Identification of Framework Residues in a Secreted Recombinant Antibody Fragment That Control Production Level and Localization in Escherichia coli

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The monoclonal antibody 5T4, directed against a human tumor-associated antigen, was expressed as a secreted Fab superantigen fusion protein in Escherichia coli. The product is a putative agent for immunotherapy of non-small cell lung cancer. During fermentation, most of the fusion protein leaked out from the periplasm to the growth medium at a level of approximately 40 mg/liter. This level was notably low compared with similar products containing identical CH1, CL, and superantigen moieties, and the Fv framework was therefore engineered. Using hybrid molecules, the light chain was found to limit high expression levels. Substituting five residues in V\(_\text{H}\) increased the level almost 15 times, exceeding 500 mg/liter in the growth medium. Here, the substitutions Phe-10 → Ser, Thr-45 → Lys, Thr-77 → Ser, and Leu-78 → Val were most powerful. In addition, replacing four V\(_\text{H}\) residues diminished cell lysis during fermentation. Therefore the product was preferentially located in the periplasm instead of the growth medium, and the total yield was more than 700 mg/liter. All engineered products retained a high affinity for the tumor-associated antigen. It is suggested that at least some of the identified framework residues generally have to be replaced to obtain high level production of recombinant Fab products in E. coli.

Antibody-based therapies are currently evaluated for treatment of several severe diseases such as cancer (1), viral infections, and autoimmunity. Recent technological improvements have made it possible to clone and produce large amounts of intact recombinant monoclonal antibodies or antibody fragments (2, 3). Using phage display technologies, high affinity intact recombinant monoclonal antibodies or antibody fragments have made it possible to clone and produce large amounts of antibody-targeted SEA can initiate a powerful T cell attack against tumor cells in vivo (12, 13).

Here E. coli production of the 5T4Fab-moiety fused to a genetically engineered superantigen chimera (14), 5T4Fab-SEch, is investigated. The murine antibody 5T4 is directed against a trophoblast-related antigen found on several solid tumor types including carcinomas in lung, breast, colon, and ovary (16, 17). The 5T4Fab-SEch has a high affinity for the antigen and targets T cells to several cancer cell lines. However, when produced as a secreted fusion protein in E. coli, the production level is 5–10-fold lower compared with several similar products. To investigate the molecular components behind this phenomenon, several amino acid residues in the Fv framework were altered. Significantly, by replacing only a few light chain residues the level of active product increased, while heavy chain substitutions affected the product distribution between growth medium and periplasmic space.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and Taq polymerase were from Boehringer Mannheim or New England Biolabs (Beverly, MA). The recombiant work was carried out mainly as described (18) in the E. coli strain HB101. Plasmid preparation was performed with Wizard\textsuperscript{TM} Midipreps DNA purification system (Promega, Madison, WI) from bacteria grown in LB medium with 50 μg/ml kanamycin. Oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). Antibodies against murine κ chain were obtained from Bio-Zac (Stockholm, Sweden) and horseradish peroxidase-conjugated antibodies against SEA from Toxin Technology (Sarasota, FL).

Cloning, Engineering, and Insertion into an E. coli Expression Vector of 5T4 Fv.—The Fv-encoding portions of 5T4 were cloned from the 5T4 hybridoma obtained from Dr. Peter Stern (CRCT, Paterson Inst. for Cancer Research, Manchester, UK). The cDNA was made from total RNA using the GeneAmp RNA PCR kit (Perkin-Elmer). The coding regions of the entire variable domains and parts of the signal sequences as well as the constant domains of the heavy and light chains were amplified by PCR. All PCR products and DNA linkers were sequenced on an ABI 373A DNA sequencer (Applied Biosystems) as recommended by the supplier. The oligonucleotides 5’-CAATTTGCTGCGATCGAGTAGGCATCCATTCTGTTTCAATTTTCTTCGTCCACCTTGCACTCTGTCGCC-3’ and 5’-ACTAGTCGACATGGGATGGAGCTATCATIT(C/T)TCGGAGCTATCATIT(C/T)TCTTT-3’ were used for primer. The amplification product was cloned into the BamH I site of the expression vector pMema; IPTG, isopropyl-β-D-thiogalactopyranoside; HPLC, high pressure liquid chromatography; CDR, complementarity-determining region.

