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Escherichia coli Maltose-binding Protein as a Molecular Chaperone for Recombinant Intracellular Cytoplasmic Single-chain Antibodies

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Recombinant single-chain antibodies (scFvs) that are expressed in the cytoplasm of cells are of considerable biotechnological and therapeutic potential. However, the reducing environment of the cytoplasm inhibits the formation of the intradomain disulfide bonds that are essential for correct folding and functionality of these antibody fragments. Thus, scFvs expressed in the cytoplasm are mostly insoluble and inactive.

Here, we describe a general approach for stabilizing scFvs for efficient functional expression in the cell cytoplasm in a soluble, active form. The scFvs are expressed as C-terminal fusions with the *Escherichia coli* maltose-binding protein (MBP). We tested a large panel of scFvs that were derived from hybridomas and from murine and human scFv phage display and expression libraries by comparing their stability and functionality as un-fused *versus* MBP fused proteins. We found that MBP fused scFvs are expressed at high levels in the cytoplasm of *E. coli* as soluble and active proteins regardless of the redox state of the bacterial cytoplasm. In contrast, most un-fused scFvs can be produced (to much lower levels) in a functional form only when expressed in *trxB*$^-$ but not in *trxB*$^+$ *E. coli* cells. We show that MBP-scFv fusions are more stable than the corresponding un-fused scFvs, and that they perform more efficiently *in vivo* as cytoplasmic intrabodies in *E. coli*. Thus, MBP seems to function as a molecular chaperone that promotes the solubility and stability of scFvs that are fused to it.

Keywords: cytoplasmic intrabodies; fusion-protein; maltose-binding protein; molecular chaperone; scFv fragments

Introduction

Antibodies are normally secreted by plasma cells and have evolved to act in the extracellular milieu of the mammalian body. The demands on stability have kept a selection pressure on immunoglobulin domains to retain disulfide bonds in all germline genes, which form in the endoplasmic reticulum during the process of protein secretion. Over the last decade, a wide variety of recombinant antibody formats have been engineered, the most popular being the single-chain Fv (scFv) that consists of the antibody variable domains connected by a flexible linker.[1,2] These antibody fragments can be produced in a variety of different hosts, ranging from bacteria to mammalian cells, usually by exploiting disulfide bond formation in the host secretory pathway. However, it is possible to express scFv or Fab fragments within the cytoplasm of eukaryotic and bacterial cells in a functional form, at least to some extent.[3–8] Sufficient functional expression of such intracellular antibodies (intrabodies) would enable them to bind to their targets and evoke specific biological effects. Assuming that the problem of

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Abbreviations used: AMEF, antibody mediated enzyme formation; EGFP, enhanced green fluorescent protein. HRP, horseradish peroxidase; Intrabodies, intracellular antibodies; MBP, *E. coli* maltose-binding protein, product of the *malE* gene; ONPG, *o*-nitrophenyl β-D-galactopyranoside; scFv, single-chain variable fragment of an antibody; V_H, heavy chain variable domain; V_L, light chain variable domain.

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inherent cytoplasmic instability and insolubility can be solved, intrabodies are currently being considered as having great potential in studies of functional genomics as the ''protein equivalent'' of antisense RNA.[5,6]

Intrabodies can be directed to all intracellular compartments by linking the corresponding intracellular trafficking signal sequence to their coding gene.[9–11] Expression in the cytoplasm (the default compartment when no sorting signals are applied) is the most difficult task, because of the reducing environment[12] that prevents the formation of disulfide bonds in antibody domains.[7,8,13–15] Since the intradomain disulfides contribute about 4-6 kcal/mol to the stability of antibody domains,[16–18] antibody fragments expressed in a reducing environment are strongly destabilized, and a smaller fraction of these fragments is likely to fold into the correct native structure, possibly accounting for their tendency to aggregate.[11,15] Nevertheless, a number of cytoplasmic intrabodies have been expressed and, in some cases, have been shown to exhibit specific biological effects.[19–23] In most of the reported cases, intrabody performance was positively correlated with *in vitro* stability, while binding affinity appeared to play only a secondary role.[24,25] However, most scFvs that have been applied as cytoplasmic intrabodies were not *a priori* considered for their stability; thus, for many applications the observed intrabody-mediated effects were insufficient, and such intrabodies would require further optimization by protein engineering.

The issue of scFv stability may be addressed by ''stability engineering'' of the variable domain framework regions by applying rational or evolutionary engineering approaches (for an excellent recent review, see Wörn & Plückthun[18]). In general, such approaches are applicable on a case-by-case basis, where every scFv has to be considered individually. Some attempts were made to identify antibody sequence motifs that would be characteristic of intrinsically stable scFvs.[26,27] However, thus far these findings did not lead to the construction of antibody repertoires that are based on ''universal stable antibody frameworks''.

An additional approach to stabilizing proteins is to express them as fusion-proteins.[28,29] Indeed, fusion of heterologous *Escherichia coli* proteins has been widely used to enhance solubility and generate affinity handles for recombinant proteins.[30] Several fusion tags have been considered as ''solubilizing agents'' owing to the fact that their fusion with an otherwise insoluble partner produces a fusion protein with enhanced solubility. There are examples of intentional expression of cytoplasmic intrabodies as fusion proteins with an antibody C-kappa domain to facilitate their stabilization and solubility.[20,31] Popular solubilizing fusion tags include glutathione *S*-transferase (GST),[32] thioredoxin (TRX)[33] and maltose-binding protein (MBP).[34,35] MBP has been shown to be the superior tag in both the periplasm [36] and in the cytoplasm (using a derivative lacking a signal peptide).[35] Indeed, MBP has been employed as a tag for affinity purification of antibody fragments.[37–40] However, in all the reported cases, the MBP-scFv fusion was exported to the bacterial periplasm where antibody disulfides may form.

Although MBP is a natural periplasmic *E. coli* protein, it is well expressed in the bacterial cytoplasm, where it enhances solubility of proteins that are fused to it.[35,36,41–44] As such, MBP was referred to as a chaperone in the context of a fusion protein.[35] An advantage of cytoplasmic expression of MBP fusions is that the expression level is much higher when compared to that of MBP fusions that are exported to the periplasm.[45] Nevertheless, the question remained of whether it would be possible to express disulfide bond-containing proteins (such as scFvs) in a soluble and functional form within the bacterial cytoplasm.

Here, we report that scFvs can be expressed efficiently as MBP fusions in the *E. coli* cytoplasm. In our examples, the MBP-scFv fusions accumulated to 20-60 % of the cell soluble fraction, regardless of the redox state of the cytoplasm. We expressed a large panel of scFvs derived from hybridomas, phage display libraries and expression libraries as MBP fusions. All of the MBP-scFvs we produced were expressed to higher levels than the corresponding un-fused scFvs. An outstanding example is the efficient production of the MBP-scFv derivative of the 4-4-20 anti-fluorescein antibody that was previously reported as a highly insoluble scFv. Furthermore, the MBP-scFvs were more stable than their un-fused counterparts. Finally, when tested as cytoplasmic intrabodies in *E. coli*, MBP-scFv fusions were more potent in activating a defective derivative of β-galactosidase.[7] Taken together, our results suggest that MBP has a chaperone-like effect on cytoplasmic intrabodies that are fused to its C terminus. We discuss the possible mechanism of scFv stabilization and the implications for potential biotechnological and therapeutic applications.

