# Research Report

# *Strep*-tag® II for One-Step Affinity Purification of Active bHLHzip Domain of Human c-Myc

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**ABSTRACT**

*The c-Myc protein, the product of the c-*myc *protooncogene, is a nuclear phosphoprotein with DNA-binding properties when heterodimerized with the Max protein. It contains an amino-terminal transcriptional activation domain and a carboxy-terminal basic helix-loop-helix leucine zipper (bHLHzip) domain that directs heterodimerization and promotes DNA binding. Here, we describe the isolation of the bHLHzip domain of human c-Myc with a technique for efficient single-step purification. Using a C-terminal* Strep-*tag® II affinity peptide and a novel Streptactin-Sepharose® matrix, elution is performed under mild conditions by competition with the biotin analog desthiobiotin. No significant influence of the affinity tag on the activity of the bHLHzip domain was observed when the fusion protein was subjected to glutathione S-transferase (GST) pull-down assays for investigating its in vitro-binding properties with GST-Max. The use of the C-terminal* Strep-*tag II was shown to be more suitable for obtaining pure product fractions than use of the N-terminal GST affinity tag.*

## INTRODUCTION

The protein c-Myc encoded by the c-*myc* protooncogene is implicated in a variety of cellular processes like proliferation, mitogenesis, differentiation and apoptosis in many different cell types (11,13–15,19). Deregulation of the gene, resulting in an increase of gene product, is associated with various types of neoplasia. Several peptide sequence motifs of the nuclear phosphoprotein have been identified that are important for its biological activity (4,18,19,31). C-terminal sequences containing *(i)* a leucine zipper (zip), *(ii)* a helix-loop-helix (HLH) dimerization motif and *(iii)* a basic (b) region, mediate dimerization and DNA binding of the protein. The amino-terminal region of c-Myc has been shown to act as a transcriptional activation domain. Heterodimerization of Myc with Max, another bHLHzip protein, is important for its DNA-binding function in vivo. The in vitro formation of Myc:Max complexes has also been demonstrated (5,6,44). In any case, the association of Myc and Max requires interaction of the HLHzip regions of both proteins. Moreover, Myc:Max heterodimers have been shown to function as sequence-specific, DNA-binding proteins and are able to activate transcription (4,6,21,30). Max homodimers bind to the same DNA sequence as Myc:Max heterodimers; however, they fail to transactivate and thus can antagonize Myc:Max function in vivo (1).

For analyzing the role of Myc:Max heterodimers in cellular processes, it is important to find substances affecting their interaction. This is possibile by means of in vitro assays requiring the isolation of active protein in sufficient amounts. A number of studies with c-Myc in several expression systems show that it is difficult to obtain the complete protein in a soluble and mature form. Yet, domains of the protein combined with peptide tags for detection and affinity purification were expressed in prokaryotic systems (2,5,10,12). Besides commonly used tags like glutathione S-transferase (GST), short tags with defined affinity properties do not necessarily interfere with the function of the protein; consequently, their removal is not needed for in vitro application (27,34). Recently, a peptide with intrinsic streptavidin-binding activity, termed *Strep*-tag, has been described (37). This nine-amino acid (aa) peptide allows efficient single-step protein purification from bacterial expression systems, but its use in fusions is restricted to the C terminus of recombinant proteins (20,26,28,38). Therefore, another streptavidin-binding tag, called *Strep*-tag® II—an octapeptide (WSHPQFEK)—has been introduced. This new tag interacts with the same surface pocket of streptavidin as *Strep*-tag and biotin, the natural ligand of streptavidin. Advantageously, *Strep*-tag II can be fused either to the C or the N terminus of recombinant proteins and allows affinity purification by means of the streptavidin derivate Streptactin® (36,42).

