The extracellular domain of the p55 TNF receptor (TNFrED) is an important therapeutic protein for targeting tumor necrosis factor-α (TNF-α). The expression level of the TNFrED is low for bioproduction, which is presumably associated with the complication of pairing 24 cysteine residues to form correct disulfide bonds. Here we report the application of the yeast display method to study expression of TNFrED, a multimeric receptor. Randomly mutated libraries of TNFrED were screened, and two mutants were identified that express severalfold higher protein levels compared with the wild type while still retaining normal binding affinity for TNF-α. The substituted residues responsible for the higher protein expression in both mutants were identified as proline, and both proline residues are adjacent to cysteine residues involved in disulfide bonds. Analysis of the mutant residues revealed that the improved level of expression is due to conformational restriction of the substituted residues to that of the folded state seen in the crystal structures of TNFrED thereby forcing the neighboring cysteine residues into the correct orientation for proper disulfide bond formation.

The ability to obtain a high level of expression of secreted recombinant proteins in yeast and mammalian cells is often found to be protein-dependent. Efforts in maximizing recombinant protein expression are often focused on increasing the levels of the mRNA of the recombinant gene. However, the rate-limiting step in the expression of certain proteins is not the level of mRNA but rather caused by inefficiencies in the folding, addition of post-translational modifications, and secretion of recombinant proteins (1–3).

Tumor necrosis factor-α (TNF-α)1 is an attractive therapeutic target for several diseases including rheumatoid arthritis and Crohn’s disease (4, 5). TNF-α initiates pleiotropic cytokine regulating effects by binding specifically to multimeric forms of the TNF receptor (9–12). However, the yeast surface display system has not been used to study multimeric proteins. We describe the isolation of two novel proline substitutions that increase the expression level of TNFrED in both yeast and mammalian cells. These mutants do not have an altered affinity for TNF-α. Analysis of structural roles of the mutants in the study has revealed an interesting structure-expression relationship.

**EXPERIMENTAL PROCEDURES**

**Construction of Yeast and Mammalian Expression Plasmids**—The TNFED-agglutinin fusion was constructed by linking the signal sequence from the invertase gene to the human TNFrED sequence encoding residues 12–172, which was then fused to the C-terminal portion of the a-agglutinin gene encoding residues 330–650. The TNFED-agglutinin fusion gene was subcloned into the pYES2 vector (Invitrogen) to generate pYES2-TNFED-Agg for expression in yeast. The human TNFED coding sequence from residue 12 to 172 was subcloned into pCMV, a mammalian expression vector containing the human cytomegalovirus immediate-early enhancer/promoter region, to generate pCMV-TNFED. The human growth hormone signal sequence is fused upstream of the hTNFED sequence. The TNFED mutations in pCMV-TNFED were generated with standard molecular biology techniques. The sequence of all constructs was verified with the Thermo Sequenase radiolabeled terminator cycle sequencing kit (Amersham Biosciences).

**Expression of TNFED on the Surface of Yeast Cells**—Saccharomyces cerevisiae strain BJ2168 (a, prc1-407, prb1-1122, pep4-3, leu1-2, trp1, ura3-52; Yeast Genetic Stock Center, Berkeley, CA) was transformed with either pYES2 or pYES2-TNFED-Agg using the lithium acetate method previously described (13). Transformed yeast cells were grown overnight with shaking in Ura medium supplemented with 2% glucose at 30 °C. Expression was induced by growing the transformed yeast overnight at 30 °C with shaking in Ura medium containing 2% galac.

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1 The abbreviations used are: TNF-α, tumor necrosis factor-α; FACS, fluorescence-activated cell sorting; TNFED, extracellular domain of the p55 TNF receptor; PDB, Protein Data Bank; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

2 W. H. Brondyk and M. Cunningham, unpublished results.
Expression of TNFrED Mutants in Mammalian Cells—Surfaces displaying polyclonal goat anti-human TNFrED were constructed by binding biotinylated antibody (BAF225 from R&D Systems) to a Sensor SA chip (PN BR-1000-32, BIACore Inc.), which was conjugated with streptavidin. Biotinylated antibodies bind to the chip with high affinity and create a stable surface for repetitive capture of TNFrED, TNF binding analysis, and regeneration. TNFrED concentrations of conditioned media containing the mutant TNFrEDs had been measured by ELISA prior to submission for binding analyses. Buffer-diluted purified TNFrED or buffer-diluted conditioned medium containing TNFrED was injected onto the chip surface at 50 nM, resulting in the formation of an antibody-TNF FrED complex. TNFrED at 1 nM (trimmer) was then injected, and the kinetics of the binding dissociation were recorded via the time course of surface plasmon resonance response. The chip surface was regenerated with 500 mM sodium citrate, pH 2.5, 50% 100 mM sodium citrate, pH 3.1, which stripped off the TNFrED and the TNF chip. This series of injections was repeated for TNFrED at 2, 5, 10, 20, 50, and 100 nM while holding the concentration of injected TNFrED or mutant at 50 nM. Each set of binding curves was fit using global analysis and a 1:1 interaction model.