Results

Cytoplasmic overexpression and purification of MBP-scFv fusions

For expression, scFvs were cloned into pMalC-NN (Figure 1) and introduced into *E. coli* BL21(DE3). Anti β-galactosidase MBP-scFvs were expressed in *E. coli* MC4100, whose entire chromosomal *lacZ* gene is deleted to prevent co-purification of β-galactosidase with the MBP-scFvs. Growth conditions, cell extract preparation and MBP-scFv purification were done as described in Materials and Methods. Upon induction, the MBP-scFvs accumulated to high levels within the cells and upon separation of the soluble and insoluble cell extracts, were found primarily in the soluble fraction. For all MBP-scFvs reported here, there was less than 5 % of MBP-scFvs in the insoluble fraction of the cell extracts (not shown). In a typical experiment, MBP-scFv accumulated to 20-60 % of the total soluble

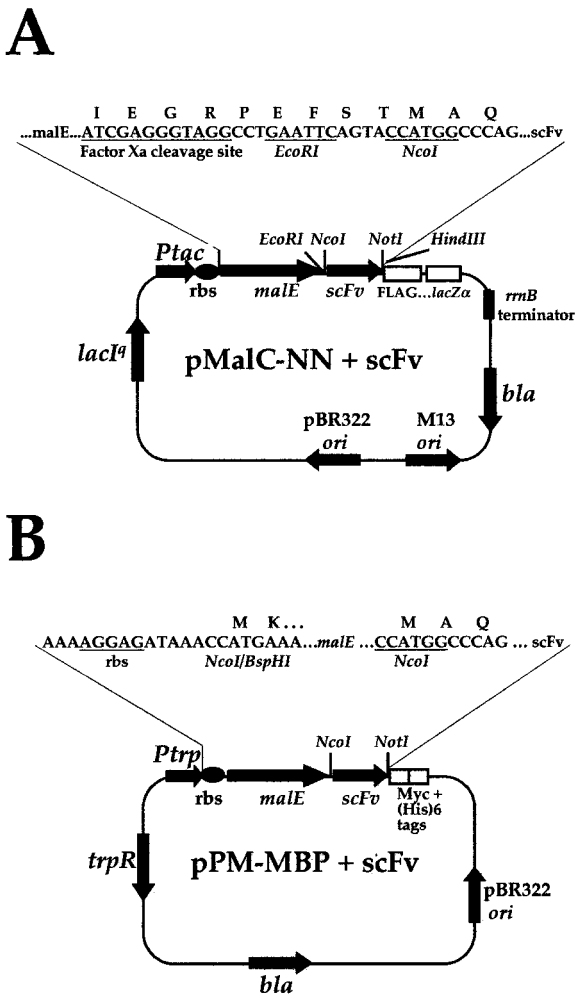

Figure 1. Maps of plasmids (a) pMalC-NN and (b) pPM-MBP used for cytoplasmic expression of MBP-scFv fusion proteins. The plasmids are represented with an scFv inserted between the *Nco*I and *Not*I restriction sites.

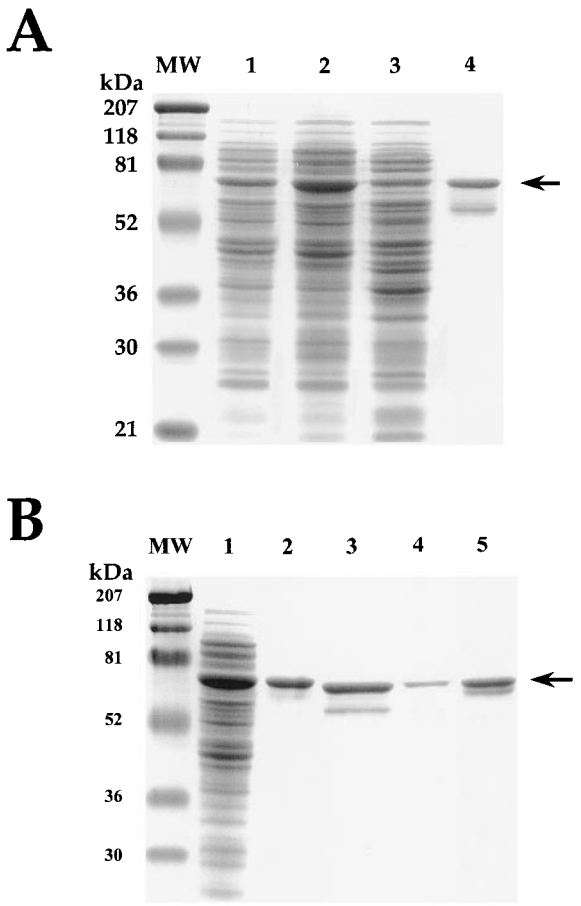

Figure 2. Expression and purification of MBP-scFv fusion proteins. (a) Aliquots of MBP-YM21(Fv): MW, molecular mass marker. Lane 1, uninduced cell extract, 20 μg of protein lane 2, induced soluble, 20 μg of protein cell extract. Lane 3, flow-through of the amylose resin column, 20 μg of protein. Lane 4, purified MBP-YM21(Fv), 2 μg of protein. (b) Aliquots of purified MBP-scFvs: MW, molecular mass marker. Lane 1, total soluble extract of MBP-scFv13R4. Lane 2, MBP-scFv13R4. Lane 3, MBP-FRP5(Fv). Lane 4, MBP-gal6(Fv). Lane 5, MBP-YM21(Fv): 20 μg of protein was loaded in lane 1, and 1-5 μg of protein was loaded in lanes 2-5. Proteins were separated on an SDS/12% polyacrylamide gel and visualized by staining with GelCode Blue. The arrows mark the position of the MBP-scFv fusion proteins.

protein as calculated by densitometric scanning of GelCode Blue-stained SDS/polyacrylamide gels. As an example, aliquots from each step of the purification of MBP-YM21(Fv) are shown in Figure 2(a). Samples of several purified MBP-scFvs are shown in Figure 2(b). When induced at 30 °C in shake flasks ($A_{600nm} = 2.0$), MBP-scFvs accumulated as soluble proteins in the cytoplasm at a level of about 200 mg/l of cell culture. Despite losses during purification, MBP-scFvs were purified to near-homogeneity at a yield of 70-120 mg/l of cells (roughly 2 g of wet bacterial paste).

We also expressed scFvs and MBP-scFvs using the *trp* promoter on plasmids pPM160 and pPM-MBP, respectively. With the exception of scFv13R4 that accumulates to about 5% of total soluble protein,[7] the expression level of scFvs was low and they could barely be detected by staining SDS/polyacrylamide gels where total cell extracts were separated. However, the scFvs could be visualized in immunoblots of such gels using an anti-myc tag antibody for detection (not shown). In contrast, MBP-scFvs accumulated to higher levels and could be detected easily in stained gels (Figure 3).

Following purification, the binding activity of the MBP-scFvs was tested in an ELISA. All the purified proteins bound their respective antigens specifically (Figure 4). The binding specificity was confirmed by the total inhibition of ELISA signal following the incubation of each MBP-scFv with an excess of its respective antigen in solution prior to its addition to the ELISA plates. In addition, we tested the binding of all MBP-scFv fusions to irrelevant antigens, where no binding could be detected. Moreover, neither MBP alone nor an MBP-green-

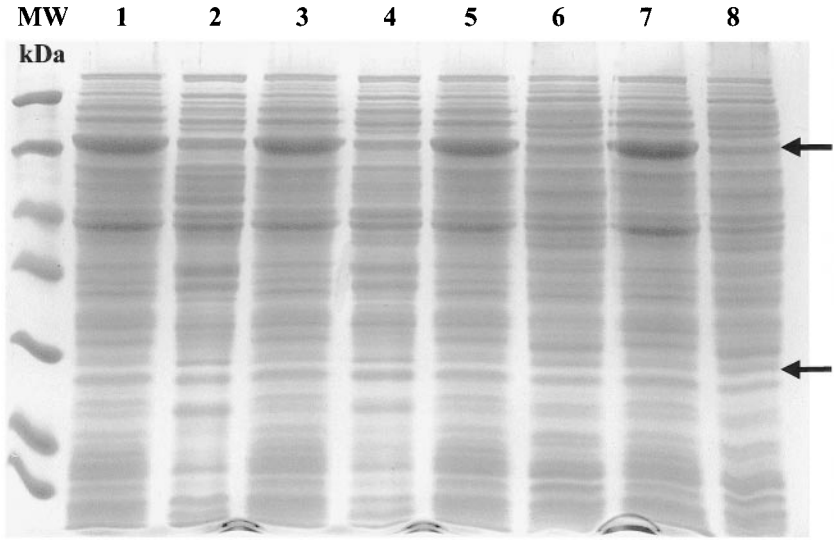

Figure 3. Expression of scFvs and MBP-scFvs under control of the *trp* promoter. Aliquots of the soluble fraction from crude extracts prepared from indole acrylic acid-induced cultures. MW, molecular mass marker. Odd-numbered lanes: MBP-scFvs, even-numbered lanes: un-fused scFvs. Lanes 1 and 2, scFv13R4; lanes 3 and 4, YM21(Fv); lanes 5 and 6, gal6(Fv); lanes 7 and 8, FRP5(Fv): 20 μg of protein was loaded in each lane of an SDS/12 % polyacrylamide gel and visualized by staining with GelCode Blue. The upper arrow marks the position of the MBP-scFv fusion and the lower arrow mars the position of the scFv.

fluorescent-protein fusion bound any of the tested antigens (not shown).