Recently, a concept for cancer therapy using recombinant fusion proteins of tumor-reactive Fab fragment and immunostimulatory bacterial superantigens was presented (11, 12). Superantigens, such as the staphylococcal enterotoxin A

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sequenced and found to be identical. DNA fragments suitable for insertion into the expression vector (12) were obtained in a second PCR step. To assemble a Fab-expression plasmid, the variable regions of ST4 were fused to sequences coding for the constant regions of the murine IgG1/k antibody C242 (12) and lacking the interchain disulfide bond. A region coding for the hybrid between SEA and staphylococcal enterotoxin E, connecting the light and heavy chains, and full-length fusion protein was detected using horse-radish peroxidase-conjugated antibodies against SEA (T.4, 6, 15). Gene segments containing the various point mutations were also combined (Table I). All constructs were verified by DNA sequencing.

Expression of 5T4Fab-SEch in the Fermenter—The Fab’s were expressed in the E. coli K-12 strain ULM 635 (xyl-7, ara-14, T4b, ΔompT) as a plasmid with a kanamycin resistance gene and lacUV5 promoter. Bacteria frozen from stock were incubated at 25 °C for approximately 21 h in shaker flasks containing (per liter) 2.5 g of (NH4)2SO4, 4.45 g of KH2PO4, 11.85 g of K2HPO4, 0.5 g of sodium citrate, 1 g of MgSO4·7H2O, 1.1 g of glucose monohydrate, 1 g of glucose monohydrate, 1 g of sodium citrate, 22 g of glucose monohydrate, 1 g of MgSO4·7H2O, 0.11 mM kanamycin, 1 ml of a decanol (Asahi Denka Rogyo K.K., Japan), and 1 ml of trace element solution (19), however, without Na2MoO4. The cells were grown to an A600 of 1–2, and 450 ml of culture medium was used to inoculate a fermenter (Chemap, Switzerland) to a final volume of 5 liters. The fermenter medium contained (per liter) 2.5 g of (NH4)2SO4, 4.45 g of KH2PO4, 11.85 g of K2HPO4, 0.5 g of sodium citrate, 22 g of glucose monohydrate, 1 g of MgSO4·7H2O, 0.11 mM kanamycin, 1 ml of a decanol (Asahi Denka Rogyo K.K., Japan), and 1 ml of trace element solution. The pH was kept at 7.0 by titration with 25% ammophos; the temperature was 25 °C and aeration was 5 liters/min. The partial pressure of dissolved O2 was controlled to 30% by increasing agitation from 300 to 1000 rpm during batch phase and regulating the feed of 60% (v/v) glucose during fed batch phase. Product formation was induced at an A600 of 50 by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After fermentation the cells were removed by centrifugation at 8000 × g for 40 min at 4 °C. The clarified medium was either analyzed and purified directly or stored at −70 °C.

Purification Procedures—DNA present in the clarified medium was removed using precipitation with 0.19% polyethyleneimine and 0.2 mM NaCl for 30 min (20). After centrifugation as above, the supernatant was collected, and the NaCl concentration was adjusted to 0.5 M. This medium was applied to a protein G-Sepharose column (Pharmacia) and equilibrated with 60 mM sodium acetate, pH 5.0, and 0.02% Tween 80. The column was then washed with 7.5 column volumes of 20 mM sodium citrate, 0.1M sodium citrate, pH 6.0, 0.05% Tween 80, 7.5 column volumes of 20 mM citric acid, 1 mM EDTA, 200 mM NaCl, 0.05% Tween 80, pH 4.7, and bound protein was eluted with 0.1 M acetic acid and 0.05% Tween 80. The protein eluate was adjusted to 15% (v/v) glycerol and 7.5% dimethylsulfoxide by concentration yielding half-maximum inhibition, IC50, was determined after linear regression of log-logit transformed binding data, and the relative affinity index was determined as the ratio between the IC50 values of competitor and wild-type 5T4Fab-SEch.