With the aim to establish an in vitro-binding assay for the screening for effectors of the Myc:Max heterodimerization, we made the HLHzip region of c-Myc, mediating the interaction with

Max, accessible to a quick and easy purification technique. In this article, we present the construction of a gene fusion coding for the bHLHzip region of human c-Myc, the affinity tag *Strep*-tag II and the OmpA sequence. The bHLHzip region of c-Myc was fused to *Strep*-tag II at its C terminus, allowing availability of the Myc protein by purification by affinity chromatography. Fusion of the recombinant protein to the OmpA sequence at its N terminus to provide secretion of the recombinant protein to the periplasmic space of *Escherichia coli* was investigated. Furthermore, the purification of the recombinant protein using the *Strep*-tag II/Streptactin Affinity System was compared to the isolation of GST-Myc using Glutathione-Sepharose®. After successful purification of the *Strep*-tagged protein, the interaction of the fusion protein with GST-Max was studied in vitro.

## MATERIALS AND METHODS

### Bacterial Strains and Plasmids

For cloning strategies, *E. coli* strains DH5α™ (Promega, Madison, WI, USA) and JM110 (46) (Stratagene, Heidelberg, Germany) were used. Fusion proteins were expressed in *E. coli* BL21 (40) (Amersham Pharmacia Biotech, Freiburg, Germany). Plasmid pGEX-4T-1-*max* containing full-length *max* as an *Eco*RI fragment was used for the expression of GST-Max under control of the *lac* promoter. Vector pFH8 (pGBT9; CLONTECH Laboratories, Heidelberg, Germany) containing MycC85 as an *Eco*RI/*Sal*I fragment (29) was used to create pGEX-4T-1-*myc*C255 (see below) for the expression of GST-MycC85. Furthermore, pASK-IBA2-*myc*C255 was constructed for the expression of MycC85-*Strep*-tag II. For that, a *myc*C255 DNA fragment (containing the 255 bp coding for the MycC85 fragment) was generated by polymerase chain reaction (PCR) (see below) and introduced into the bacterial expression vector pASK-IBA2 (IBA, Göttingen, Germany). With this method, MycC85 (aa 354–439) was fused to the OmpA signal peptide at its N terminus and to the *Strep*-tag II affinity tag at its C terminus. Vector pASK-IBA-*azurin* (IBA) was used as a source for the recombinant protein *Strep*-tag II-Azurin. All pASK-IBA expression cassettes are under transcriptional control of the *tetA* promoter.

### Construction of pGEX-4T-1-*myc*C255

All manipulations on recombinant DNA were performed following standard procedures (33). Both plasmids pGEX-4T-1 and pFH8 were digested with *Eco*RI and *Sal*I. The derived DNA fragments of pFH8 were separated on an agarose gel, and *myc*C255 was isolated by using the NucleoSpin® Extract Kit for the extraction of nucleic acids from agarose gels (Macherey-Nagel, Düren, Germany). Finally *myc*C255 was ligated with pGEX-4T-1, resulting in pGEX-4T-1-*myc*C255.

### Construction of pASK-IBA2-*myc*C255

Vector pASK-IBA2-*myc*C255 encoding for the fusion protein OmpA-MycC85-*Strep*-tag II was derived from pASK-IBA2 by means of PCR. The DNA fragment *myc*C255 was amplified using a standard PCR protocol and the primers NZ-MYC-01, 5′-AAA TTA GGT CTC AGG CCA AGA GGC GAA CAC ACA ACG TCT TGG-3′ and NZ-MYC-02, 5′-AAT TAT GGT CTC AGC GCT TCC ACC ACC CGC ACA AGA GTT CCG TAG CT-3′ (MWG-Biotech, Ebersberg, Germany). Plasmid pSP65-c-*myc*II/Awt (obtained from M. Eilers, University of Marburg, Germany) containing the cDNA sequence of full-length human c-*myc* was used as a template. The MycC85-encoding fragment of the human c-Myc derived from PCR was cleaved with *Eco*31I and introduced into the *Eco*31I site of pASK-IBA2, resulting in pASK-IBA2-*myc*C255.