It has been reported that several proteins that were expressed as MBP fusions accumulated in a soluble, but inactive form in the cytoplasm of *E. coli*.[46–48] In addition, a fusion-protein between a scFv with a 26-amino acid N-terminal fragment of human interleukin-2 was also reported to be partially soluble, but completely inactive in the bacterial cytoplasm.[49] We performed an analytical affinity chromatography to investigate what fraction of our soluble MBP-scFvs is actually active. Anti β-galactosidase MBP-scFvs were first purified on amylose resin columns as described in Materials and Methods, as was the fluorescein-specific MBP-4-4-20(Fv). Next the MBP-scFvs were mixed with β-galactosidase that was covalently attached to Sepharose beads. After an hour of incubation, the un-bound fraction was recovered by centrifugation and analyzed in an immuno-dot-blot alongside the input material. As shown in Figure 5, >90 % of the tested β-galactosidase-specific MBP-scFvs were retained on the affinity matrix. In contrast, no MBP-4-4-20(Fv) was retained on the affinity matrix, indicating that binding was specific.

A number of additional scFvs were expressed as MBP fusions and binding was tested using crude cell extracts. In all cases, the MBP-scFvs were soluble in the *E. coli* cytoplasmic fractions. All the MBP-scFvs bound their antigens specifically. We found that such crude extracts maintain most of the binding activity for several weeks of storage at 4 °C with no appreciable degradation (not shown). A few examples include two anti ErbB2 scFvs that were isolated using DIP selection,[50] an additional anti β-galactosidase scFv, N9 (I.B., unpublished results) and the scFv of the anti myc 9E10 hybridoma.[51] A notable case is the anti fluorescein

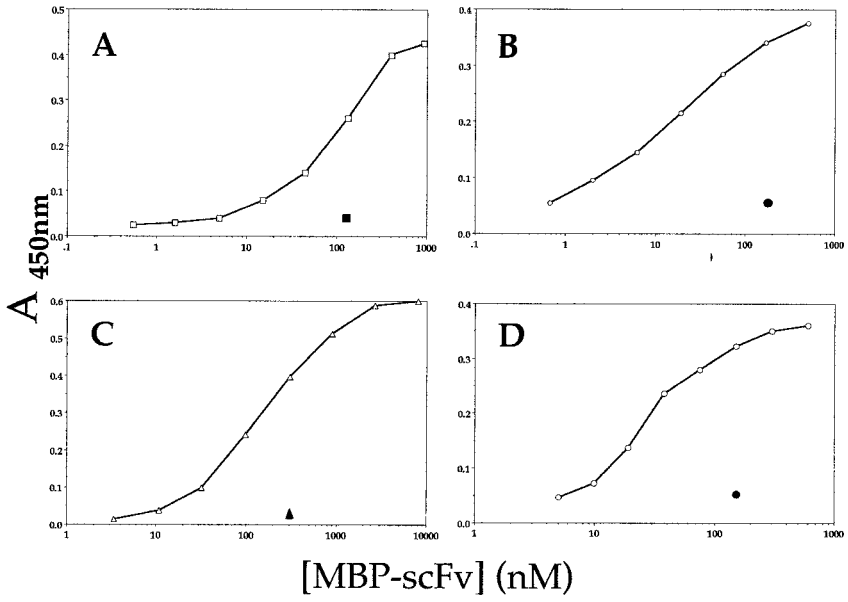

Figure 4. Analysis of antigen binding by purified MBP-scFvs in ELISA. (a) MBP-scFvR4 tested for binding β-galactosidase. (b) MBP-FRP5(Fv) tested for binding with ErbB2-GST. (c) MBP-gal6(Fv) tested for binding β-galactosidase. (d) MBP-YM21(Fv) tested for binding β-galactosidase. Filled symbols correspond to ELISA signals obtained following competition of binding with an excess of antigen.

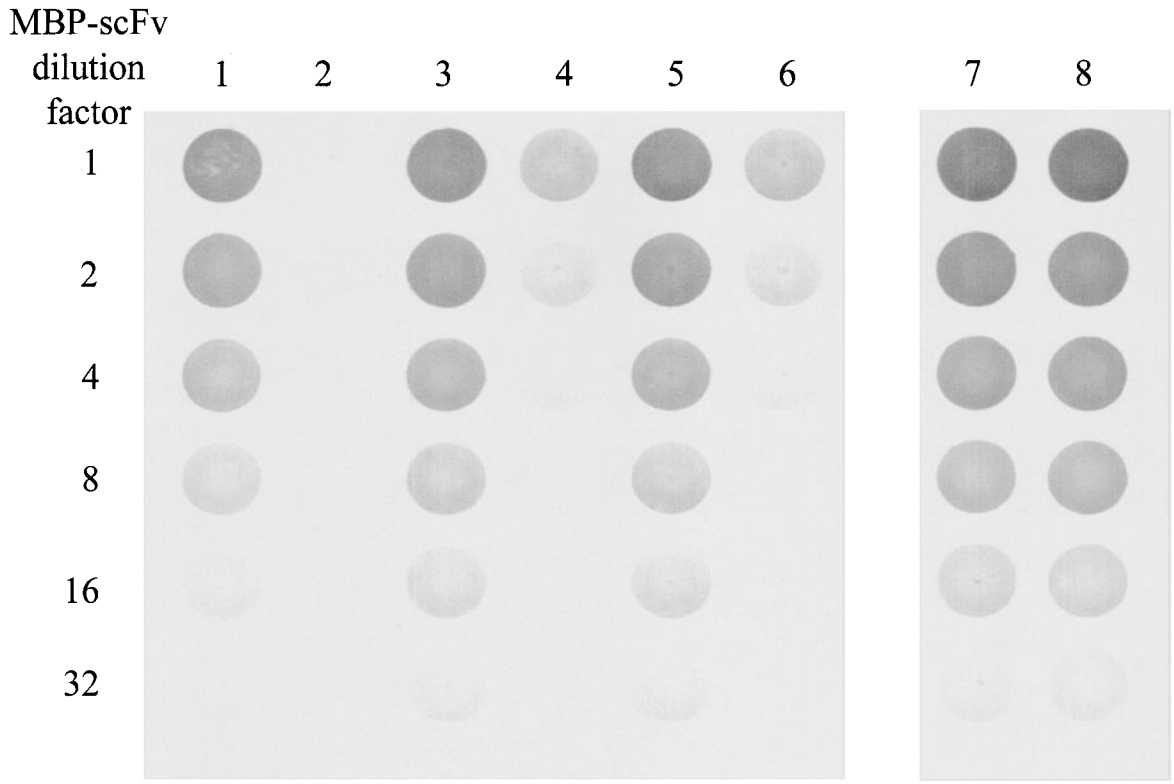

Figure 5. Analysis of the fraction of active MBP-scFvs by affinity chromatography and immuno-dot-blot. MBP-scFvs were mixed with a β-galactosidase affinity matrix and the un-bound fraction was recovered as described in Materials and Methods. Serial twofold dilutions of the MBP-scFvs before chromatography (odd lanes) and of unbound fractions (even lanes) were spotted onto a nitrocellulose membrane and detected as described in Materials and Methods. The β-galactosidase-specific MBP-gal6(Fv) (lanes 1 and 2), MBP-YM21(Fv) (lanes 3 and 4) and MBP-YM14(Fv) (lanes 5 and 6) are shown. The fluorescein-specific MBP-4-4-20(Fv) (lanes 7 and 8) serves as a specificity control.

