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Production and epitope characterization of mAbs specific for translation factor IF1

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Abstract

Initiation of protein synthesis in bacteria relies on the presence of three translation initiation factors, of which translation initiation factor IF1 is the smallest having a molecular weight of only 8.2 kDa. In addition to its function in this highly dynamic process, the essential IF1 protein also functions as an RNA chaperone. Despite extensive research, the exact function of IF1 in translation initiation has not yet been determined, and the research in the function of the factor has in some areas been impeded by the lack of monoclonal antibodies specific for this protein. Several attempts to induce immune response in mice with wild-type IF1 for the production of antibodies have failed. We have now succeeded in producing monoclonal antibodies specific for IF1 by applying a new immunization strategy involving an antigen combination of IF1 coupled to glutathione S-transferase (GST) and a recombinant dimer of IF1. This resulted in the generation of 6 IgG, 2 IgM, and 1 IgA anti-IF1 antibodies, which can be used in ELISA screening and Western immunoblots. We also provide a mapping of the functional epitopes of the generated anti-IF1 monoclonal antibodies by screening the antibodies for binding to IF1 proteins mutated at single amino acid positions.

Keywords: Monoclonal antibody; Translation initiation factor IF1; Immunization; Glutathione S-transferase; Epitope mapping; IF1 mutants

Bacterial translation initiation factor IF1 is a small 8.2 kDa protein, which is involved in initiation of protein synthesis [1]. It has been shown to influence several steps in this process. IF1 stimulates formation of the 30S pre-initiation complex [2–4] and inhibits premature docking of 50S subunits to 30S subunits lacking initiator tRNA [5]. Being positioned at the ribosomal A-site, IF1 occludes tRNAs from this site, accelerates binding of initiator tRNA to the P-site [6] and enhances the accuracy of initiator tRNA selection [7]. Removal of IF1 reduces initiation efficiency seven-fold as shown by kinetic studies [5], so IF1 is clearly an important factor even though a single specific function has not yet been assigned to it.

IF1 also displays RNA chaperone activity [6,8]. During cold shock conditions the level of IF1 is moderately increased [9], and IF1 mutants confer cold sensitivity to cells [10]. Furthermore, the RNA chaperone activity has been linked to transcription antitermination [8], an activity also ascribed to cold shock proteins [11]. Mutating the amino acid residue responsible for the IF1 transcription antitermination activity does not affect cell viability, why it has been presumed that the essential function of IF1 is in translation and not in cold shock response. However, recent results indicate an essential role for IF1 during cold adaptation [12].

The structure of bacterial IF1 was determined by NMR spectroscopy in 1997 [13], and it revealed a remarkably rigid antiparallel five-stranded β-barrel. This structure is referred to as an oligonucleotide- or oligosaccharide-binding fold (OB-fold) and is also found in cold shock proteins

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CspA and CspB. The IF1 structure also includes a single short 3_{10} helix loop and disordered N-terminal and C-terminal regions.

The structures of eukaryotic and archaeal IF1 homologues, eIF1A and aIF1A, also include OB-folds and are highly similar to IF1 (Fig. 1). In addition to a 3_{10} helix between β-strands 3 and 4, eIF1A contains a long α-helix and two ordered extended strands C-terminal to the β-barrel. However, since the β-barrel structure constitutes the main part of IF1, there are barely any structural characteristics to separate the bacterial IF1 from human eIF1A. The structure of eIF1A in *Mus musculus* has not yet been determined, but the amino acid sequence differs in only 2 positions from that of the human eIF1A (Fig. 2), so the structures of human and mouse eIF1A are expected to be highly similar.

Surface structures of *Escherichia coli* IF1 are shown in Fig. 2. In this representation, amino acid residues identical or similar to sequence positions in mouse eIF1A are highlighted, clearly revealing the surface structure similarities. The close structural similarity may be the reason for the lack of immune response experienced when attempting to produce monoclonal antibodies against IF1 by immunizing mice with the wild-type monomeric protein. Another reason for the difficulties in provoking an immune response in mice to bacterial IF1 may be the small size of the protein.

