fig. 3 and Table 1). With the exception of K33 H, all of these residues are accessible in the homology model of anti-TS1 and the residue positions have an antigen binding frequency between 33–73% (Fig. 4 and Table 1).

It has been demonstrated that 3–6 residues in the ACS contribute to most of the interaction energy and these important residues tend to cluster together in the center of the ACS.35–43 When a residue contributes to the binding, it must be protected from the solvent and therefore ACS usually contains hydrophobic regions.
that desolvate the interacting interface.\textsuperscript{35} The homology model of anti-TS1 indicates that the molecule is mainly polar, but displays a centrally located region that is hydrophobic. This hydrophobic region involves CDR3 in the heavy chain, but also to some extent the CDR3 in the light chain. Residues Y32, K50, V94, and Y96, as well as K33, Y52, and D100, are positioned adjacent to this hydrophobic region (Fig. 4).

Collis et al. compared the residue distribution in CDR loops and loops of other proteins, and tyrosine (Y) and tryptophan (W) were demonstrated to be over-represented in CDR loops in antibodies that bind proteins, while cysteine (C), aspartic acid (D), glutamic acid (E), glycine (G), lysine (K), proline (P) and valine (V) were demonstrated to be significantly under-represented.\textsuperscript{18} We have demonstrated that, besides Y and D, K and V in anti-TS1 are also important for the binding to TS1. Interestingly, lysine (K) and valine (V) are residues that are under-represented as specificity determining residues in antibodies,\textsuperscript{22} but it has been demonstrated that approximately 50% of the binding energy derived from the antigen is attributed to the residues arginine (R), lysine (K), asparagine (N) and aspartic acid (D).\textsuperscript{44} In our previous study, chemical modification indicated that K was not important for the interaction with TS1.\textsuperscript{35} The reason for these different results could be explained by the fact that chemical modification adds a chemical group to the side chains while the alanine substitution reduces the side chain to a methyl group.\textsuperscript{45}

Many studies have demonstrated that the shape of the ACS varies with the antigen. Antibodies that bind protein antigen are usually flatter, while often a groove can be seen in antibodies that bind peptides.\textsuperscript{21} The ACS of TS1 and anti-TS1 are mainly flat as expected; however, the light chain CDR1 is protruding in both antibodies and TS1 contains a groove that forms a C-shape around the CDR3 in the heavy chain.\textsuperscript{33} In addition to this geometric fit, both the groove in TS1 and the protruding parts of anti-TS1 are partly hydrophobic, which make hydrophobic interactions possible between these sites.\textsuperscript{10} Using chemical modification, it was demonstrated that histidine was important for TS1 interaction with anti-TS1 and not CK8 and the only histidine found in the ACS is located in this groove.\textsuperscript{33} Interestingly, despite the fact that anti-TS1 and CK8 compete for the TS1 binding, no resemblance could be seen between the ACS of anti-TS1 and the epitope of CK8.

The model also displays several positively charged areas, and some negative, that are scattered over the surface. The surface of anti-TS1 was colored by electrostatic potential (data not shown) using the Swiss-Pdb, Deep View version 3.7 program, which illustrated the entire Fv region as positively charged, while TS1 is more negatively charged. If the desired result of a mutation is to increase the association rate, then it is most likely that the long range electrostatic interactions should be altered. Relatively fast association rates have been achieved for several protein complexes by optimizing the electrostatic complementarity of their binding sites.\textsuperscript{46,47} Replacing four residues with alanine as single-mutants, Y32H, D56H, H30A, and R54L, increased the association between 36–56 times; these residues are positioned in the periphery of the ACS of anti-TS1 (Fig. 4). For the Y32A mutuation (Fig. 4), the increased association could occur because the tyrosine side chain hides a large part of the positively charged residue R94 (and also to some extent D101). Mutant anti-Id antibodies with similar