### Gene Expression

Transformation of *E. coli* was performed as previously described (33). Transformants were selected on LB medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L sodium chloride) supplemented with 100 µg/mL ampicillin. *E. coli* BL21 cells harboring either plasmid pGEX-4T-1-*max* or plasmid pGEX-4T-1-*myc*C255 were grown at 37°C in shake flasks overnight containing 16 g/L yeast extract, 10 g/L tryptone, 5 g/L sodium chloride and 100 µg/mL ampicillin. The overnight cultures were diluted 100-fold into shake flasks of the same medium and grown at 37°C. Expression of the recombinant protein was induced at the mid-log phase [optical density $(OD)_{600\ nm}$ = 0.5] by the addition of isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich, Deisenhofen, Germany) to a final concentration of 100 µM. Cells were harvested 4 h after induction by centrifugation at 2400× *g* at 4°C for 10 min. The pelleted cells were resuspended in 25 µL of ice-cold phosphate-buffered saline (PBS) (33) per 1 mL culture volume containing the Complete™ Protease Inhibitor Cocktail (Boehringer Mannheim GmbH, Mannheim, Germany). Cells were lysed using a Mini-BeadBeater® (BioSpec Products, Bartlesville, OK, USA) and 18-mm-diameter glass beads (Braun, Melsungen, Germany). Cell debris and insoluble material were separated by centrifugation at 14 000× *g* at 4°C for 20 min. The supernatant containing the GST fusion protein was further processed.

In the case of the *Strep*-tag II fusion protein, *E. coli* BL21 cells harboring plasmid pASK-IBA2-*myc*C255 were cultivated in LB medium supplemented with 100 µg/mL ampicillin at 30°C. Expression was induced at the mid-log phase by the addition of anhydrotetracycline (Sigma-Aldrich) to a final concentration of 0.2 mg/L. Cells were harvested as described above. For obtaining the periplasmic extract, the pelleted cells were resuspended in Buffer W (100 mM Tris-HCl, pH 8.0, 1 mM EDTA) supplemented with sucrose (Sigma-Aldrich) to a final concentration of 500 mM and incubated on ice for 30 min. For obtaining the crude cell extract, the pelleted cells were resuspended in Buffer W, and lysis of the cells was achieved by the use of a Mini-BeadBeater as described above. In both cases, the supernatant was further processed.

### Protein Purification

The cell extract containing GST-Max was incubated with 100 µL pre-washed Glutathione-Sepharose 4B

suspension (Amersham Pharmacia Biotech) per 100 mL culture volume at 4°C with gentle agitation overnight. After packing the matrix into a column, the Sepharose beads were washed with PBS. By incubating the matrix with one bed volume of Glutathione Elution Buffer (20 mM glutathione in 100 mM Tris-HCl, pH 8.0) at room temperature for 10 min, elution of the fusion protein was achieved. Isolation of the *Strep*-tag II-fusion proteins was carried out by affinity chromatography using a bed volume of 1 mL of Streptactin-Sepharose (IBA) per 100 mL culture volume. The column was washed with Buffer W, and competitive elution was performed by the addition of Buffer W containing 2.5 mM desthiobiotin (Sigma-Aldrich). The eluted fusion proteins were quantified according to the method of Bradford using bovine serum albumin as a standard (7,9).

### GST Pull-Down Assay for In Vitro Protein-Protein Interaction

Cell extract containing either GST or GST-Max was incubated with prewashed Glutathione-Sepharose 4B at 4°C overnight. The matrix was pelleted by centrifugation at 500× *g* for 5 min, and the supernatant was discarded. After washing the Sepharose beads with ice-cold PBS, the cell extract containing the interacting protein was added to the pre-loaded matrix and incubated with gentle agitation at 4°C for 1 h. The Sepharose was packed into a column and washed, and elution was performed by incubating the matrix with one bed volume Glutathione Elution Buffer at room temperature for 10 min. Interaction of the proteins was studied by western blot analysis.