A recombinant IF1 dimer ($IF1_2$) consisting of two IF1 molecules connected by a short linker (Fig. 1) was constructed for the use as antigen in an immunization protocol for the production of monoclonal anti-IF1 antibodies. This protocol included the IF1 dimer as well as IF1 conjugated to a glutathione S-transferase (GST) tag, which has previously been used alone in immunization protocols [14]. An immune response was generated, and monoclonal antibodies specific for *E. coli* IF1 was obtained. The binding of these monoclonal antibodies to IF1 mutant proteins with single amino acid substitutions were tested by ELISA screening, and the results were used to map the functional epitope of each antibody. The amino acid residues that are part of the functional epitopes are also part of the structural epitopes, which are expected to include mostly the residues that do not align to the eIF1A sequence.

Materials and methods

Strains and plasmids. A recombinant dimer of IF1 molecules was designed with a short linker between the two monomers and a C-terminal His-tag. Construction of a plasmid encoding this dimer was performed by PCR amplification of *infA* sequences using two sets of primers: 5′GCG ATAACCATGGCCAAAGAAGACAATATTGAAAT (forward primer, underscored: restriction site, boldface: start codon) and 5′GCGATA GGATCCGCCGCGACTACGGAAGACAATGC (reverse primer), 5′G ATAGGATCCGGCGGGATGGCCAAAGAAGACAATA (forward primer) and 5′GCGATAAAGCTTGCGACTACGGAAGACAATGCG GC (reverse primer). The two sequences were inserted into pET24d vectors (Novagen) using the restriction sites NcoI, BamHI, and HindIII. Competent JM109 and BL21 (DE3) cells (Novagen) were prepared using Transformation and Storage Solution, essentially as described by Chung et al. [15]. The JM109 transformants obtained were subjected to sequence analysis to verify the presence of the expected sequences. The constructed pET24d*infA*$_2$ plasmid was isolated and transformed into BL21 (DE3) cells for protein expression.

Protein expression and purification. $IF1_2$ protein was expressed in BL21 (DE3) cells, which were cultivated in 2xTY medium (16 g/L peptone from casein (AppliChem), 10 g/L yeast extract and 5 g/L NaCl) containing 50 μg/mL kanamycin. The cultures were incubated at 37 °C with constant shaking, until the optical density at 550 nm reached 0.8. IPTG was added to a final concentration of 0.1 mM to induce protein expression, and the cultures were incubated at 37 °C with constant shaking for 5 h. The cells were harvested by centrifugation at 9500*g* for 10 min followed by a wash in 0.9% NaCl and centrifugation at 4700*g* for 25 min.

The pelleted cells were resuspended in 2 mL of buffer A (50 mM Tris–HCl, pH 7.6, 500 mM NaCl, 0.1 mM PMSF, 5 mM imidazole) per gram of cells. The cells were disrupted by sonication and ultracentrifuged at 250,000*g* and 4 °C for 75 min. The filtered supernatant was loaded onto a nickel-charged His-Bind IMAC column (all columns were from GE Healthcare), and the His-tagged $IF1_2$ protein was eluted using an increasing gradient of imidazole (5–500 mM). The fractions containing the protein of interest were pooled and dialysed against H(0) buffer (50 mM Hepes, pH 7.6, 10 mM $MgCl_2$, 15 mM NaN_3) in a membrane tubing with 6–8 kDa molecular weight cut-off (MWCO). The protein was further purified on an SP-Sepharose HP column using buffer H(0) and an increasing NaCl gradient (0–1000 mM). The protein was dialysed (6–8 kDa MWCO) against 50% buffer H(100) (50 mM Hepes, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, and 15 mM NaN_3) and 50% glycerol and stored at −20 °C.

The IF1 mutant proteins were purified as described previously [6,16].

Generation and screening of monoclonal antibodies. Purified GST-IF1 (H.P. Sørensen, unpublished) and $IF1_2$ were used to immunize a BALB/c mouse. GST-IF1 was used for the first and second immunization, and 1:1 mixtures of GST-IF1 and $IF1_2$ were used for all the following immunizations. Monoclonal antibodies were produced by the hybridoma technique, screened for isotype and purified on Protein G Sepharose 4B as described previously [17]. Clones were screened by ELISA for their specificity to IF1 using purified IF1 (H.P. Sørensen, unpublished). Western immunoblots were prepared as described [18] using the purified antibodies as primary antibodies and HRP-conjugated rabbit anti-mouse antibodies (DAKO) as secondary antibodies.