4-4-20/212 scFv.[1] This particular scFv has been reported to be extremely insoluble even when exported to the bacterial periplasm.[52,53] We expressed a MBP-4-4-20(Fv) fusion and recovered it from the soluble cell extract. MBP-4-4-20(Fv) was recovered as >90% pure protein (Figure 6(a)) at a yield of 50 mg/l of cells (at $A_{600nm} = 2$). The recovered protein was active as determined in an ELISA where binding to fluorescein conjugated to BSA was tested (Figure 6(b)). MBP-4-4-20(Fv) did not bind a number of irrelevant antigens, indicating that its fusion with MBP did not affect its binding specificity.

Several MBP-scFvs were expressed in parallel in a *trxB*$^-$ isogenic strain of BL21(DE3) where cytoplasmic disulfide bond formation may occur to some extent.[7,14,54–56] Both the production yield and the binding activity of the MBP-scFvs in this background were similar to those produced in the wild-type BL21(DE3) strain (not shown). In the experiments reported above, the MBP-scFvs may have been air-oxidized upon lysis of the overproducing cells that could contribute to their activity. To investigate whether fusion to MBP renders the scFvs completely independent of disulfide bond formation, we carried out the following experiment. ScFvs and MBP-scFvs were prepared as described above but, following induction, half of the culture was treated with iodoacetamide to block the free cysteine residues before extraction.[54] Binding was measured in an ELISA as described above. As shown in Figure 7, the MBP-scFvs recovered from iodoacetamide-treated cells were as active as the corresponding untreated MBP-scFvs. In contrast, un-fused scFvs that were recovered from iodoacetamide-treated cells were totally inactive (not shown), confirming similar published observation.[8,52]

Stability of MBP-scFvs

The functional stability of MBP-scFvs in comparison to their corresponding un-fused counterparts was assessed by measuring residual binding activity following incubation at 37 °C. The assay was done using both crude soluble extracts of induced cells containing plasmids pPM160 (scFvs) or pMalC-NN (MBP-scFvs) and purified scFvs and MBP-scFvs. The results are shown as percentage residual activity in Table 1A. As shown, all the tested antibodies were more stable as MBP-scFvs than the corresponding scFvs. The un-fused scFvs were primarily inactive following an incubation for

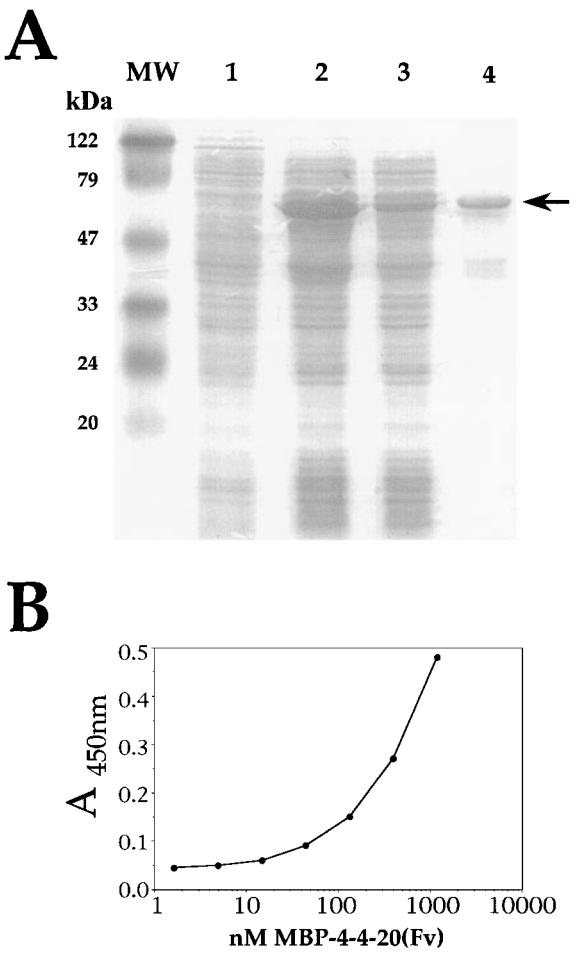

Figure 6. (a) Expression and purification of MBP-4-4-20(Fv): MW, molecular mass marker. Lane 1, uninduced cell extract. Lane 2, induced soluble cell extract. Lane 3, flow-through of the amylose resin column. Lane 4, purified MBP-4-4-20(Fv): 20 μg of protein was loaded in lanes 1-3 and 2 μg in lane 4. Proteins were separated on an SDS/12% polyacrylamide gel and visualized by staining with GelCode Blue. The arrow marks the position of the MBP-scFv fusion proteins. (b) Analysis of fluorescein-BSA binding by purified MBP-4-4-20 in ELISA.

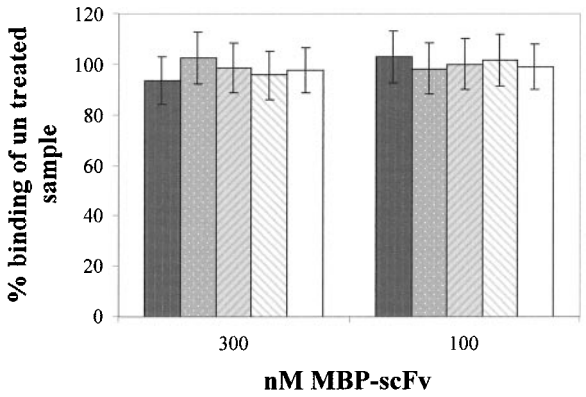

Figure 7. Analysis the effect of iodoacetamide alkylation of free cysteine residues on antigen binding by MBP-scFvs. The binding activity of MBP-scFvs recovered from iodoacetamide-treated cells was compared to that of the corresponding untreated MBP-scFvs. Results are plotted as activity in the treated fractions as percentage of the activity in the untreated fractions. MBP-gal6(Fv) (filled bars), MBP-YM21(Fv) (stippled bars), MBP-YM14(Fv) (grey bars with diagonal stripes), MBP-YM40(Fv) (open bars with diagonal stripes) and MBP-FRP5(Fv) (open bars) were analyzed. The error bars represent standard deviations of three independent experiments.

eight hours at 37 °C. In addition, we observed that the un-fused scFv extracts became turbid by two hours at 37 °C, indicating that the onset of protein precipitation had begun. In contrast, the MBP-scFvs were stable for much longer, maintaining partial binding activity after 20 hours at 37 °C (not shown). No appreciable precipitation was observed in these fractions for up to eight hours at 37 °C. We used an immunoblot to qualitatively determine residual MBP-scFvs in the fractions at different time-points. As shown in Figure 8, fractions obtained after eight hours at 37 °C still contained most of the soluble MBP-scFv. Similar results were obtained when purified MBP-scFvs were used in place of crude extracts.

To assess the possible involvement of the maltose-binding site of MBP in its stabilizing effects, we tested the stability of MBP-scFvs in the presence or absence of 10 mM maltose in the extract. The stability of the tested MBP-scFvs was not affected by the presence of maltose (Table 1B and Figure 8). These results clearly indicate that MBP-scFvs are more soluble and stable than the corresponding un-fused scFvs, and that the ligand-binding site of MBP most likely does not participate in its ability to stabilize scFvs that were fused at its C terminus.

To further assess the contribution of MBP to scFv stability, we de-stabilized the β-galactosidase-specific gal6(Fv)[57] by mutating a V_L cysteine residue to alanine. As a result, the intramolecular disulfide bond in the V_L domain cannot form. The mutated derivative, gal6(Fv) V_LC23A, was expressed as an un-fused Fv from pPM160 and as an MBP-scFv fusion from pMalC-NN. We then compared the functional stability of the destabilized scFv to that of the wild-type scFv following incubation at 37 °C as described above. As expected, the un-fused mutated scFv was less stable than the wild-type scFv (Table 1A). However, under our assay conditions, during eight hours at 37 °C only a small difference in stability between MBP-gal6(Fv) to MBP-gal6(Fv) V_LC23A was observed. This further demonstrates that expression of the scFv as a MBP fusion results in increased stability that compensates for the stability loss resulting from the absence of the disulfide bonds.