Structure-Expression Relationship Analysis—The structures were downloaded from the May 2002 release of the Protein Data Bank (PDB) (17) at the Research Collaboratory for Structural Bioinformatics (www.rcsb.org/pdb/). The PDB codes of the three crystal structures containing TNFrED molecules are 1ext (release 1.59), 1inf (19), and 1tm (20). The angles of interesting residues in these structures were calculated and were compared with the angles of the same residue type in a large set of representative structures in the PDB. A total of 4930 representative polypeptide chains were selected based on the Astral list (release 2.4). SCOP domains using an identity filter of ≈50% (astral.stanford.edu/scopseq-1.59.html) to remove closely related structure folds or very similar sequences (21). The structures in the list were then limited to those with resolution 2.4 Å and better and R factor < 22%, which narrowed them down to 2674 structure domains. Residues in the selected domains were further subjected to B factor restrictions. Only those with an average B factor of main-chain atoms (Cα, C, O, and N) between 1.0 and 25.0 are qualified for Ramachandran plots. Each Ramachandran plot was divided into 10° × 10° grids, resulting in 36 × 36 pixels. The conformation in a pixel is considered as accessible if the number of the data points in the pixel exceeds the expected value, which is defined as the ratio of the total number of data points to the total number of pixels.

The formula of estimating the approximate entropy gain of protein folding for a mutation from residue X to residue Z was adopted from Ref. 22. The contribution to the entropy of folding residue Z from unfolded states to its folded structure relative to residue X in the mutation of X → Z is given by,

$$\Delta S_{X \rightarrow Z} = -R \ln \left( \frac{A_Z}{A_X} \right)$$

(1.8)

where $R$ is the gas constant, $A_X$ is the accessible conformational area in the Ramachandran conformational plot for residue X, and $A_Z$ is that for residue Z.

RESULTS

Expression of Functional TNFrED on the Surface of S. cerevisiae—TNFrED was fused to the C-terminal domain of α-ag-
gglutinin and expressed in the *S. cerevisiae* strain BJ2168 (a, prc1-407, prb1-1122, pep4-3, leak2, trp1, ura3–52) using the pYES2 vector. The C-terminal portion of α-agglutinin is tightly anchored in the cell wall and serves as a scaffold to present TNFrED on the cell surface (23). The TNFrED-agglutinin fusion gene was under the regulation of the inducible GAL1 promoter. In a flow cytometric analysis with polyclonal antibodies directed against TNFrED we found that in an induced culture ~70% of the yeast-expressed TNFrED-agglutinin on the cell surface (Fig. 1A). To determine whether TNFrED on the yeast cell surface was folded correctly and could bind TNF-α, we performed a flow cytometric analysis using biotinylated TNF-α as a probe and FITC-labeled avidin as the detection reagent. Yeast expressing the TNFrED-agglutinin fusion gene bound more biotinylated TNF-α than did yeast containing the pYES2 vector (Fig. 1B). Adding excess unlabeled TNF-α (data not shown) reversed the shift in the histogram seen with yeast expressing the TNFrED-agglutinin fusion gene.

**Selection of Mutant TNFrED Clones with Enhanced Biotinylated-TNF-α Binding**—A modification of the mutagenesis approach previously described (14) was used to generate random mutant libraries. In this approach mutant oligonucleotides are generated by spiking a predetermined level of the wild-type TNFrED, mutant clone 6 or 11. This experiment was repeated three times and similar results were obtained. A single representative experiment is shown.