For the epitope mapping, ELISA screening was performed using the IF1 mutant proteins and hybridoma cell supernatant from each of the six chosen monoclonal antibodies.

Results and discussion

A plasmid encoding a recombinant dimeric IF1 protein was constructed and used for the expression and purification of this chimeric protein for mouse immunizations. Our strategy involved initial immunizations of the BALB/c mouse with GST-IF1 to ensure an efficient immune response. The GST-tag is rather large compared to IF1, so for the following immunizations we used 1:1 combinations of GST-IF1 and $IF1_2$ to create a better exposure of the IF1 surface areas to the immune system of the mouse.

Hybridoma cells were obtained from fusion of SP2/0-AG14 mouse myeloma cells and mouse spleen cells. Repeated screening of hybridoma cell supernatants for specificity towards IF1 and cloning resulted in the identification of 9 monoclonal hybridoma cell lines, and the antibodies were class-characterized as 6 IgG, 2 IgM, and 1 IgA by isotype analysis. The IgG antibodies were affinity purified on a Protein G Sepharose 4B column, whereas the IgM

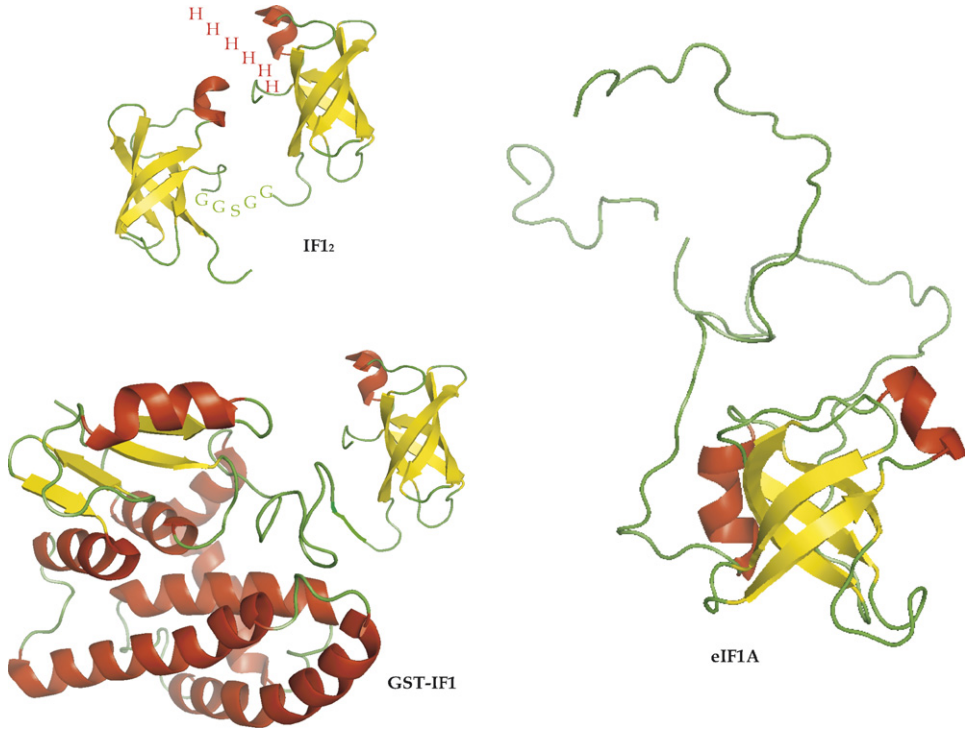

Fig. 1. IF1 structures. Recombinant $IF1_2$ is composed of two IF1 molecules connected by a linker and a C-terminal His-tag. The other antigen used is a fusion between GST and IF1. The IF1 homologue, eIF1A, is shown on the right to illustrate the structural similarity between prokaryotic/bacterial IF1 and eukaryotic/mammalian eIF1A. The structures are not to scale.

and IgA antibodies were used directly from the hybridoma cell supernatant. The antibodies were screened for efficiency in Western immunoblots (Table 1).

The sequences of *E. coli* IF1 and eIF1A from *Mus musculus* are aligned in Fig. 2. The central part of eIF1A clearly shows a high degree of sequence homology to *E. coli* IF1, and this homology persists in the secondary structures (Fig. 2) (as mentioned previously, the structure of *M. musculus* eIF1A is predicted to be virtually identical to the human eIF1A structure).