### SDS-PAGE and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using standard protocols (3). Samples containing GST fusion proteins were separated according to the method of Laemmli (22), and, in the case of *Strep*-tag II-MycC85, electrophoresis was carried out using Tris-Tricine buffer systems (35). For subsequent western blotting, the gel was soaked in Anode Buffer (0.3 M Tris in 20% methanol) after electrophoresis. Filter papers (3 MM; Whatman International, Maidstone, Kent, UK) were soaked either in Anode Buffer or in Cathode Buffer (3 g/L Tris and 5.3 g/L ε-amino-*n*-caproic acid in 20% methanol; before use, 2.5 mL of 20% SDS/L were added). The proteins were transferred to a polyvinylidene difluoride (PVDF) Transfer Membrane (NEN Life Science Products, Köln, Germany) by the semi-dry method using the Trans-Blot® SD Semi-Dry Transfer Cell (Bio-Rad, München, Germany). In the case of alkaline phosphatase (AP)-conjugated streptavidin (IBA) for the detection of *Strep*-tag II (see below), western blotting was carried out according to the manufacturer's instructions. Otherwise, the membrane was blocked with TBS-Tween® (10 mM Tris, 50 mM NaCl, 0.1% Tween-20) supplemented with 5% milk powder at room temperature for 1 h. After washing, the membrane was incubated with the primary antibody (diluted in TBS-Tween) for 45 min and was thoroughly washed and incubated with the labeled secondary antibody for another 45 min. *Strep*-tag II-Myc was detected either by using AP-conjugated streptavidin (diluted 1:2500) (IBA) or by using a