**TABLE I**

| Sample     | DNA | TNFrED | Fold over wild-type |
|------------|-----|--------|---------------------|
|            | ng/ml |        |                     |
| Wild-type  | 230 ± 5.0 | 3 |                     |
| H34P       | 724 ± 40 | 3 |                     |
| S57I       | 190 ± 19 | 0.8  |                     |
| S87P       | 361 ± 21 | 1.5  |                     |
| H34P, S57I | 614 ± 54 | 2.6  |                     |
| H34P, S87P | 711 ± 33 | 3 |                     |
| H34P, S57I, S87P | 704 ± 26 | 2.9 |                     |

**TABLE II**

| Sample | $k_m \times 10^5$ | $k_s \times 10^{-3}$ | $R_{max}$ | $K_d$ | $\chi^2$ |
|--------|------------------|-------------------|-----------|-------|---------|
|        | $\mu s^{-1}$ | $s$ | RU | $\mu \times 10^{-9}$ |       |
| Wild-type (spike) | 2.1 | 2.6 | 70.1 | 1.0 | 1.95 |
| H34P | 2.0 | 3.0 | 71.0 | 1.5 | 0.96 |
| S57I | 2.2 | 2.5 | 66.7 | 1.1 | 2.41 |
| S87P | 2.2 | 3.0 | 61.5 | 0.9 | 2.4 |
| H34P, S57I | 2.4 | 3.0 | 79.1 | 1.2 | 1.84 |
| H34P, S87P | 2.2 | 2.8 | 76.9 | 1.3 | 1.62 |
| H34P, S57I, S87P | 2.1 | 3.3 | 76.1 | 1.6 | 1.32 |

**FIG. 2.** Saturation binding analysis of yeast expressing wild-type TNFrED, mutant clones 6 or 11. The solid square (■) represents TNFrED control, solid triangle (▲) represents clone 11, and open inverted triangle (▽) represents clone 6. Triplicate samples of yeast were incubated with increasing concentrations of 125I-TNF-α. Binding experiments were repeated 2–3 times with similar results. For wild-type TNFrED, the $K_d$ is $2.0 \pm 0.2$ nM and $R_{max}$ is 0.064 ± 0.003, for clone 11 the $K_d$ is $1.3 \pm 0.1$ nM and $R_{max}$ is 0.127 ± 0.004, and for clone 6 the $K_d$ is $1.7 \pm 0.2$ nM and $R_{max}$ is 0.334 ± 0.014.
cleotides at each position. The level of contamination of the wrong nucleotides was adjusted to generate either an average of two or three random point mutations per oligonucleotide. Each mutant library covered between 40 and 105 base pairs of TNFrED. Ten random clones from each library were sequenced. The type and position of mutations were random, and the regions were found to contain the anticipated average number of mutations. The size of each library was between $10^5$ and $10^6$ independent mutant clones. Each library was transformed into the strain BJ2168, and $10^6$ independent transformants were selected for binding to both biotinylated-TNF-α and polyclonal antibodies directed against the TNFrED. The window of the two-dimensional fluorescence histogram that was used to select for the subpopulation of yeast expressing active TNFrED is shown in Fig. 1C. This window was originally chosen to select for mutants with an increased affinity for TNF-α. The selected subpopulation of yeast was grown and reselected with two-color sorting. After several rounds of two-color sorting the population of yeast in the selected window was enriched (Fig. 1C). Following three to four rounds of cell sorting, individual clones were analyzed by examining binding of biotinylated-TNF-α to the TNFrED on the cell surface. A majority of the clones analyzed from each sorted library appeared to bind higher levels of biotinylated-TNF-α. The mutant TNFrED plasmid was recovered from each yeast clone and re-transformed into the BJ2168 strain and then reanalyzed by flow cytometry. The vast majority of yeast clones were false positives as the recovered plasmids did not confer higher levels of biotinylated-TNF-α binding. When false positive yeast clones were analyzed in the absence of biotinylated TNF-α and avidin-FITC, they were shifted as compared with the parental BJ2168 strain. These false positive yeast clones are $30\%$ larger compared with the parental BJ2168, which is presumably what gives rise to the shift in the baseline absorbance. However two mutant clones, 6 and 11, from different libraries, were identified after re-transformed into the BJ2168 strain as conferring higher levels of biotinylated TNF-α binding and therefore are true positives (Fig. 1D).

Characterization of Mutant Clones 6 and 11—The TNFrED coding region in mutant clones 6 and 11 was sequenced and, as anticipated, the mutations for each clone were only found in the sequence region mutated for that specific library. In mutant clone 11 there was a point mutation that resulted in Ser → Pro change at position 87, and in mutant clone 6 there were two point mutations that resulted in an His → Pro change at position 34 and a Ser → Ile change at position 57. To determine whether the mutations increase binding of biotinylated TNF-α through increased expression of TNFrED or by increasing the affinity for TNF-α, we performed a saturation binding experiment on yeast expressing either mutant clone 6 or 11. Analysis of saturation binding experiments (Fig. 2) indicated that yeast expressing either mutant clone 6 or 11 express higher levels of

![Ramachandran plots of the substituted residues in mutant clones 6 and 11 and the corresponding residues in the wild type.](image-url)