Computer Modeling of the 5T4Fab Variable Region—The individual VH and VL domains were built by homology modeling to known structures using the COMPOSER module in SYBYL 6.22 (Molecular modeling program SYBYL 6.22, Tripos Associates, St. Louis, MO). A family of structurally homologous molecules with sequence identities of at least 60% to the modeled chains were used as templates. For the heavy chain, 7 immunoglobulin fragments were selected, while for the light chain, 22 fragments were used. The structurally conserved regions were built by averaging the template structures according to the COMPOSER algorithm. The remaining LOOP regions were built using template loop fragments found among Fab fragments in the protein structure data base of the COMPOSER module. The individual VH and VL domains were docked to each other to form the Fv fragments. A structural alignment between the individual VH and VL chains and the crystallographic structure of a murine Fab, entry 1MCP in the Protein Data Bank (23), was made using the ALIGN procedure of the ICM program (24). Finally, hydrogen atoms were added, and the structure was refined by a regularization algorithm in the ICM algorithm (24). RESULTS

Cloning and Expression of Recombinant 5T4Fab-SEch Constructs—The variable regions of the antibody ST4 were cloned using PCR and introduced into an expression vector (12) coding for a Fab product with a superantigen linked to the C terminus of the heavy chain. The plasmid was transformed into an ompT strain of E. coli, UL635, and expression of the recombinant product was induced with IPTG. The product was secreted as amide gel electrophoresis was performed on precast Tris/glycine gels (NOVEX, San Diego, CA) containing 12% polyacrylamide. The products were analyzed as both reduced and non-reduced samples using the methods recommended by the supplier. Isoelectric focusing was performed using precast gels (Servalyte Precotes, Serva, Heidelberg, Federal Republic of Germany) with a resolving power between 15 and 30 using the methods recommended by the supplier. Mass spectrometry was carried out on a MALDI-TOF MS (Hewlett-Packard), and amino acid analysis was performed using a Beckman 6300 essentially as described (19).

Cytotoxicity Assay—Cytotoxicity was measured in a 3Cr release assay after 4 h using the 5T4 antigen-positive Colo205 cultured in combination with 5000 cells (21) as target cells and human SEA-reactive T cell lines (12) at an effector to target ratio of 30:1. 3Cr-labeled target cells were used in the assay at 2500 cells/200 μl of tissue culture medium in V-bottomed microwell plates. 5T4Fab-SEch fusion proteins were added at various concentrations, and 3Cr release was measured in a γ-counter. Specific cytotoxicity was calculated as 100 × (cpm experimental release – cpm background release)/(cpm maximal release – cpm background release).

Determination of Antigen Binding Characteristics—The human cancer cell lines Calu-1 (ATCC HTB 54) and ME-180 (ATCC HTB 33), both expressing high levels of 5T4 antigen as demonstrated by fluorescence-activated cell sorter staining, were cultivated in tissue culture medium as above. Adhered cells were detached from the flasks using nonenzymatic cell dissociation solution (Sigma), washed twice in a CO2 independent medium without l-glutamine (Life Technologies, Inc.) containing 10% fetal calf serum, and finally suspended in that medium at a density of 6 × 105 cells/ml.

The 5T4Fab-SEch was radiolabeled with the lactoperoxidase technique using Enzymobeads (DuPont NEN). The reaction was stopped with 0.05% Na2S2O3, and the labeled protein was desalted by gel filtration (PD-10, Pharmacia) using culture medium as elution buffer. Conditions were chosen to obtain a ratio of iodine to protein of 2:1.

In a direct binding assay, 3 × 106 cells in 50 μl of solution were mixed with 50 μl of serially diluted radiodinated fusion protein in a protein polycarbonate tube in triplicate and incubated for 2 h at room temperature with intermittent mixing. Each tube was washed using 9 ml of phosphate-buffered saline containing 1% fetal calf serum, which was removed by centrifugation for 5 min at 1000 × g. After the final wash, cell-bound radioactivity was determined in a γ-counter. The apparent dissociation constant and number of binding sites at saturation were calculated (22) after subtraction of nonspecific binding (i.e., binding after incubation in the absence of cells). This method was modified to an inhibition assay. Here serially diluted fusion proteins competes with wild-type 5T4Fab-SEch at a concentration corresponding to the K50 value determined in the direct assay. The concentration yielding half-maximum inhibition, IC50, was determined after linear regression of log-log transformed binding data, and the relative affinity index was determined as the ratio between the IC50 values of competitor and wild-type 5T4Fab-SEch.
Using an enzyme-linked immunosorbent assay method the level in the growth medium and total yield in a sonicated mixture of growth medium and bacteria were determined. The IC_{50} values were obtained from affinity measurements to 5T4 antigen-positive cells, and the biological activity was determined using a cytotoxicity assay. The variants of the 5T4 heavy chain contain the following substitutions: H41P, S44G, I69T, and V113G (mutant 1 (m1)) and H41P, S44G, and V113G (mutant 2 (m2)).