Table 1. Functional stability of scFvs and MBP-scFvs

scFv clone	Residual binding activity (%) following incubation at 37°C for 2 h	4 h	8 h
A. *No maltose*			
FRP5(Fv)	60	30	~5
MBP-FRP5(Fv)	100	90	80
scFv13R4	100	60	40
MBP-scFv13R4	100	80	50
YM21(Fv)	60	30	~5
MBP-YM21(Fv)	100	90	30
gal6(Fv)	70	30	10
MBP-gal6(Fv)	100	95	80
gal6(Fv) V_L C23A	30	10	~5
MBP-gal6(Fv) V_L C23A	90	80	70
B. *Maltose (10 mM)*			
MBP-scFv13R4	100	80	50
MBP-YM21(Fv)	100	90	30
MBP-gal6(Fv)	100	92	85

A, Functional stability of scFvs was estimated by measuring residual antigen binding in ELISA following their incubation at 37 °C for various periods. The percentage binding activity remaining was calculated by comparing ELISA signals of the fractions to the signal obtained with un-treated proteins. Results shown with the ~ (approximation mark) are of residual binding that is significant but below the linear range of the ELISA.

B, Functional stability MBP-scFvs measured as in A but in the presence of 10 mM maltose.

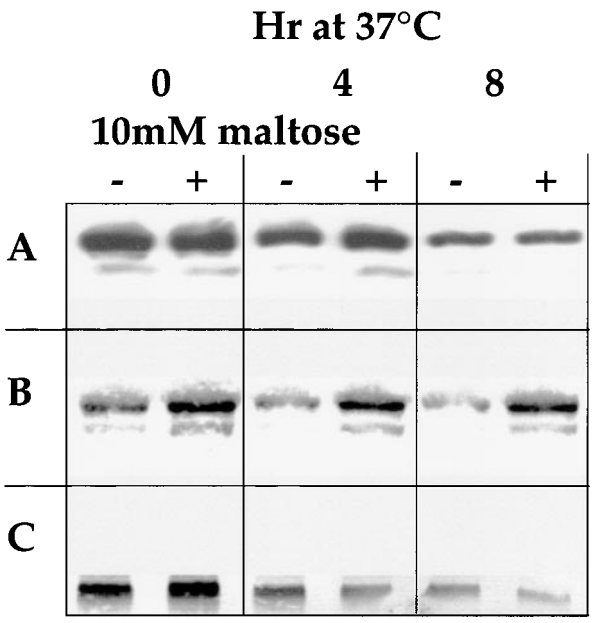

Figure 8. Functional stability of MBP-scFvs in the presence (+) or absence (−) of maltose. Samples of (a) MBP-scFvR4, (b) MBP-gal6(Fv) and (c) MBP-YM21(Fv) were each incubated at 37 °C for various times in the presence or absence of 10 mM maltose. At each time-point, an aliquot was removed and spun for ten minutes at 20,000 *g* to remove precipitated protein. Aliquots were analyzed by SDS/polyacrylamide gel electrophoresis and immunoblotting as described in Materials and Methods, and the intensities of the specific bands were compared in order to calculate the remaining soluble antibody at each time-point.

In vivo activation of AMEF β-galactosidase

All the AMEF β-galactosidase activating scFvs we isolated were identified initially as pPM160[7] clones as light blue colonies (weak Lac^+ phenotype) on minimal lactose plates supplemented with 10 μg/ml of indole acrylic acid and X-gal. Control non-activating clones form white colonies on such plates. The potent activators scFvR4[7] and YM21(Fv) form darker colonies on the indicative plates. These observations are in agreement with results published by Martineau.[7] When the scFvs were transferred to pPM-MBP (Figure 1B) and plated side by side with the corresponding pPM160 clones, all but scFvR4 showed a stronger Lac^+ phenotype (formed darker blue colonies on the indicative plates) (not shown). To evaluate the contribution of MBP to the functionality of scFvs in the reducing environment of the cytoplasm quantitatively, the following experiment was conducted. Activation of AMEF β-galactosidase is a sensitive probe of the concentration and functionality of activating scFvs that interact with it.[7] Therefore, we compared the β-galactosidase activity of intact *E. coli* AMEF 959 cells that contained pPM160 (unfused scFvs) and pPM-MBP (MBP-scFvs expressed at the same transcription level as the former). We applied an ONPG hydrolysis assay, in which the cells are treated with low concentrations of chloroform and SDS prior to addition of the substrate. This gentle treatment does not lyse the cells, but makes the membranes permeable, allowing the ONPG to diffuse into the cells and become accessible to the enzyme.[58] Indeed, no β-galactosidase activity, scFvs or MBP-scFvs could be recovered from cell-free supernatants following the chloroform/SDS treatment (not shown). The results of the β-galactosidase assays are shown in Figure 9.

With the exception of scFv13R4, all the tested scFvs were more potent activators of the AMEF β-galactosidase when expressed as MBP-scFvs. This may be attributed primarily to the fact that there was a higher accumulation of MBP-scFvs in a soluble and active form than the corresponding un-fused scFvs (see Figure 3). One explanation for the exceptional behavior of scFv13R4 in both plating on indicative plates and in the quantitative assay may be explained by the fact that it has been evolved for efficient expression in the cytoplasm.[7,8] Therefore, its expression level as an un-fused scFv is already sufficient to achieve the maximal possible activation of AMEF β-galactosidase with further increase in its intracellular concentration having no added effect. In contrast, our activating scFvs (YM14, YM21, YM40 and the AS clones) have not been subjected to such a process and their accumulation as un-fused scFvs in the cytoplasm is limited. This limitation may be overcome by expressing them as MBP fusions. The increase in β-galactosidase activity is not due to a direct effect

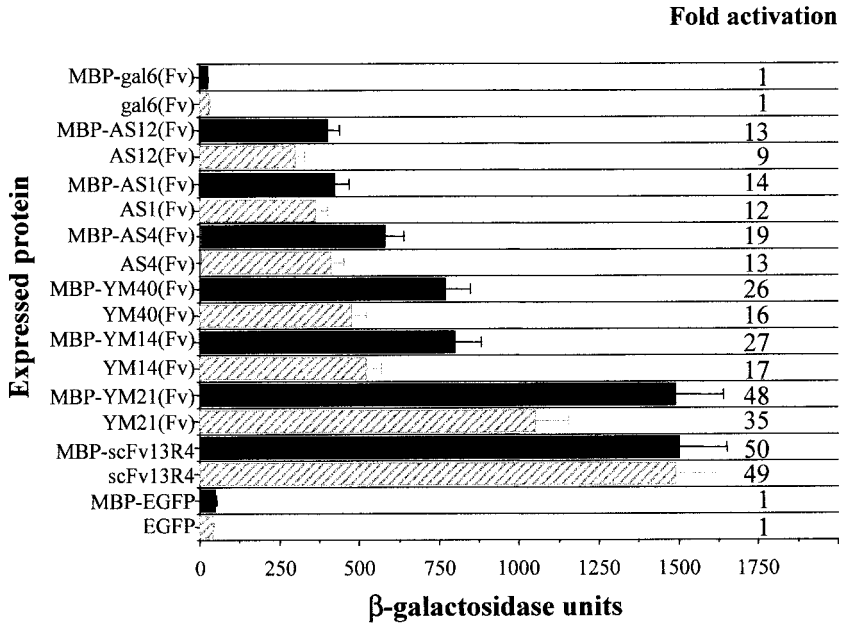

Figure 9. *In vivo* activation of AMEF β-galactosidase by cytoplasmic intrabodies. *E. coli* AMEF 959 cells carrying pPM160 derivatives (scFvs) or pPM-MBP derivatives (MBP-scFvs) were induced with indole acrylic acid and β-galactosidase activity was measured as described in Materials and Methods. The anti-β-galactosidase, non-activating binder gal6(Fv) and EGFP serve as negative controls. Error bars represent the standard deviation of the data.

of MBP on the AMEF β-galactosidase itself. This is evident from the activities in cells that express the negative controls gal6(Fv) and EGFP either as MBP fusions or as un-fused proteins (Figure 9).