**Fig. 3.** Ramachandran plots of the substituted residues in mutant clones 6 and 11 and the corresponding residues in the wild type. The darkness of each $10^5 \times 10^5$ $\phi-\psi$ pixel indicates the density of the conformation population. The lightest region indicates density higher than the expected value, the next level is $e^{9}$-fold higher than the expected value and the darkest is $e^{12}$-fold higher than the expected value. The $\phi-\psi$ angles of the corresponding residue in the TNFrED crystal structures are shown in red squares (1ncf.pdb, TNFrED dimer structure, 1.85 Å resolution), red diamonds (1ext.pdb, TNFrED dimer structure, 2.85 Å resolution), and red triangles (1tnr.pdb, TNF/TNFrED complex structure, 2.85 Å resolution). The top panel in each column is the Ramachandran plot for the wild-type residue, and the lower panel is for the mutant residue.
Expression of Mutated TNF\(\alpha\)EDs in Mammalian Cells—We next determined whether the mutant clones derived in yeast had similar expression characteristics in mammalian cells. The TNF\(\alpha\)ED mutants were transiently expressed in HEK293-EBNA cells and the amount of secreted TNF\(\alpha\)ED was measured with an ELISA specific for TNF\(\alpha\)ED. The results (Table I) indicated that either mutation H34P or S87P increased the expression level of TNF\(\alpha\)ED. Moreover, the relative increase in expression is similar to what was seen when these mutants were expressed in yeast (Fig. 2). The S57I mutation alone did not alter the expression level of TNF\(\alpha\)ED (Table I). The effects of these mutations do not appear to be additive because the presence of both H34P and S87P on the same construct did not increase the level of TNF\(\alpha\)ED secreted in comparison to that found with H34P alone.

Steady-state RNA levels from transiently transfected HEK293-EBNA cells were analyzed by real-time PCR. The RNA levels of TNF\(\alpha\)ED for the six mutants were found to be 28–73\% of the level of wild-type TNF\(\alpha\)ED RNA indicating that the presence of the described mutations does not increase RNA levels.

The association and dissociation kinetics of TNF\(\alpha\) binding to the mutated TNF\(\alpha\)EDs were directly comparable to those of the wild-type protein (Table II). The difference in TNF\(\alpha\) affinity between wild-type and mutated TNF\(\alpha\)EDs was not found to be significant, which is consistent with our binding analysis with the yeast transformants.

Analysis of Accessible Conformations of the Mutations—The residue substitutions in mutant clone 6 and 11 and their adjacent residues include proline, cysteine, and glycine. As these three residues are known to have a significant role in protein structure, we analyzed potential functions of the substitutions or the adjacent residues in their protein-folding processes. We first compared the accessible conformations of the substituted residue types before and after the mutations as seen in the PDB structures. As shown in Fig. 3, the conformation of a proline residue is more restricted than the other three residues, and a serine residue is less restricted than an isoleucine residue. Moreover, the conformation of Ser87 and His34 in TNF\(\alpha\)ED crystal structures (red-filled marks) lies in the most abundantly accessible conformational region of the proline residues. Following the same method as described by Matthews et al. (22), we estimated the contribution of the entropy of protein folding before and after the mutations: 6.1 J/mol/degree for S87P, 7.1 J/mol/degree for H34P, and 1.66 J/mol/degree for S57I. In all three cases, each mutated residue contributes favorably to the entropic contribution to protein folding (Fig. 3).

We next analyzed the accessible conformational space of the adjacent residues affected by the mutations. Conformational restriction has little effect for a residue following proline (compare Fig. 4, A and B). In contrast, conformational restriction of a residue preceding proline is particularly profound due to the steric hindrance, as illustrated in Fig. 4, C and D. The rigidity of the pyrrolidine ring of the proline residue constrains the \(\psi\) angle of its preceding non-glycine residue to a positive value in the top left quarter of its Ramachandran plot (refer to Fig. 5B). Glycine is another residue with a noticeable shift of accessible conformational space when it is adjacent to bulky residues. When it follows the bulky asymmetric residue, isoleucine, the accessibility of glycine to conformational space of the small \(\psi\) angle region is notably reduced (compare Fig. 4, E and F and refer to Fig. 5C).

To assess the effect of adjacent residues in the choice of conformation preferences by the substituted residue, we analyzed the accessible conformational space of the same neighboring residues in the PDB structures as those in clone 6, clone.

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**Fig. 4. Effects of mutations on accessible conformations of the adjacent residues.** Shown are the sequences from wild type at residue positions 87–88 (A), 33–34 (C), and 57–58 (E), and the corresponding sequences from the mutants S87P (B), H34P (D), and S57I (F). \(\psi\) angles that would cause steric hindrance are labeled in D and F.