**TABLE I**

| Variant | Light chain replacements | Heavy chain | Yield | Medium | Total | IC_{50} | Activity |
|---------|--------------------------|-------------|-------|--------|-------|--------|----------|
| F10S    |                         | 5T4         | 39    | 48     | 1.5   | 100    |
| T45K    |                         | 5T4         | 39    | 59     | ND*   | 0      |
| R68S    | C215                     | 5T4         | 224   | 297    | 1000  | 0      |
| Y67S    | X^b                      | 5T4         | 92    | 126    | 1.2   | 100    |
| F73L    |                         | 5T4         | 224   | 297    | 1000  | 0      |
| T77S    | X                        | 5T4         | 39    | 52     |       |        |
| L78V    | X                        | 5T4         | 93    | 136    | 2.9   | 100    |
| L85A    | X                        | 5T4         | 39    | 44     |       |        |
| V10     | X X X                    | 5T4         | 57    | 86     |       |        |
| V9      | X X X                    | 5T4         | 53    | 77     |       |        |
| V11     | X X X                    | 5T4         | 214   | 250    | 2.6   | 100    |
| V12     | X X X                    | 5T4         | 586   | 701    |       |        |
| V13     | X X X                    | 5T4         | 512   | 470    | 2.0   | 100    |
| V14     | X X X                    | 5T4 (m1)    | 578   | 560    | 1.4   | 100    |
| V15     | X X                      | 5T4 (m2)    | 288   | 730    | 1.9   | 100    |
| V16     | X X                      | C215-(1–23)-5T4 | 250  | 560    |       |        |
| wt      |                         | 5T4         | 110   | 124    | 5.4   | 100    |

^a ND, not detected.  
^b X, substitution introduced in the respective variant.

two separate polypeptide chains that assembled to a heterodimeric product in the *E. coli* periplasm. During fermentation, a significant amount of the two-chain product is excreted to the growth medium and usually connected by a significant cell lysis. Normally the levels of Fab supernatant products range from 100 to 400 mg/liter in the growth medium (data not shown). However, for 5T4Fab-SEch the production level was around 40 mg/liter in the growth medium and less than 10 mg/liter in the periplasm, as determined with enzyme-linked immunosorbent assay (Table I). To increase the yield, several parameters in the fermentation procedure were varied such as time point and level of induction, temperature, and medium composition, but no further improvement in production level was achieved.

**Construction and Investigation of Hybrid Molecules between 5T4-SEch and C215Fab-SEch**—To determine whether one of the two polypeptide chains dominated the production problem, hybrid molecules of 5T4Fab-SEch and C215Fab-SEch were made. C215 is a murine antibody recognizing a colon cancer epitope (25), and fusion proteins between C215Fab and SEA-based superantigens are normally secreted at levels up to 400 mg/liter in *E. coli*. Fermentation of variant V1 with the 5T4 light and C215 heavy chain yielded 39 mg/liter product in the growth medium, while variant V2 with C215 light and 5T4 heavy chain yielded 224 mg/liter product (Table I). For V1 and V2, most of the product was found in the growth medium instead of the periplasm (Table I). Hence, replacing the heavy chain of 5T4 did not affect the low production level, while replacement of the light chain resulted in a more than 5-fold increase.

One further observation was made during fermentation. For the 5T4 wild-type construct, as well as variant V2 that contained the C215 light chain, the A_{abs} started to decline and the cell viability decreased more than 10-fold within a few hours after induction (Fig. 3), followed by an increased DNA level in the growth medium. The variant V1 that contained the C215 heavy chain behaved differently. The amount of viable *E. coli* cells was more than 10-fold higher for V1 than for V2 with 5T4 heavy chain when fermentation was terminated. In addition, the final cell density was much higher (Fig. 3).