Since IF1 structurally resembles eIF1A in the mouse, its immune system may not recognize IF1 as a foreign protein, hence normally not produce antibodies against it. Immunizing a mouse with GST-tagged IF1 and a recombinant IF1 dimer provoked an immune response and production of antibodies, which means either (i) that IF1 in these chimeric proteins assumes a partly new conformation, which induces antibody production targeting epitopes that is recovered in the wild-type IF1 or (ii) that the simple size increase has induced an immune response targeting surface areas of residues not conserved in eIF1A.

The structural epitope of a protein usually covers a 5–10 nm^2 (500–1000 $Å^2$) surface area involving 14–20 amino acid sidechains. Only few of these sidechains are responsible for the majority of the binding affinity, and the functional epitope may only involve 4–14 antigen sidechains. Testing the binding efficiency of antibodies to antigens with amino acid substitutions is one way to determine the functional epitope, from which the position of the structural epitope may be considered.

In the sequence alignment (Fig. 2), vertical arrows indicate the residues substituted in the IF1 mutants. The mutants contain single amino acid substitutions distributed over the entire length of the IF1 sequence, and the substitutions replace both conserved and unconserved amino acid residues. Only one IF1 mutant protein contains a double amino acid substitution, viz. Arg69Leu + Arg71Leu. Except for Val12Ala and Ser36Pro, all amino acid substitutions involve a change in the electric charge of the residue, either between positive/negative or charged/uncharged.

The 3AE12 IgG and the two IgM antibodies show low efficiency in ELISA, which complicates the determination of the effect of the amino acid substitutions, so they have not been considered in the mapping of functional epitopes. The results of the ELISA screening of each of the 6 remaining monoclonal antibodies (mAbs) are shown in Fig. 3 and listed in Table 1. The ability of the antibodies to recognize IF1 is clearly affected by the different mutations.

Amino acid substitutions in positions 12, 36, 40, 45, and 50 result in significantly reduced binding of both mAb 1AD9 and 3CB9. These residues are found in the top of the protein as depicted in Fig. 4, and these residues are close to or part of the 3_{10} helix loop comprising residues 36–49. Except for the Arg40Leu substitution, these residue substitutions in IF1 all lead to significantly or moderately reduced growth rates of their host cells [10], suggesting a functional importance of these residues, which may be structurally related. Especially for Ser36Pro, which is thought to involve changes in the three-dimensional orientation of the following 3_{10} helix [10]. Only residues 36 and 45 are unconserved residues com-