It was previously reported that AMEF β-galactosidase-activating scFvs are expressed better in *trxB*$^-$ than in *trxB*$^+$ cells.[7] In contrast, when we tested AMEF β-galactosidase activation by MBP-scFvs, no difference in expression or in AMEF β-galactosidase activation was detected in cells of *E. coli* 959 *trxB*$^+$ and those that were *trxB*$^-$ (not shown).

Discussion

Functional scFvs have been produced in the cytoplasm of *trxB*$^-$ mutants, where the intracellular environment is less reducing.[7,54–56] However, even in *trxB*$^-$ cells, soluble scFvs can be expressed only at low levels, as increasing the expression level by applying a strong promoter results in precipitation of the scFvs in the form of inclusion bodies ([56] and our unpublished results). The result presented here show that both the stability and solubility of cytoplasmically expressed scFvs are improved significantly when they are expressed as C-terminal fusions with the *E. coli* maltose-binding protein. The improved solubility is evident from the fact that overexpressed MBP-scFvs are found in soluble cell extracts from which they are recovered in high yields (Figures 2 and 3). In addition, the MBP-scFvs that are recovered are over 90% active in antigen binding (Figure 5). The solubilizing effect of MBP is most striking in the case of the MBP-scFv derivative of the anti fluorescein scFv 4-4-20/212 (Figure 6). The improved stability of our MBP-scFvs is evident from the following. (a) Crude extracts (prepared in the presence of protease inhibitors and stored at 4 °C) containing MBP-scFvs retain significant binding activity for several weeks (up to three months so far). In contrast, the corresponding un-fused scFvs lose most of their binding activity within a week. (b) MBP-scFvs that are alkylated with iodoacetamide to prevent air-oxidation of disulfide bonds following cell lysis are as active as untreated ones (Figure 7). In contrast, the corresponding un-fused scFvs are inactivated by such treatment. This suggests that the added stability resulting from fusion to MBP fully compensates for the loss of stability due to the absence of the conserved disulfide bonds. (c) MBP-scFvs that are incubated at 37 °C remain active in antigen binding for significantly longer than the corresponding un-fused scFvs (Table 1). (d) The β-galactosidase-specific scFv gal6 is de-stabilized by mutating one of the conserved cysteine residues to alanine. However, the corresponding MBP-gal6(Fv) is nearly as stable as the wild-type MBP-scFv (Table 1A). Our interpretation of these observations is that MBP has a ''chaperone-like'' activity in promoting the solubility of scFvs that are fused to it. Indeed, MBP was dubbed ''a chaperone'' in previous studies where it was found to promote the solubility of proteins to which it was fused.[35] MBP was reported to stabilize and promote the solubility of several proteins, thus increasing their yield in the bacterial cytoplasm.[41–44,59] In other cases, where the fusion partner contained disulfide bonds (for example, the receptor CD4) the fusion proteins were exported to the periplasm.[45,60] scFvs were expressed as MBP fusions with reported improvements of production yield.[38–40,61–63] In all these reports, export of the MBP-scFvs to the periplasm was used to facilitate disulfide bond formation. We demonstrate here for the first time that the chaperone-like activity of MBP may be harnessed for the efficient functional

expression of scFvs in the reducing environment of the bacterial cytoplasm.

It has been proposed that for the expression of correctly folded proteins that rely on disulfide bond formation, exposure to oxidizing conditions may be required.[35,45] This may have been the case with our purified MBP-scFvs that could become oxidized upon lysis of the overexpressing bacteria. However, the fact that MBP-scFvs remained fully active following iodoacetamide-alkylation of the free cysteine residues suggests otherwise. In addition, the fact that AMEF β-galactosidase-activating MBP-scFvs were more potent *in vivo* activators than the corresponding un-fused scFvs indicates that MBP-mediated stabilization made the scFvs no longer dependent on disulfide bond formation for productive folding. It has been proposed that ''stability engineering'' may facilitate the efficient expression of intracellular cytoplasmic scFvs, where stabilizing the scFv by mutations compensates for the stability loss due to the absence of the disulfide bonds.[64] It was further proposed that stability is probably the limiting factor for folding of an scFv in the cytoplasm;[18] thus, in principle, a disulfide-free fully functional form of any scFv can be obtained, as long as the disulfide-containing scFv has a high enough thermodynamic stability.[64] Our results are in full agreement with these predictions, since all the scFvs we tested were more stable and were produced more efficiently as MBP fusions. In this regard, it should be noted that there was no difference in AMEF β-galactosidase activation measured in $trxB^+$ and $trxB^-$ cells.

There is an apparent discrepancy between the fact that MBP-scFvs are produced so much more efficiently than the corresponding un-fused scFvs, while their AMEF β-galactosidase activation is less than twofold better. Some of our unpublished observations may clarify this issue. When un-fused anti β-galactosidase scFvs are expressed in lac^+ or AMEF *E. coli* cells, the β-galactosidase protein (expressed from the single chromosomal copy) is co-purified with the scFvs. Concomitantly, the extracts are depleted of 30-60 % of the β-galactosidase activity as measured by ONPG hydrolysis. This indicates that a significant portion of β-galactosidase is in complex with the scFvs, although they are not expressed at high levels. When the corresponding MBP-scFvs are expressed in lac^+ or AMEF *E. coli* cells, the β-galactosidase protein is co-purified with the MBP-scFvs. However, in this case, the extracts are almost completely depleted of the β-galactosidase activity. Evidently, the MBP-scFvs are present in the cell at a large excess over the β-galactosidase protein. Assuming that activating scFvs or MBP-scFvs form stochiometric complexes with β-galactosidase,[7,65] this large excess does not result in a more potent activation.

Kapust & Waugh reported that MBP seems to be capable of functioning as a general molecular chaperone in the context of a fusion protein.[35] Direct elucidation of the mechanism by which MBP acts as a chaperone awaits structural studies of MBP fusion proteins. Currently, chaperones are divided into two groups.[66–68] Small chaperones of the stress proteins DnaJ and DnaK families bind as monomers to short, extended runs of hydrophobic residues as they appear on elongating nascent polypeptide chains. This binding is transient, and serves both as a delaying device to prevent premature folding before a complete folding unit has been synthesized, and as an anti-aggregation device to prevent interaction with hydrophobic residues on adjacent nascent chains. On the other hand, large oligomeric chaperones of the chaperonin (GroEL/ES) family act to prevent protein aggregation inside an *E. coli* cell after the complete polypeptide is released from the ribosome. Together, the main task of both groups of chaperones is to prevent aggregation of proteins in the crowd intracellular milieu of the cell cytosol.[66–68] We speculate that the monomeric MBP may function like the small chaperones but, since it does not release the protein that is fused to it, its stabilizing and solubilizing effects persist.