The fusion proteins were purified using protein G affinity and then ion exchange chromatography to remove degraded forms. The purified products were analyzed with SDS-polyacrylamide gel electrophoresis, reverse phase HPLC, mass spectrometry, isoelectric focusing, and amino acid analysis. The latter technique was also used to determine protein concentrations. All of these assays indicated that the main product contained and constituted 85–95% of the expected characteristics. However, both products showed a strongly reduced affinity for the 5T4 antigen (16), and only variant V2 with the 5T4 heavy chain had measurable affinity. The IC_{50} value was lowered approximately 1000-fold, and the products were at least 1000-fold less potent in cytotoxic activity (Table I).

Three important conclusions could be made from these data. The low yield of product was mainly associated with the 5T4 light chain; the high cell lysis during fermentation was primarily associated with the 5T4 heavy chain, and both chains contain residues important for binding to the 5T4 antigen.

**Molecular Modeling of 5T4 Fo**—To explain the low production level of 5T4, a model was built. Here information regarding exposed hydrophobic residues, structural identification of the complementarity-determining regions, CDRs, and insights into the structural environment of the residues described below was obtained. The high sequence identity of more than 60% to a relatively large number of template structures ensured that the overall accuracy of the model was good. The most uncertain regions are those modeled as LOOP regions, *e.g.* either not structurally conserved within the family of template structures or not highly homologous to them. However, for these LOOP regions such templates, which fitted well to the structurally conserved regions, were found among other immunoglobulin structures. Of the residues investigated only Tyr-67 in the light chain and Val-113 in the heavy chain were situated in the structurally conserved regions, were found among other immunoglobulin structures. Of the residues investigated only Tyr-67 in the light chain and Val-113 in the heavy chain were situated in the structurally conserved regions. Therefore, the model most likely correctly predicts whether the residues studied were exposed or buried and whether a certain residue is needed to support the CDR loop structure.

**Engineering of the 5T4Fab-SEch Construct**—Based on the finding that V1 replacement in 5T4Fab efficiently increased the production level but affected the binding properties, the molecule was modified to identify residues that hampered high level production. Hydrophobic residues, suggested to be on the Fab surface by computer modeling (Fig. 2), were replaced by serine...
residues. Selected residues differing from the equivalents in more readily produced Fab fragments such as C215 were exchanged for the latter (Fig. 1). To minimize putative effects in affinity and specificity, residues in the CDRs were not altered. The CH1 and CL regions were identical in all antibodies studied. The chosen substitutions were Phe-10→Ser, Thr-45→Lys, Ile-63→Ser, Tyr-67→Ser, Phe-73→Leu, Thr-77→Ser, Leu-78→Val, and Leu-83→Ala in the light chain. In addition, to identify heavy chain residues that could affect the yield or cell lysis as suggested by the hybrid studies, the substitutions His-41→Pro, Ser-44→Gly, Ile-69→Thr, and Val-113→Gly were investigated. The positions of these residues and a sequence alignment between the Fv regions of 5T4 and C215 are shown in Figs. 1 and 2. In the model, Phe-10, Thr-45, Ile-63, and Thr-77 in the light chain are exposed side-chain residues. Consequently, the replacements Phe-10→Ser, Ile-63→Ser, and to a lower degree Thr-77→Ser should make the product less hydrophobic. The substitutions Phe-73→Leu and Leu-78→Val were made in the completely buried hydrophobic core of the light chain. The light chain residue Tyr-67 is in a loop close to the CDRs. Replacing this residue may therefore change the binding properties of the molecules. The heavy chain substitutions His-41→Pro and Ser-44→Gly involved exposed side chains positioned at the N and C terminus, respectively, of a sharp turn connecting two framework β strands. Both proline and glycine residues are important in stabilizing sharp turns in proteins. The substitutions Leu-83→Ala in the light chain and especially Val-113→Gly in the heavy chain may affect the interactions with the constant domains. Although not modeled, these residues are in the domain-domain interface of structural homologues. Finally, to find out if other residues in framework affected the yields, a variant of 5T4 containing the 23 N-terminal residues of the C215 light chain instead of the wild-type ones, was constructed. The effects of the different substitutions were investigated as single or combined amino acid replacements. In a reverse phase HPLC system (Fig. 4), the variant chains of 5T4 are much more hydrophilic than the wild-type chains.