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functional TNF\(\alpha\)ED than wild-type TNF\(\alpha\)ED and that the presence of these mutations does not affect the affinity of TNF\(\alpha\)ED for TNF-\(\alpha\). The number of receptors/cell for yeast expressing mutant clone 6, mutant clone 11, and wild-type TNF\(\alpha\)ED was 3930, 1490, 740, respectively.
11, and the wild type. The sequences surrounding the mutated residues are Ser\textsuperscript{86}-Pro\textsuperscript{87}-Cys\textsuperscript{88}, Cys\textsuperscript{33}-Pro\textsuperscript{34}-Lys\textsuperscript{35}, and Glu\textsuperscript{56}-Ile\textsuperscript{57}-Gly\textsuperscript{58}, where the superscript numbers denote residue numbers in the protein. The corresponding sequences in wild-type TNFrED are Ser\textsuperscript{86}-Ser\textsuperscript{87}-Cys\textsuperscript{88}, Cys\textsuperscript{33}-His\textsuperscript{34}-Lys\textsuperscript{35}, and Glu\textsuperscript{56}-Ser\textsuperscript{57}-Gly\textsuperscript{58}. Fig. 5 compares the Ramachandran plots of neighboring residues before and after the mutations. The mutation from Ser to Pro at residue 87 markedly limited the accessible conformations of the preceding serine residue to the neighborhood of the observed serine conformation in the TNFrED crystal structures. Similarly, the mutation from His to Pro at residue 34 also restricts the accessible conformational space of the preceding cysteine residue to those surrounding the folded conformation. This indicates that nearly all of the possible conformations of this cysteine residue in unfolded states are similar to the folded conformation. In contrast, the mutation from Ser to Ile at residue 57 reduces accessibility to small \(\psi\) angle conformations of the following Gly residue, although the small \(\psi\) angle is its folded conformation.

**DISCUSSION**

Through screening of mutant TNFrED clones with the yeast display system we were able to identify two mutations of TNFrED that conferred a higher expression level in both yeast and
mammalian cells when compared with the wild type. Analysis of transiently transfected cells revealed that the presence of the two mutations does not increase RNA levels of TNFrED indicating that these mutations increase TNFrED expression levels through a post-transcriptional mechanism.

In each mutant clone, we showed that the residue responsible for the higher protein expression levels is a proline substitution next to a cysteine involved in a disulfide bond. Structural examination of the TNFrED structures revealed that these residues apparently are not involved in ligand binding, glycosylation, or structural roles of intra- or intermolecular interactions by side-chains. Using an approach similar to that described by Matthews et al. (22) who analyzed the contribution of entropy change to protein folding before and after a mutation, we demonstrated that the entropy change of the preceding residues in the mutations S87P, H34P, and S57I contributes favorably to the protein folding of the mutants as compared with the wild type. The degree of contributions is: H34P > S87P >> S57I.

In addition, we showed that the accessible conformational space of the residues adjacent to the mutated ones contribute to the protein folding either favorably or unfavorably. In the mutant S87P, the accessible conformational space of the preceding Ser residue is limited, and this limitation favors the protein folding of the mutant relative to the wild type. The higher expression level of S87P is most likely the result of the positive entropy contribution of the mutated residue per se and the favorable accessible conformations of the preceding Ser residue. In the mutant H34P, the Cys-Pro combination restricts the conformation of the preceding Cys residue to or near the folded conformation. The correct orientation of the cysteine presumably facilitates proper disulfide bond formation and results in the higher yield of correctly folded molecules that lead to the higher expression. In the mutant S57I, the bulky Ile residue reduces the number of small $\phi$ angle conformations accessible to the following Gly residue, even though the small $\psi$ conformation is the folded structure. This negative contribution from the following Gly residue eliminates the positive contribution of the entropy change from the mutated residue in protein folding of the mutant. This is consistent with the observed expression level of S57I, which is actually slightly lower than the wild type. We also noticed that the expression level increase is non-additive when both H34P and S87P are present on the same construct, possibly because the two cysteine residues are not disulfide bridged to each other.

In summary, this study provides a step forward toward the solution of expressing a potentially important therapeutic molecule, TNFrED. It is also the first application, to our knowledge, of the yeast display system to study multimeric proteins. Moreover, this work presents the first analysis of the structure-expression relationship by demonstrating protein conformation is a determinant of expression.

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Structure-Expression Relationship of Tumor Necrosis Factor Receptor Mutants That Increase Expression

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