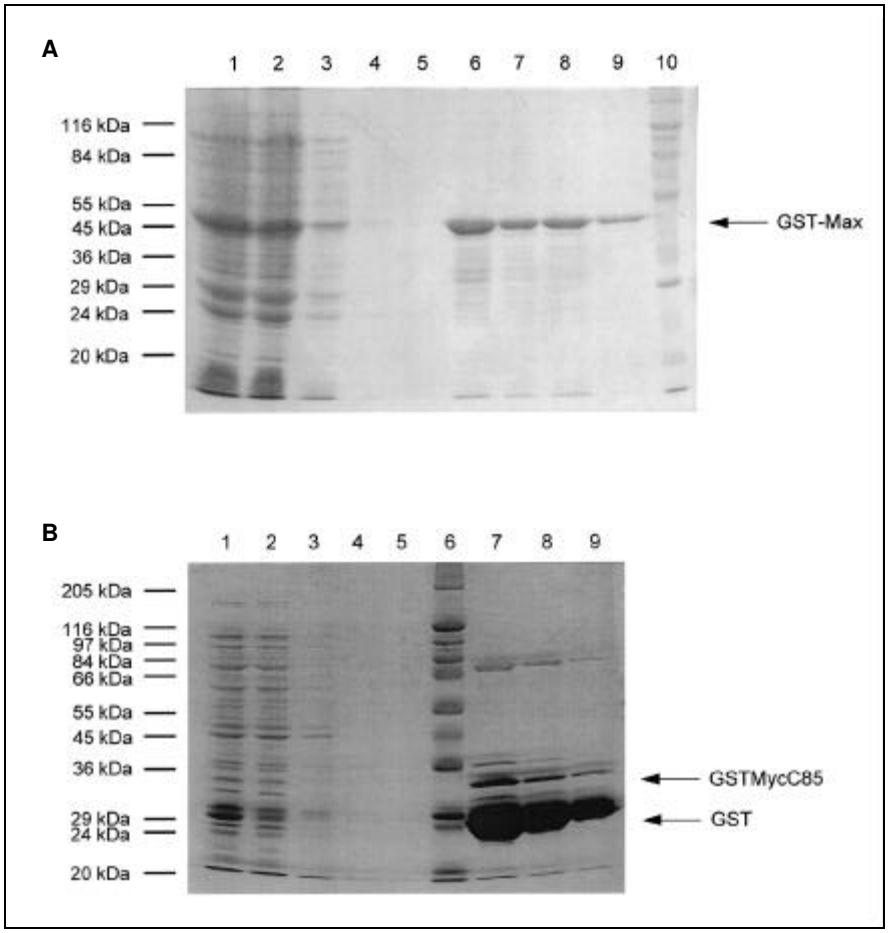


**Figure 1. Purification of GST-Max and GST-MycC85.** Samples were taken from each step of isolation and subjected to SDS-PAGE and subsequent Coomassie® Blue staining. After subjecting the cytosolic fraction (lanes 1) to glutathione-Sepharose, the matrix was washed 3× (lanes 3–5). (A) Purification of GST-Max. A 46-kDa protein band could be observed in the eluted product fractions (lanes 6–9) that was identified as GST-Max. Additional protein bands were observed in the elutions that could be identified as smaller fragments of the fusion protein. The eluate (lane 2) contains all the proteins that did not bind to the matrix. Lane 10 shows the distribution of the molecular weight marker. (B) Purification of GST-MycC85. A 32-kDa protein band could be observed in the eluted product fractions (lanes 7–9) that could be identified as the fusion protein GST-MycC85. The main product in the elutions was found not to be GST-MycC85 but GST itself. The eluate (lane 2) contains all the proteins that did not bind to the matrix. Lane 6 shows the distribution of the molecular weight marker.

mouse monoclonal 9E10 primary antibody (100 µg/mL, diluted 1:100; Santa Cruz Biotechnology, Heidelberg, Germany) and a peroxidase-conjugated, anti-mouse secondary antibody (400 µg/mL), which was diluted 1:1000 (Santa Cruz Biotechnology). Detection of GST-Max was performed using a goat monoclonal anti-GST antibody (5 mg/mL, diluted 1:1000; Amersham Pharmacia Biotech) and an AP-conjugated, anti-goat secondary antibody (diluted 1:2000; Sigma-Aldrich). *Strep*-tag II-Azurin was detected by using AP-conjugated streptavidin. In the case of peroxidase-conjugated secondary antibodies, the blot was subsequently developed by enhanced chemiluminescence detection (ECL™; Amersham Pharmacia Biotech, Braunschweig, Germany) according to the manufacturer's instructions. Immunocomplexes containing AP-conjugated antibodies were visualized by using a standard protocol (7).

## RESULTS

### Isolation of GST Fusion Proteins

MycC85 and Max were expressed as GST fusion proteins in *E. coli* BL21 and purified on Glutathione-Sepharose. This purification technique allows affinity selection and purification of complexes containing the GST tag under non-denaturing conditions (39). Cultivation and purification procedures as described above were controlled by SDS-PAGE (Figure 1) and subsequent western blotting. Besides the major 48-kDa protein band of GST-Max, additional minor bands of 22–42 kDa were found, which were identified by western blot to be smaller fragments of the GST-Max fusion protein (data not shown). According to SDS-PAGE analysis and quantification, the GST-Max fusion protein was estimated to represent approximately 10% of the total cell protein extract. In the case of GST-MycC85, additional bands besides the fusion protein could also be observed (Figure 1). Here, the main product of the isolation was found not to be GST-Myc, but GST itself.

### Isolation of OmpA-MycC85-*Strep*-tag II

For investigating Myc:Max association in vitro, we chose a construct containing the carboxy-terminal bHLHzip domain of human c-Myc for our studies. This region contains all motifs required for sequence-specific DNA binding and protein dimerization in vitro and in vivo. Moreover, it can be easily isolated from prokaryotic expression systems in an active form without denaturation and renaturation. Recently, for vertebrates, it has been demonstrated that Myc proteins have short half-lives of approximately 25 min (16,41, 43). To avoid the exposition of recombinant MycC85 to prokaryotic proteases, the OmpA signal peptide was fused to the N terminus of c-*myc*C255. This usually leads to secretion of the protein to the periplasmic space of *E. coli*, which is advantageous for separating the peptide from cytosolic proteases. The OmpA signal peptide itself is selectively cleaved off by the *E. coli* signal peptidase after the secretion step. For the production of *Strep*-tag II-Myc, pASK-IBA2-*myc*C255 was transformed into the protease-deficient *E. coli* BL21 strain. Cultivation and isolation procedures were carried out as described above. Myc could only be detected in the total cell protein extract and in the spheroplasts, but not in the periplasmic extract (Figure 2). The formation of inclusion bodies interfering with the export could not be observed. Purification of the OmpA-MycC85-*Strep*-tag II fusion protein from the crude cell extract was achieved through the *Strep*-tag II/Streptactin Affinity System. Elution of the protein was performed in a single step by competition with the biotin analog desthiobiotin. The efficient extraction of the recombinant protein was confirmed by SDS-PAGE (Figure 3) and subsequent western blotting (data not shown). A 20–21-kDa band was detected, representing the recombinant c-Myc still associated with the OmpA signal peptide. No co-eluting proteins were found by SDS-PAGE or by western blotting.