| CDR | Position | Amino acid | A-LA | ΔΔG Kcal/mol | K_a red | SDR |
|-----|----------|------------|------|--------------|---------|-----|
| HCDR1 | 32 | Y | A | 2.3 | 48 | 8/19 |
| | 33 | K | LA | 2.5 | 78 | 9/19 |
| HCDR2 | 52 | Y | A | 2.6 | 96 | 14/19 |
| | 56 | D | A | 0.2 | 1.6 | 12/19 |
| HCDR3 | 97 | F | A | 1.4 | 11 | 12/17 |
| | 100 | W | A | 1.6 | 17 | 6/14 |
| | 100a | D | A | 3.9 | 800 | 4/12 |
| | 101 | D | LA | 5.6 | 12,200 | - |
| LCDR1 | 24 | R | LA | 1.4 | 11 | - |
| | 30a | H | A | 1.0 | 5 | 2/8 |
| | 32 | Y | A | 3.0 | 164 | 14/19 |
| | 34 | H | LA | 2.5 | 75 | - |
| LCDR2 | 50 | K | A | 2.6 | 85 | 13/19 |
| | 54 | R | A | 0.3 | 2 | 1/19 |
| LCDR3 | 93 | H | A | 1.6 | 15 | 10/19 |
| | 94 | V | A | 2.3 | 56 | 11/19 |
| | 96 | Y | LA | 4.3 | 1610 | 8/19 |

A, accessible; LA, low accessibility; ΔΔG, changes in free binding energy; K_a red, reduced affinity; SDR, specificity determining residues, the number of antibodies that involve that particular residue position in antigen binding out of 19 anti-protein antibodies studied;\textsuperscript{22} -, no antigen binding observed; HCDR, heavy chain CDR; LCDR, light chain CDR.

Table 1. For each amino acid residue position selected for mutagenesis, the table presents the approximate relative accessibility, the reduction in binding energy, and the antigen binding frequency for antibodies that bind protein antigens.\textsuperscript{22}
affinity as the wild type, but with higher association and dissociation rate constants as mutants D56A, and R54A, are expected to bind faster to TS1 in the circulation and compete differently with the antigen at the tumor site. This could possibly cause a further increase on the tumor uptake of TS1 scFv in pre-formed scFv complexes studied earlier.9 The present work has functionally mapped the ACS of anti-TS1 and generated a large panorama of anti-Id antibodies with different interacting properties, which may help in the design of a system with improved clearing and tumor delivery.

Material and Methods

Hybridoma cell lines and monoclonal antibodies. Hybridoma cell lines producing monoclonal antibody TS1,48 which targets cytokeratin 8 (CK8) and its anti-Id, anti-TS1,48 were cultured as described.48 The mAbs were purified from cell culture media using a Protein G column (Pharmacia Biotech) and eluted with 0.1 M glycine/HCl buffer pH 2.3. Fractions were neutralized and using a Protein G column (Pharmacia Biotech) and eluted with 0.1 M glycine/HCl buffer pH 2.3. Fractions were neutralized and stored at -20°C.

Site-directed mutagenesis. Site-directed mutagenesis of 17 amino acid residues was performed on purified pET 26 plasmid with the anti-TS1scFv gene,49 gene bank accession numbers AJ884574 (anti-TS1 heavy chain variable region) and AJ884575 (anti-TS1 light chain variable region), using Quick-Change Site-Directed Mutagenesis kit as described by the manufacturer (Stratagen). Four tyrosine (Y), three histidine (H), two lysine (K), three aspartic acid (D), two arginine (R), one tryptophan (W), one phenylalanine (F) and one valine (V) residues were selected for mutagenesis based mainly on the chemical modification results (Y, R and D), surface exposure33 and statistical importance of actual amino acid position in the Fv part (K, H, W, V and F).47 Each of the 17 amino acids selected for mutagenesis was replaced with the small and hydrophobic amino acid alanine, using mutagenic primers, designed according to the manufacturer (Stratagen, La Jolla, CA, USA). Mutated plasmids were systematically verified by sequencing of the antibody fragments on both strands using T7 promoter and T7 terminator primers (Novagen). For the sequencing, the Big Dye Terminator cycle sequencing kit (PE Biosystems) and the ABI Prism 310 Genetic Analyzer (PE Biosystems) were used. Each mutated plasmid was transformed into the E. coli strain Rosetta DE3 (Novagen).

Expression and purification. Expression of the scFv was performed by culturing the transformed E. coli Rosetta DE3 strains in 400 ml LB with kanamycin 30 μg/ml and chloramphenicol 75 μg/ml for approximately 16 h at 30°C to an OD₆₀₀ value between 3–5. The expression vector with the scFv gene contains a pelB leader to enable transportation of the scFv to the periplasmic space. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to induce expression and glycine and Triton X-100 were added to a final concentration of 2 and 1% respectively, to release the scFv into the culture media.46 The expression of scFv into the media was performed at 20°C overnight. Cell cultures were centrifuged and culture supernatant were obtained, concentrated approximately 200 times and dialysed against 20 mM Na-phosphate buffer pH 6.5.