Other investigators have speculated on this property of MBP and other periplasmic ligand-binding proteins of Gram-negative bacteria.[35,69] It has been proposed that the intracellular concentration of these ligand-binding proteins far exceeds what is required for their role in ligand transport and chemotaxis, which may imply that they have additional roles in protein folding and protection from stress in the periplasm.[69] Kapust & Waugh[35] suggested that the ligand-binding cleft of MBP may be involved in its interaction with the fusion partner. On the other hand, Richrarme *et al*.[69] found that the chaperone activity of periplasmic ligand proteins is unaffected by the presence of ligand, and concluded that the ligand-binding sites of these proteins are not responsible for their chaperone-like activity. This is further supported by a recent report where MBP mutants that do not bind maltose still function just as well in promoting the solubility of fusion proteins.[70] Our results are in agreement with such an interpretation, as demonstrated by the similarity of the functional stability of MBP-scFvs when measured in the presence or absence of maltose (Table 1B and Figure 9). In addition, the fact that MBP-scFvs are efficiently bound to an amylose affinity matrix and released by maltose from that matrix indicates that the maltose-binding site is unoccupied by any ligand that would otherwise diminish its affinity to maltose. Another possibility is that the interaction between MBP and its fusion partner does involve the maltose-binding cleft but is transient. In such a scenario, the binding site interacts with hydrophobic patches of the fusion partner that become accessible during folding (i.e. of a nascent polypeptide chain) or unfolding. According to this model, the interaction is relieved once the fusion partner is in the fully folded state, in a mechanism similar to that of small chaperones.[66–68,71,72] In addition to the maltose-binding site, MBP has sites for interaction with

other components of the *E. coli* maltose import system. Specifically, MBP interacts with the membrane components of the transport receptor, MalF, MalG and MalK, which are complexed as $MalFGK_2$.[73] The possibility exists that MBP exerts its chaperone-like activity by utilizing that binding site. It would be intriguing to test whether MBP mutations that modify its maltose binding or $MalFGK_2$ binding[73,74] would affect its chaperone-like activity.

The effects that fusion to MBP has on scFv stability and solubility may be related but not totally overlapping. The solubility of scFvs is affected by the fact that residues of the variable/constant domain interface (in the context of an intact immunoglobulin) become solvent-exposed. Many of the interfacial residues are hydrophobic and their exposure to the solvent limits the solubility of the scFv.[18,53] During expression in *E. coli*, MBP emerges from the ribosome and may fold before the fusion partner linked at its C terminus has been translated in full. It is at that stage where MBP can presumably bind to its fusion partner and shield it from interacting with other cell constituents that would promote its aggregation mainly by interacting with the solvent-exposed hydrophobic residues. That would account for the increased production yield of MBP-scFvs. However, classical small chaperones release their passenger protein once it has adopted its folded conformation;[66–68,71,72,75–77] MBP, on the other hand, stays attached to its fusion partner and further protects it from denaturation, as is evident from our stability assays (Table 1).

There are several practical implications to our results. First, the expression of MBP-scFvs may become the method-of choice for the efficient production of recombinant antibodies in bacteria. The production yields we report, 70-120 mg of purified MBP-scFv from 2 g of wet bacterial paste exceed the best periplasmically expressed scFv[78] and parallels the production level we achieve from insoluble cytoplasmic inclusion bodies by matrix-assisted refolding.[79] It is conceivable that, with further optimization of growth and induction conditions (medium composition, inducer concentration, growth temperature),[7,42] our system can be made even more efficient. In addition, as we show here, certain scFvs that are very insoluble when expressed in bacteria, such as the anti-fluorescein scFv 4-4-20/212, can be produced as a soluble MBP-scFv with very little effort (Figure 6). Expression as MBP-scFv fusions can be a rapid means of obtaining large quantities of such molecules for analysis in a high-throughput screening system. The increased stability of MBP-scFvs should make them better analytical tools and diagnostic reagents.

A future possibility of a therapeutic application of MBP-scFvs may be their application as cytoplasmic intrabodies.[3–6] In mammalian cells, the intrinsic thermodynamic stability and solubility should not differ from those of the same scFv expressed in bacteria. However, the cytosolic milieu is quite different, which may affect the solubility of the MBP-scFvs. Currently, of particular interest would be the expression of MBP-scFvs in the cytosol of mammalian cells and the comparison of their performance to that of unfused scFvs. Experiments along these lines are in progress.

Materials and Methods

Bacterial strains

E. coli strain TG-1 (Amersham Pharmacia, Sweden) was used for molecular cloning and plasmid propagation. BL21(DE3) and BL21(DE3) *trxB*$^-$*:kan* (Novagen, USA) were used for protein overexpression. MC4100 (New England Biolabs, USA) whose chromosomal *lacZ* gene is deleted was used for overexpression of anti β-galactosidase scFvs. *E. coli* 959 carries an AMEF *lacZ* gene.[80] AMEF 959 and its *trxB*$^-$ derivative[7] were used for intrabody-mediated enzyme activation studies.

MalC1	GGTGCGGATATCTCGGTAGTG
MalC2	GGCGTTTTCCATAGTGGCGGCAAT
MalC3	ATTGCCGCCACTATGGAAAACGCC
MalC4	CAGTGCCAAGCTTGCCTAGTCCTTGTAGTCT
	GCGGCCGCTCCATCCATGGTACTGAATTCAGGCCTACCCTC
MalE-BspHI-Back	CCCTAATCATGAAAACTGAAGAAGGTAAA
$Gal6V_LC23A$-Back	GTCACCATGACCGCCAGTGCCAGCTCA
$Gal6V_LC23A$-For	GCTGGCACTGGCGGTCATGGTGACCTT
CBD-AS	GAATTCAACCTTCAAATTGCC
Uni-Sfi-Back	ATCTATGCGGCCCAGCCGGCCATG
Fd-Seq1	GAATTTTCTGTATGAGG

Oligonucleotides

Plasmid vectors

Plasmid pPM160 for cytoplasmic expression of scFv under the *E. coli trp* promoter and its derivative pPM163R4 that carries an AMEF β-galactosidase-activating scFv that was evolved for efficient cytoplasmic expression have been described.[7,8]

Plasmid pMalC (New England Biolabs, USA) for expression of proteins fused to the C terminus of MBP in the cytoplasm under the strong *tac* promoter was modified as follows: the *malE* gene carried on pMalC was amplified in two separate reactions. One reaction using primers MalC1 that overlaps with the unique *Eco*RV site of the plasmid and MalC2 that introduces a silent mutation that knocks out an internal *Nco*I site within the coding sequence of *malE*. In the second PCR reaction, primers MalC3, which overlaps with MalC2, and MalC4, which modifies the polylinker located 3′ to *malE*, were used. The PCR products were combined and re-amplified using primers MalC1 and MalC4, which corresponds to the full-length *malE* gene. The products of the second PCR reaction was digested with *Eco*RV and *Hin*dIII, and ligated into a vector fragment recovered following digestion of pMalC with the same enzymes. The resulting plasmid was named pMalC-NN (Figure 1(a)). The modified polylinker in pMalC-NN allows the cloning of scFvs as *Nco*I-*Not*I fragments, which is common in scFv expression and phage display vectors. In addition, a FLAG epitope[81] is appended to the C terminus of the scFvs.

Plasmid pPM-MBP for cytoplasmic expression of MBP-scFv fusions under the *E. coli trp* promoter was constructed as follows: the *malE* gene with its modified 3′ end polylinker was PCR amplified using pMalC-NN as template with primers MalE-BspHI-Back and MalC4. The PCR product was digested with *Bsp*HI and *Not*I, and ligated into a vector fragment recovered following digestion of pPM160 with *Nco*I and *Not*I. scFvs are cloned into both pMalC-NN and pPM-MBP (Figure 1(b)) as *Nco*I-*Not*I fragments. The scFvs described below were cloned into the two plasmids and into pPM160 in this manner.

Single-chain Fvs

The anti ErbB2 FRP5(Fv), derived from the FRP5 hybridoma,[82] the anti β-galactosidase scFv gal6(Fv),[57,79] the anti-fluorescein scFv 4-4-20/212(Fv) derived from the 4-4-20 hybridoma[1] and the AMEF β-galactosidase-activating scFv13R4[7,8] have been described.