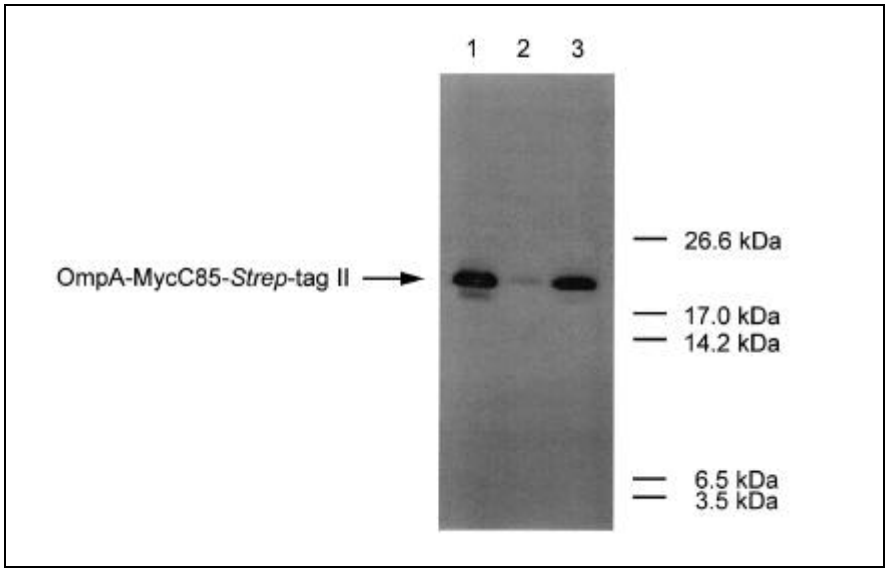


**Figure 2. Isolation of *Strep*-tag II-MycC85.** Samples from each step of purification were subjected to Tris-Tricine SDS-PAGE and subsequent western blotting. MycC85 was detected in the total cell protein extract (lane 1) and in the soluble fraction of the sonicated spheroplasts (lane 3) enlarged by the amino-terminal OmpA-sequence; it was not in the periplasmic extract (lane 2). Distribution of the molecular weight marker is shown on the right. Arrow marks the position of the fusion protein OmpA-MycC85-*Strep*-tag II.

### In Vitro Association of GST-Max and OmpA-MycC85-*Strep*-tag II

As mentioned above, it has been demonstrated in several studies that the bHLHzip region of c-Myc exhibits Max-binding activity. Since the fusion of a peptide tag might induce conformational changes of the recombinant protein, it was confirmed that the recombinant Myc fragment associated with *Strep*-tag II still shows Max-binding activity. This was done by isolating the fusion protein OmpA-MycC85-