**Impact of Engineering on Production Levels**—The hybrid variants of 5T4 and C215 suggest a replacement of critical light chain residues in 5T4 to obtain a higher production level. Indeed, enzyme-linked immunosorbert assay measurements
after fermentation showed that individual substitutions had substantial impact on the yields. Notably, a single substitution Phe-10 → Ser, variant V3, increased the level from 39 to 92 mg/liter in the growth medium (Table I). Further substitutions increased the production levels continuously and by introducing five or seven point mutations in the light chain, variants V11–V13, the growth medium levels exceeded 500 mg/liter. For these variants, the V₄ moiety may no longer be the limiting component. Phe-10 → Ser was the most important replacement, followed by similar and almost additive effects from Thr-45 → Lys, Thr-77 → Ser, and Leu-78 → Val. Furthermore, Leu-83 → Ala also enhanced the yield but was not studied in combination with the others. Except for Phe-10 → Ser, replacing the complete framework I did not drastically alter the level as seen in variants V5 and V16.

Significant cell lysis was observed during cultivation of wild-type 5T4Fab-SEch and the most product was found in the periplasm. However, in variant V14 with seven light chain and four heavy chain substitutions, the level was 288 mg/liter in the growth medium and almost 450 mg/liter in the periplasm (Table I). Subsequently, combined with a suitable light chain, the heavy chain replacements increased the total level of fusion protein to 30%. The DNA levels in the growth medium, reflecting the cell lysis, showed that variant V13 contained more than 1 g of DNA/liter, while V14 contained less than 0.2 g. A hybrid molecule, V15, with C215 light chain and 5T4 heavy chain with the replacements His-41 → Pro, Ser-44 → Gly, and Val-113 → Gly, was also investigated. This molecule gave approximately the same yield of product in the growth medium, 250 mg/liter, as hybrid V2. However, similarly to variant V14, cell lysis was less pronounced with this heavy chain, indicating that the substitution Ile-69 → Thr was less important for increased cell viability.

Thus, replacing a few residues in the 5T4 light chain increased the yield almost 15-fold and was further augmented by heavy chain substitutions. The heavy chain replacements altered the phenotype of the E. coli cells during fermentation, and less lysis was observed. Subsequently, most of the product was found in the periplasm instead of the growth medium.

Analysis of the Mutated Forms of 5T4—Similar to the 5T4 and C215 hybrids, the variants of 5T4Fab-SEch were purified, and biochemical analyses showed approximately 85–95% of the main component (Fig. 4) with expected characteristics. The minor products seen on reverse phase HPLC are isomers of the light or heavy chains that are not separated from the wild-type chains on SDS-polyacrylamide gel electrophoresis.

To investigate whether the replacements affected biological properties, the different products were analyzed for binding to the 5T4 antigen, and since the constructs were aimed for immunotherapy, a functional in vitro assay was also performed. None of the substitutions seemed to have a significant effect on cytotoxic activity (Table I), but replacement of Ile-63 and Tyr-67 with serine residues as in variants V4, V5, and V10 resulted in a reduced affinity for the antigen by approximately 50% (Fig. 5 and Table I). Surprisingly, this effect was reversed by the light chain substitutions Phe-73 → Leu, Thr-77 → Ser, and Leu-78 → Val in variants V12 and V13. The variant V16 containing the 23 N-terminal residues from C215 combined with the substitutions Ile-63 → Ser and Tyr-67 → Ser had an affinity of approximately 30% compared with the wild-type 5T4Fab-SEch (Fig. 5 and Table I). This indicated that unknown residues in framework 1 of 5T4 stabilized the antigen binding site, and if replaced by the equivalents from C215, the binding properties were affected. These effects were not studied further.

In conclusion, none of the replacements resulted in a dramatic alteration in either affinity or cytotoxic activity of the 5T4Fab-SEch molecule. However, some of the substitutions slightly changed the binding properties.