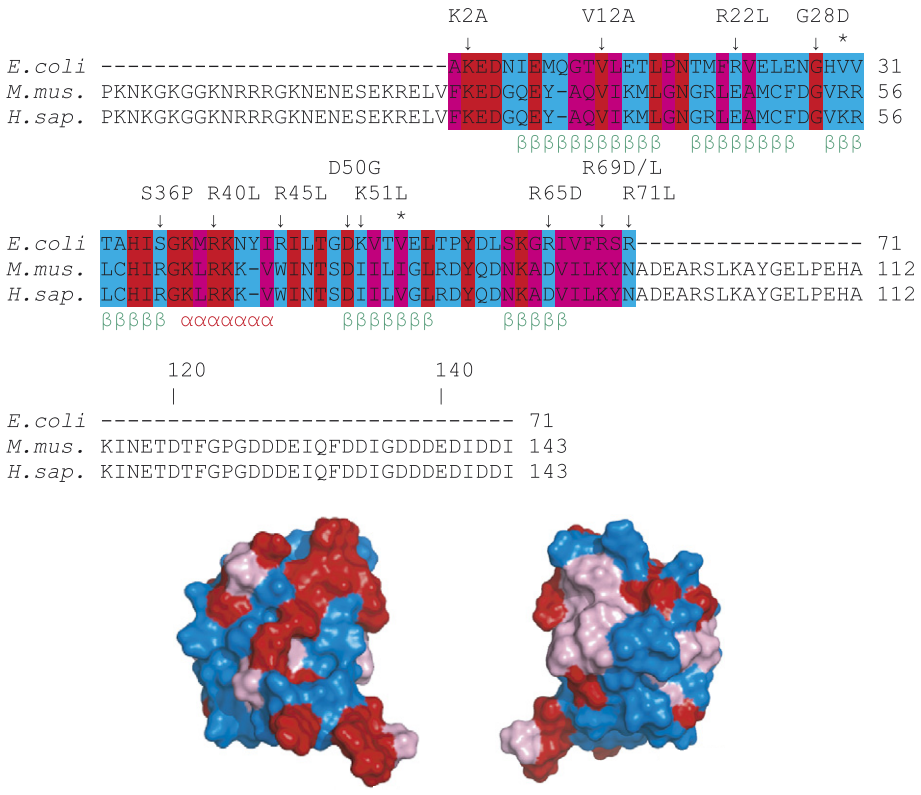

Fig. 2. Comparing IF1 to eIF1A. (Top) The sequences of *E. coli* IF1 and eIF1A homologues from *M. musculus* and *H. sapiens* are aligned and coloured according to the following colour code: Identical positions, red; similar positions, magenta; nonsimilar positions, blue. The 3_{10}-helix and the β-strands of *E. coli* IF1 are marked by α and β characters, respectively. The two asterisks mark the only sites, at which the amino acid sequences of eIF1A from *M. musculus* and *H. sapiens* differ. The positions mutated for epitope mapping are marked by vertical arrows. (Bottom) Ventral and dorsal view of *E. coli* IF1 surface structure. The colour-coding is the same as of the IF1 sequence alignment.

pared to *M. musculus* eIF1A, but the mAb 1AD9/mAb 3CB9 epitope may also include the adjacent unconserved residues Asn42 and Leu47, which have not been tested here. At least, they will most likely be part of the structural epitope for these two mAbs.

Only mAbs 1BD3 and 3AE12 seem unaffected by the V12A substitution, which is puzzling in that this valine residue is situated in the middle of the β-barrel of the OB-fold and is barely visible on the IF1 surface. However, the cavity created by replacing alanine for this residue may lead to a destabilization of the β-barrel and a conformational change, which affects the epitopes of most of the mAbs in this study. The only observation that seems not to correspond with this explanation is the moderate effect of this substitution on the growth rate of host cells of this IF1 mutant protein [10].

Table 1
Monoclonal antibodies specific for *E. coli* IF1

mAb	Isotype	Western	ELISA	Residues of func. epitopes
1AD9	IgG_1	++	+++	12, 36, 40, 45, ~50
1BD3	IgG_1	+	+++	~45, ~50
1CE7	IgG_1	++	++	12, 28, 36, 40, 45, 50, 65, 69
3AE12	IgG_1	+	+	ND
2EF10	IgG_{2b}	++	+++	12, ~36, 50
3CB9	IgG_{2b}	++	+++	12, 36, 40, 45, 50
1BB12	IgM	÷	+	ND
1EE6	IgM	÷	+	ND
2CD12	IgA	+	++	~12, ~65

The antibodies were tested in Western immunoblots and evaluated by visual inspection of the blot intensities (÷, none; +, low intensity; ++, high intensity). The binding efficiencies of the mAbs in ELISA were evaluated based on the relative OD measurements of their binding to wild-type IF1. 0–0.2: +; 0.2–0.4: ++; >0.4: +++. The residues, whose substitution leads to loss of or reduced binding of the tested mAb (Fig. 3), are listed in the column to the right. The functional epitopes for the fourth IgG and the two IgM antibodies were not determined.

The binding of mAb 1BD3 is only appreciably affected by the substitution of the unconserved Arg45 for aspartic acid and to a lesser degree of the substitution of the conserved Asp50 for glycine. So these two residues might only be in the periphery of the functional epitope, which might stretch towards the top and include the unconserved Asn42 and Leu47.