The dialysed samples (corresponding to approximately 200 ml culture supernatant) were filtered through a 0.45 μm filter (Acrodisc syringe filter, PALL, Gelman laboratory), applied to a cation exchange chromatography column (Hi Trap Sp HP, Amersham Biosciences) in 20 mM Na-phosphate buffer pH 6.5 and eluted with a continuous NaCl gradient.

SDS-PAGE. The cation exchange chromatography purified wild type and scFv mutants, as well as Ni-NTA affinity and cation exchanged purified wild-type anti-TS1 scFv, used in quantitative ELISA were concentrated five times with trichloroacetic acid and analysed on SDS-PAGE (4% stacking and 12% separating gel) performed according to Laemmli.51 The SDS-PAGE gels were stained with Coomassie brilliant blue.

Quantitative ELISA. Microtiter plates (Nunc) were coated overnight at 4°C with 100 μl/well of polyclonal goat anti mouse Fab (SIGMA) in 50 mM Tris pH 7.4, 0.5 M NaCl (TBS) at a concentration of 2.5 μg/ml. The plates were washed three times for five minutes with TBS, pH 7.4 with 0.05% Tween 20 (TBST), before adding the cation exchange chromatography purified scFv mutants in duplicate, undiluted and serially diluted 1:3 in ten steps. Sample incubation was performed overnight at 4°C. The plates were washed with TBST as before and polyclonal goat anti-mouse Fab conjugated with alkaline phosphatase (SIGMA) at a concentration of 2.5 μg/ml was added and incubated over night at 4°C. After washing as before, the plates were developed with 3 mM 5-5-nitrophenyl phosphate in 50 mM 2-aminm-2-methyl-1-propanol, 1 mM MgCl₂ and pH 10.0. The absorbance was read at 405 nm and the samples were quantified using a standard curve of the wild type anti-TS1 scFv with a measuring range from 0.4–40 nM.

Kinetic studies using BIAcore®. A BIAcore® 2000 with the BIAcore 2000 control software version 3.2 (BIAcore, Uppsala, Sweden) was used for the kinetic studies. For the evaluation of the sensorgrams at concentrations higher than 25 nM, the Langmuir model was used. For concentrations below 25 nM, binding with the mass transfer model was used. Local Rmax was used to correct for the bulk effect seen in some samples. For all kinetic measurements, the CM5 sensor chip and the amine coupling kit with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysuccinimide (NHS) was used. Immobilization of TS1 to approximately 1,500 resonance units (RU) on the sensor chip CM5 was performed with 30 μg TS1 IgG/ml Na-acetate buffer pH 4.5 and standard procedures using the amine coupling kit. The samples (wild type and the 17 mutant scFvs) were dialyzed against running buffer and, because of large variation in concentrations, all samples were analyzed undiluted and diluted 1:3, 1:9, 1:27 and 1:81 with a continuous flow rate of 30 μl/min in HBS-EP pH 7.4 (Hepes 10 mM, NaCl 150 mM, EDTA 3.4 mM, P20 surfactant 0.05%) as running buffer. A negative control with ion exchange purified culture supernatant from E. coli with pET 26b without a scFv insert was included in the experimental setup to study the effect of impurities in the BIAcore system. The association and dissociation time between immobilized TS1, the wild type and the 17 mutant scFvs (cation exchange chromatography purified) were measured for 180 seconds and 600 seconds, respectively. The changes in free energy
\((\Delta G = \text{kcal/mol})\) for each anti-TS1 mutant interacting with TS1 were calculated using the equilibrium dissociation constant \(K_{D}[\text{kd/ka (M)}]\), the ideal gas constant \(R\) (1.989 cal/Kmol), the temperature \(T\) (298 K) and the following equation \(\Delta G=(-RT\ln K_{D}\text{mutant})-(-RT\ln K_{D}\text{wild type})\).

**Surface analysis.** A three-dimensional model of the variable region (Fv) of anti-TS1 wild type was generated as described previously. Using the Deep View version 3.7, the 17 anti-TS1 mutations were made in the model, energy minimized and the modeled mutant under investigation was compared to the wild type model. The same software was also used to color the surface of the anti-TS1 wild type homology model by polarity and charge and by accessibility.

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