*Strep*-tag II from the crude cell extract by affinity chromatography, making use of GST-Max immobilized on Glutathione-Sepharose. Recombinant GST-Max and interacting proteins were competitively eluted with Glutathione Elution Buffer. By western blotting with an anti-Myc antibody, it was confirmed that the recombinant OmpA-MycC85-*Strep*-tag II fusion protein is present in the eluted product fractions and exhibits GST-Max-binding activity (Figure 4). As a control, the crude cell extract containing *Strep*-tag II-Azurin was treated in the same manner for confirming that binding to GST-Max is mediated by the Myc fragment and not by *Strep*-tag II. Using peroxidase-labeled streptavidin, no *Strep*-tag II-containing proteins could be detected in the elutions (data not shown). This finding demonstrates that association of the Myc fusion protein with GST-Max is not mediated by the affinity tag *Strep*-tag II. In addition, we performed a second control experiment by carrying out a pull-down assay with immobilized GST. Using the MycC85 containing crude cell extract, we confirmed that the recombinant Myc fusion protein does exclusively interact with Max and not with GST. As the western blot demonstrated, no recombinant OmpA-MycC85-*Strep*-tag II fusion protein was present in the elutions (Figure 4). Therefore, binding of the Myc fusion protein to GST-Max is not mediated by the GST-tag. In combination, our results demonstrate that the bHLHzip domain of c-Myc fused to *Strep*-tag II still exhibits Max-binding activity.

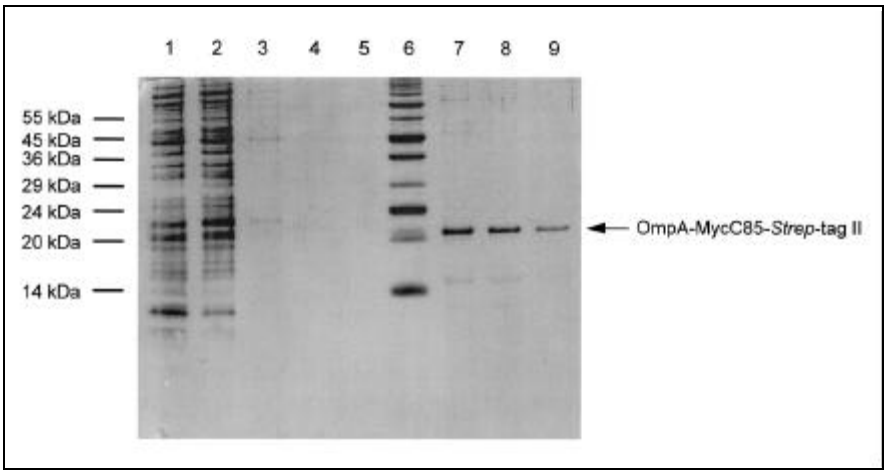


Figure 3. **Purification of OmpA-MycC85-*Strep*-tag II.** Samples were taken from each step of isolation and subjected to Tris-Tricine SDS-PAGE and subsequent silver staining. After subjecting the cytosolic fraction (lane 1) to Streptactin-Sepharose, the matrix was washed 3× (lanes 3–5). A 21-kDa protein band could be observed in the eluted product fractions (lanes 7–9) that could be identified as the fusion protein OmpA-MycC85-*Strep*-tag II. The eluate (lane 2) contains all the proteins that did not bind to the matrix. Lane 6 shows the distribution of the molecular weight marker.