All amino acid substitutions result in significantly reduced mAb 1CE7 binding, but the effect is most significant for residues 12, 28, 36, 40, 45, 50, 65, and 69. This includes the same residues as the epitope for mAb 1AD9 and 3CB9. Except for Gly28, all these residues are found at the top of the protein as depicted in Fig. 4, but the surface area positions of these residues indicate a rather large epitope compared to the others. It is puzzling that the Arg69Leu substitution, involving the removal of a positive charge, decreases mAb 1CE7 binding efficiency more than

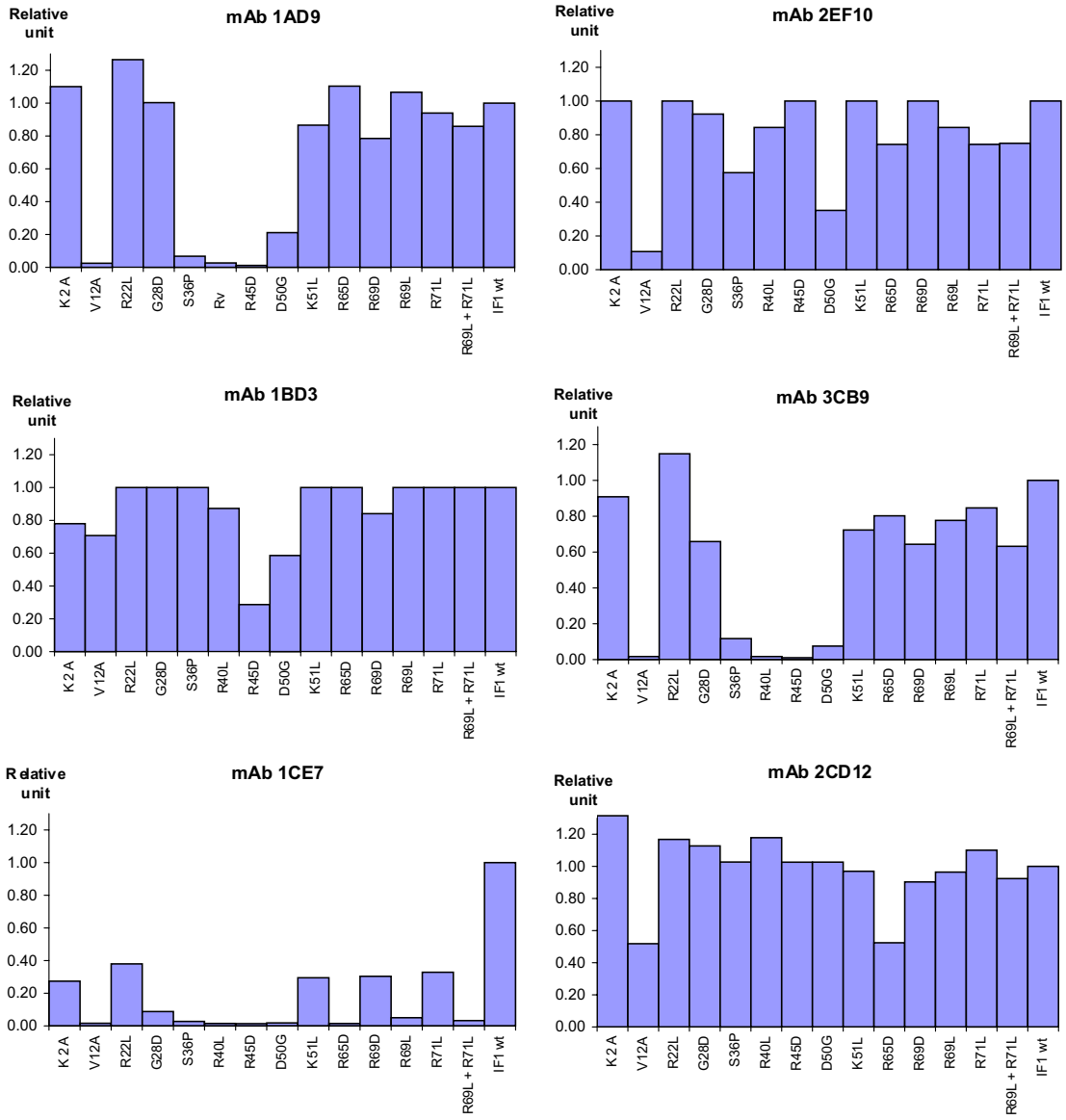

Fig. 3. Epitope mapping using ELISA screening. Each mAb, except the fourth IgG and the two IgM antibodies, was tested for its binding to 14 different IF1 mutants as well as wild-type IF1 by ELISA screening. The OD_{492}-measurements were normalized to the measurement for wild-type IF1 in each assay.

the Arg69Asp substitution, involving the replacement of a positive charge for a negative. Maybe the carbonyl oxygen found in both arginine and asparagine is especially important for mAb 1CE7 binding.