AMEF β-galactosidase activating scFvs AS1,AS4 and AS12 were isolated as follows: the Griffin 1 human synthetic phage display library† was subjected to two affinity selection cycles using β-galactosidase (Sigma, Israel) as a capturing antigen. β-Galactosidase was immobilized onto polystyrene tubes (Immunotubes, Nunc, Sweden) at 10 μg/ml in 50 mM $NaHCO_3$ (pH 9.6). Affinity selection (biopanning) was carried out according to the protocols that accompany the library at the MRC web site‡. Following the second affinity selection cycle, the scFv inserts was recovered from the enriched phage population by PCR amplification of phagemid DNA using primers Uni-Sfi-Back and Fd-Seq1. The PCR product was digested with *Nco*I and *Not*I, and ligated into a vector fragment recovered following digestion of pPM160 with the same enzymes. DNA of the resulting scFv expression library was introduced into *E. coli* AMEF 959 cells that were plated on M9-lactose-agar plates supplemented with X-gal and 10 μg/ml indole acrylic acid to induce the *trp* promoter that controls transcription of the scFvs in the pPM160 vector. The subsequent isolation and characterization of AMEF β-galactosidase-activating scFvs was performed as described.[7]

The AMEF β-galactosidase-activating scFvs YM14, YM21 and YM40 were isolated from an immune expression library where the source for antibody genes was mRNA isolated from spleens of three mice that we had immunized with β-galactosidase. scFv gene construction was done by conventional RT-PCR using the Amersham Pharmacia (Sweden) RPAS scFv module according to the protocols provided with the kit. The scFvs were inserted as *Nco*I-*Not*I fragments into the pPM160 vector for intracellular scFv expression. Several potent AMEF β-galactosidase scFvs were identified as described above for the AS scFvs. YM14(Fv), YM21(Fv) and YM40(Fv) are three of these clones.

Mutagenesis of gal6(Fv)

The first V_L cysteine (codon 23, Kabat numbering scheme)[83] of the anti β-galactosidase scFv gal6(Fv) was mutated to alanine to de-stabilize the scFv as follows: DNA of phagemid pCC-gal6(Fv)[57] was used as template in two PCR reactions. In one reaction we used primers Uni-Sfi-Back and Gal6VLC23A-For, and in the second reaction primers Gal6VLC23A-Back and CBD-AS. The PCR products were combined and assembled into the complete scFv in a second PCR amplification using primers Uni-Sfi-Back and CBD-AS. The product of the second PCR reaction was digested with *Nco*I, and *Not*I and cloned into pPM160 and pMalC-NN as described above.

Protein expression, purification and analysis

E. coli cells transformed with expression plasmids were grown in 200 ml of LB medium supplemented with 100 μg/ml ampicillin and 1 % (w/v) glucose. When the cultures reached A_{600} of 0.5-0.8 they were induced for protein overexpression. Un-fused scFvs were expressed in pPM160 by induction with 10 μg/ml indole acrylic acid at 30 °C for 20 hours. Cell extracts were prepared and scFvs were purified by Ni-NTA agarose chromatography as described.[7] MBP-scFv fusions were expressed in pMalC-NN by induction with 0.5 mM IPTG at 30 °C for three hours. Cell extracts were prepared in 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 200 mM NaCl and a protease inhibitors cocktail (Sigma, Israel) by freeze-thawing followed by a brief sonication. The extracts were clarified by centrifugation at 20,000 *g* and stored at 4 °C. MBP-scFv fusions were purified by passing the extracts over an amylose resin column (New England Biolabs, USA) and recovered by elution with 10 mM maltose in the same buffer. In some experiments the induced cells were incubated on ice with 0.1 M iodoacetamide to block the free cysteine residues and prevent their oxidation following cell lysis as described.[54] Purified scFvs and MBP-scFvs were stored at 4 °C. Protein concentration was determined using the Pierce Coomassie plus (Pierce, USA) reagent with BSA as a standard.

† www.mrc-cpe.cam.ac.uk/ ~ phage
‡ www.mrc-cpe.cam.ac.uk/ ~ phage/g1p.html

ScFv or MBP-scFv containing fractions were analyzed on SDS/12% polyacrylamide gels that were stained with GelCode Blue (Pierce, USA). For immunoblots, the proteins were electro-transferred onto nitrocellulose membranes and scFvs were detected with anti myc 9E10 (Sigma, Israel), while MBP-scFvs were detected with an anti-MBP monoclonal antibody (Sigma, Israel). HRP-conjugated rabbit anti mouse antibodies (Jackson Laboratories, USA) were used as a secondary antibody. The immunoblots were developed using the Rennaissance Western Blot Chemiluminescence Reagent (NEN, USA) according to the supplier's instructions.

ELISA

ELISA plates were coated with antigens diluted in 50 mM $NaHCO_3$ buffer (pH 9.6) at 4 °C for 20 hours and blocked with 2% (v/v) non-fat milk in PBS for two hours at 37 °C. All subsequent steps were done at room temperature. *E. coli* β-galactosidase (Sigma, Israel) was coated at a concentration of 2 μg/ml. GST-ErbB2 fusion-protein[50] and FITC-albumin (Sigma, Israel) were coated at a concentration of 5 μg/ml. ScFvs or MBP-scFvs were applied onto the plates at various concentrations. For competition ELISAs, 20 ng of scFvs or MBP-scFvs was mixed with a 100-fold excess over the coating concentration of their corresponding antigen and incubated for one hour in 0.05% (v/v) Tween 20 (Sigma, Israel) in PBS (PBST) before adding them to the plates. scFvs were detected with anti myc 9E10 (Sigma, Israel) while MBP-scFvs were detected with an anti-MBP monoclonal antibody (Sigma, Israel). HRP-conjugated rabbit anti mouse antibodies (Jackson Laboratories, USA) were used as a secondary antibody. The ELISAs were developed using the chromogenic HRP substrate TMB (Sigma, Israel) and color development was terminated with 1 M H_2SO_4. The A_{450nm} signal obtained was proportional to the scFv or MBP-scFv concentration between 5 and 60 μg/ml.

Analytical affinity chromatography and immuno-dot-blot

β-Galactosidase was coupled to cyanogen bromide-activated Sepharose (Sigma, Israel) at a concentration of 1 mg/ml swollen beads. Coupling and subsequent blocking of free binding sites with glycine were done according to the manufacturer's instructions. Then 10 μg of each tested MBP-scFv was added to 100 μl of the resulting affinity matrix in PBS, placed in a microfuge tube and incubated for one hour at room temperature. Following incubation, the tubes were spun and the supernatant (unbound fraction) was recovered. Serial twofold dilutions of the unbound fraction were immobilized onto a nitrocellulose filter using a dot-blot apparatus (Milliblot system, Millipore). MBP-scFvs on the blot were detected with an anti MBP monoclonal antibody (Sigma, Israel). HRP-conjugated rabbit anti-mouse antibodies (Jackson Laboratories, USA) were used as a secondary antibody. The immuno-dot-blot was developed using the HRP chromogenic substrate 3,3′-diaminobenzidine.

Functional stability assay

The functional stability of the scFvs and MBP-scFvs was estimated by incubating crude cell extracts or purified proteins at 37 °C for varying periods. At each time-point, an aliquot was removed and spun for ten minutes at 20,000 *g* to remove precipitated protein. The percentage binding activity remaining was calculated by comparing ELISA signals of the fractions to the signal obtained with un-treated proteins. Fractions of the aliquots were analyzed by immunoblotting as described above, and the intensities of the specific bands were compared for calculation or the remaining soluble antibody at each time-point.

In vivo activation of AMEF β-galactosidase

E. coli AMEF 959 cells containing plasmids pPM160 or pPM-MBP that express various scFvs or MBP-scFvs, respectively, were grown in 20 ml of lactose-M9 minimal medium[7] and supplemented with 10 mM indole acrylic acid (to induce the *trp* promoter that controls the transcription of the clones genes in these plasmids). Following 20 hours of induction at 30 °C, cells were collected by centrifugation and re-suspended in 1 ml of Z-buffer/ONPG,[7] and the cell membrane was gently permeated by addition of chloroform/SDS[58] to allow diffusion of the OPNG substrate into the intact cells. The reaction was stopped after ten minutes at room temperature with 0.5 vol. 1 M Na_2CO_3 and the absorbance was recorded at 405 nm. β-Galactosidase units were calculated according to Miller.[58] β-Galactosidase activity was compared to the basal activity obtained with the β-galactosidase-binding, but non-activating scFv gal6 and EGFP to calculate the level of activation resulting from expression of the activating scFvs or MBP-scFvs within the AMEF bacteria.

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