**DISCUSSION**

It was recently demonstrated that particular amino acid residues in the CDRs of recombinant antibodies can influence the level of secreted product in *E. coli* (26, 27). Here that finding was extended showing that Fv framework substitutions significantly enhanced the yield of a secreted Fab-fusion protein in *E. coli*. Two approaches were used to design variants of the antibody 5T4. Hydrophobic residues, likely to be on the framework surface according to molecular modeling, were replaced with Ser or Ala, and a few less frequent residues were replaced by those of an antibody that can be produced at relatively high yields in *E. coli*. To minimize the risk of changing the binding properties, CDR engineering was not performed. Using only five light chain substitutions, the product level in the growth medium increased approximately 15 times without significantly modifying the affinity for the antigen. This level was higher than for the model antibody C215. The high producing variants V11–V13 all reach a level above 500 mg/liter, and here the V₄ part may not be the limiting component. The hydrophobic light chain residue Phe-10, which is totally exposed in the model (Fig. 2), was very limiting for high level production. In variant V3, Phe-10 was replaced with Ser, which resulted in a 2.5-fold increase in the production level. In addition, the substitutions Thr-45 → Lys, Thr-77 →
Ser, Leu-78 → Val, and Leu-83 → Ala increased the yield especially when Phe-10 was replaced as observed with the variants V5 and V7–V13 (Table I).

While the light chain substitutions had a tremendous impact on the final yield, the heavy chain replacements primarily affected product localization (Fig. 3, Table I). Thereby, a tool that enables targeting of recombinant antibody fragments to either the periplasm or the growth medium might have been identified. Whether it is optimal to obtain a recombinant product in the periplasm or growth medium can be questioned, but for downstream processing there are definite advantages to recovering a product from the growth medium. In accordance with this study, a recent investigation of heavy chain loop substitutions show that residues controlling production level and periplasmic leakiness may differ (26). A Pro as residue 40 led to a higher leakiness compared with Ala, while the substitutions Ser-61 → Ala and Ala-62 → Asp (using the 5T4 positions) lead to higher production levels. Notably, Pro-40 resided in the corresponding turn that appeared to be important for periplasmic leakiness in this study. By comparing previously reported yields with our results, it seems possible that the residues studied here generally determine the production level for secreted antibody fragments. For instance, one antibody reported to be produced very poorly in E. coli contains a Phe at position 10 (27). Also, the humanized Fab secreted in approximately 1 g/liter (7) contains most of the optimal residues like Ser-10, Lys-45, and Ser-77 as well as those found important by Knappik and Plückthun (26). The heavy chain substitution Ile-69 → Thr seems to have less impact on product levels and localization (Table I) (26).

There are several possible explanations for the drastic differences observed. For instance, compared with the engineered variants, wild-type 5T4Fab may have poor folding properties, lower solubility of the unpaired chains, a higher tendency for aggregation, a higher formation rate of unproductive light chain dimers (28), or a lower stability toward proteolysis (29). Also the wild-type mRNA could have a low stability, or less likely there may be problems with the translocation initiation process (30). The periplasmic folding process has been suggested to be the major limitation for secretion of recombinant antibodies in E. coli (31, 32). Furthermore, replacing residues identified as limiting indeed improved in vitro refolding of reduced and denatured Fv molecules (26), and proline isomerization was rate-limiting for that folding process (33). It is therefore likely that at least some of the substitutions in 5T4 caused an effect that facilitated proper folding. Replacing hydrophobic residues on protein surfaces with more hydrophilic ones has yielded products with suppressed tendencies for aggregation or dimer formation during production (34). Therefore, the variant light chains of 5T4, which are more hydrophilic than the wild-type chain, are probably less prone to aggregate. Preliminary analyses on the amount of light chain dimers indicate that in no case does the level exceed that of Fab (data not shown). Recently, a folding model for recombinant antibodies in E. coli was suggested (33). Here, the light chain acts as a folding template for the heavy chain that would otherwise aggregate. Our data do not contrast that model. Thus, the final yield was probably determined by the folding and aggregate-forming properties of the light chain and the time needed for the heavy chain to find its partner chain before precipitation, which may induce stress to the host cell. None of the substitutions in 5T4 resulted in a drastic change in affinity for the antigen. According to the model (Fig. 2) only Tyr-67 is positioned close to the CDRs. Combining the substitutions Ile-63 → Ser and Tyr-67 → Ser or replacing the light
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