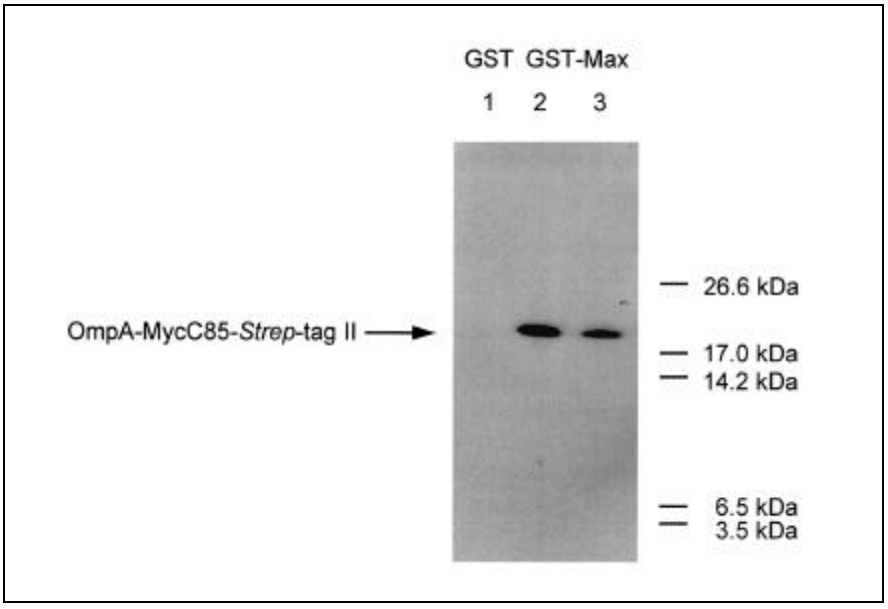


**Figure 4. Interaction of MycC85 with GST-Max in GST pull-down assays.** Either GST-Max or GST were immobilized on Glutathione-Sepharose 4B and incubated with the crude cell extract of *E. coli* BL21 pASK-IBA2-mycC255 for 1 h. Elution was carried out with Glutathione Elution Buffer. Samples were subjected to SDS-PAGE and western blotting. MycC85 was detected in the crude cell extract of *E. coli* BL21 pASK-IBA2-mycC255 (lane 2) and in the elution in the case of immobilized GST-Max (lane 3). As expected, no MycC85 could be detected in the elution when GST was immobilized (lane 1). Distribution of the molecular weight marker is shown on the right.

## DISCUSSION

In several studies, difficulties were encountered in obtaining full-length Myc in a soluble form from both eukaryotic and prokaryotic expression systems. Thus, many investigations on Myc:Max association were carried out with specific domains of the Myc protein. Fusion of the peptides to affinity tags enables protein isolation by affinity chromatography; however, it might lead to conformational changes of the desired peptide, resulting in loss of activity. For this reason, small tags like *Strep*-tag II implemented in our experiments are used, having no or only little influence upon its fusion partner. Our investigations have demonstrated that the carboxy-terminal 85-aa fragment of human c-Myc, containing the bHLHzip domain, fused to *Strep*-tag II associates with Max in vitro. Heterodimerization was proved to be independent of both *Strep*-tag II and GST, the affinity tag of the Max fusion protein. Using the *Strep*-tag II/Streptactin Affinity System, Myc was purified to near homogeneity from overexpressing *E. coli* cells. In contrast to these findings, the purification using GST-Sepharose resulted in additional protein bands in the case of GST-

Myc85, which were observed in the elutions. The main product of the isolation here was found not to be GST-Myc but GST itself. This leads to a reduced amount of full-length recombinant protein and might require additional intensive purification procedures.

Our strategy of translocating the bHLHzip domain of human c-*myc* to the periplasmic space of *E. coli*, mediated by the OmpA sequence, failed. In general, proteins attached to the OmpA-leader sequence are transported across the inner membrane through the *sec*-dependent pathway. However, the attachment of a signal sequence to a cytoplasmic or nuclear protein does not necessarily result in export of the protein. The efficiency of translocation of a polypeptide depends on the rate of its folding in relation to the rate of its export. The presence of a leader sequence usually slows down folding of precursor polypeptides and thus favors the pathway leading to export. However, if retarding the speed of intrinsic folding activity is not sufficient, folding of the protein is favored against its export (17,25). In addition to rapid folding, the nature of the early mature sequence (first 10 aa) of a protein is also critical for the export efficiency. The presence of too many basic amino acids in this region can result in blocking protein translocation (8,23,24,32,45). The fragment MycC85 contains 21 positively charged aa, with four of them located in the early mature region, which might be responsible for inhibiting protein translocation. Our results show that the ability of a protein to be exported by attachment of a signal sequence, in general, is difficult to predict and has to be tested in each individual case.

In summary, heterodimerization of GST-Max and *Strep*-tag II-Myc was demonstrated, and the interaction was proved to be mediated by the in vitro interaction of both MycC85 and Max. The use of the *Strep*-tag II system was shown to provide highly pure product fractions containing the bHLHzip domain of the Myc protein in an active form and proved to be more suitable than the introduction of the N-terminal GST tag. These findings now form the basis of establishing an in vitro-binding assay for the screening of molecules affecting Myc:Max heterodimerization.

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