The binding of mAb 2EF10 is mostly affected by the Val12Ala substitution and weakly by Ser36Pro and Asp50Gly. Since this binding is almost unaffected by the other substitutions, the functional epitope for mAb 2EF10 is indicated to be smaller than the epitopes for mAb 1AD9, 1CE7, and 3CB9, which also include these three residues.

Finally, only substitutions of residues 12 and 65 affect binding of mAb 2CD12. A considerable part of the surface area around residue 65 remains uninvestigated in this study, but since substituting residues 36 and 40 has no affect on mAb 2CD12 binding, the epitope is not expected to spread towards the top of the protein but rather towards the bottom, according to the protein orientation in Fig. 4. As it is, this area is populated with unconserved residues, and Arg65 is itself an unconserved residue.

It is rather surprising that no mAbs react strongly to substitutions 51 and 71, which are highly exposed unconserved residues. Investigations on the interactions of IF1 with translation initiation factor IF2 on the 30S ribosome using the IF1-specific monoclonal antibodies as interaction inhibitors are currently in progress. These antibodies may also be used as a tool for investigating interactions of IF1 with the ribosome or with mRNA. Combinations of the monoclonal antibodies could potentially be applied for precipitation of IF1. For a more detailed investigation of the functional epitopes

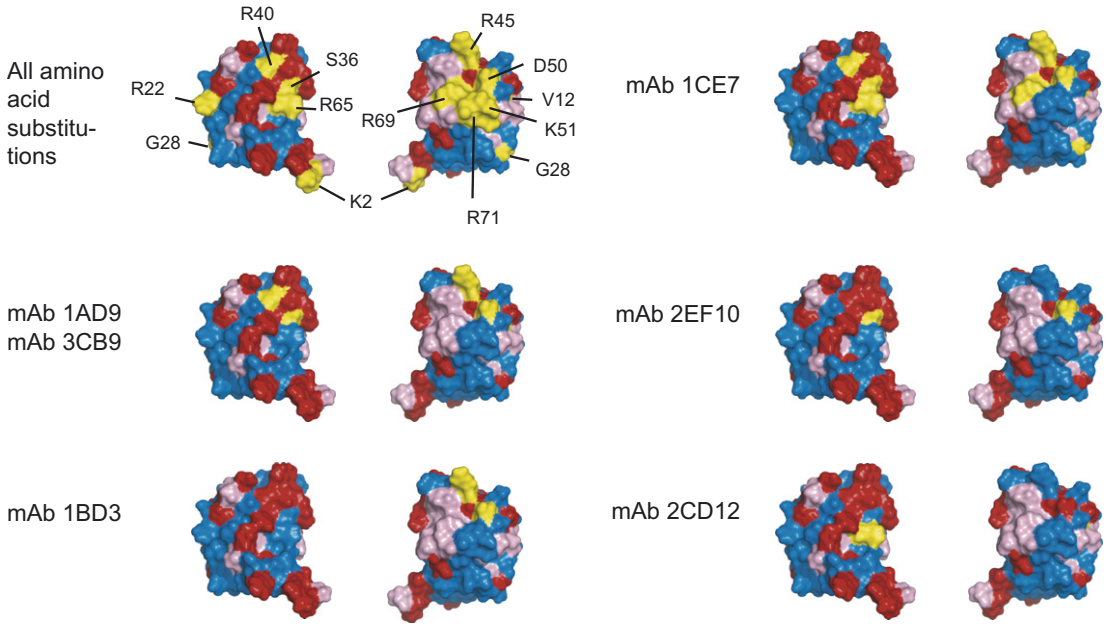

Fig. 4. Epitope mapping. The panel displays ventral and dorsal views of *E. coli* IF1 surface structures with the same colour-coding as in Fig. 2. For each of the six monoclonal antibodies, the amino acid substitutions affecting antibody recognition are shown in yellow. At the upper left, the positions of all the tested amino acid substitutions are indicated.

of these antibodies, more IF1 mutant proteins with single amino acid substitutions are needed, but this study gives indications as to the positions of the functional epitopes of the IF1-specific monoclonal antibodies. In studies including IF1 proteins with amino acid substitutions, knowledge of the epitopes may be useful in choosing the proper mAb for identification of these proteins in Western blots.

Acknowledgments

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