Peptides from the SARS-associated coronavirus as tags for protein expression and purification

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ABSTRACT

Protein tagging with a peptide is a commonly used technique to facilitate protein detection and to carry out protein purification. Flexibility with respect to the peptide tag is essential since no single tag suites all purposes. This report describes the usage of two short peptides from the SARS-associated coronavirus nucleocapsid (SARS-N) protein as protein tags. Plasmids for the generation of tagged proteins were generated by ligating synthetic oligonucleotides for the peptide-coding regions downstream of the protein coding sequence. The data show recognition of prokaryotically expressed HIV-1 Gag/p24 fusion protein by Western blot and efficient affinity purification using monoclonal antibodies against the tags. The SARS peptide antibody system described presents an alternative tagging opportunity in the growing field of protein science.

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Detection and purification of proteins usually involves complicated and time-consuming protocols. To facilitate analysis, solubility and handling, proteins are commonly fused to large fusion partners such as the *Escherichia coli* maltose binding protein [1] or the glutathione-*S*-transferase [2] or to small oligopeptides. Protein tagging with a short peptide to which an antibody is available has first been described by Munro and Pelham [3]. It allows detection and affinity purification of a protein in the absence of a specific antibody against the protein itself. Use of a tag is of particular advantage if no specific antiserum is available for instance with proteins that are poorly immunogenic [4] or when it is necessary to distinguish expression of a transgene from protein production from endogenic loci [5].

One example for protein detection and purification with an oligopeptide tag is immobilized metal affinity chromatography, where binding occurs between an oligohistidine (His) tag at the protein and polyvalent cations on the affinity column [6]. Another peptide tag which was first described in the context of detecting and purifying recombinant lymphokines from lysates of yeast and *E. coli* [7] is the FLAG epitope. A short sequence from the human *c-myc* gene [8] is also being used as a peptide tag for detection of recombinant proteins in different expression systems [9–11].

Despite the availability of these and several other tagging systems, there is no ideal and universal tag for any application or purpose. For instance, the presence of a series of histidine-containing products can make purification of the desired His-tagged protein difficult since the eluate may contain impurities from less specific binding events [12]. For the same reason, use of an anti-His antibody for detection of recombinant proteins in immunoblot or immunofluorescence analysis can be problematic as non-specific reactions might occur. In addition, purification may be inefficient if protein folding prevents accessibility of the His tag [13]. The anti-FLAG antibody preferentially recognizes its epitope when expressed at the N-terminus of a protein [14]. This limits the use of the system for certain purification procedures. C-myc antibodies may cross-react with other structures and bind non-specifically [12]. In addition, Fan et al. [15] observed detection of c-myc-tagged proteins in immunofluorescence analysis that could not be confirmed by Western blot. Finally, peptide tags may be problematic in certain expression systems [12,13,15,16] or affect the function of the protein depending on the size and amino acid composition of the tag [17]. Therefore, efforts are constantly being made to provide alternative peptide tagging systems [18,19].

We have recently generated murine monoclonal antibodies (mAbs)[1] directed against the nucleocapsid (N) protein of the SARS coronavirus (CoV) and identified peptides of ten amino acids as epitopes [20]. In the present study we characterized the minimal epitope sequences and examined the application of the peptides as tags for protein expression in *E. coli*. In addition, we tested the efficiency of purification of tagged proteins by affinity chromatography.

Materials and methods

Plasmid construction

The mAb AMII/8G7B2C (8G7) recognizes the amino acid sequence GNSRNSTPGS (amino acid positions 193 to 202). The mAb AMII/2G8D4 (2G8) binds to the peptide KKKKTDEAQP (amino acids 373 to 382). To generate expression plasmids, we

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[1] *Abbreviations used:* mAbs, monoclonal antibodies; N, nucleocapsid; CoV, coronavirus; MBP, maltose binding protein.

used oligonucleotides for the coding and non-coding strand of the SARS-N peptides with the following sequences: epitope of 8G7: 5′-*CTAGA*GGTAATTCAAGAAATTCAACTCCTGGCAGC**TAG***CTGCA*-3′ (sense) and 5′-*G*CTAGCTGCCAGGAGTTGAATTTCTTGAATTACC*T*-3′ (antisense) (sequences in italics mark the XbaI and PstI overhang, stop codon is indicated in bold); epitope of 2G8: 5′-*CTAGA*AAAAAG AAAAAGACTGATGAAGCTCAGCCT**TAG***CTGCA*-3′ and 5′-*G*CTA AGGCT GAGCTTCATCAGTCTTTTTCTTTTT*T*-3′. Oligonucleotides were hybridized and cloned in the XbaI and PstI sites of vector pMAL^TMc2X (New England Biolabs) for C-terminal fusion with the protein of choice.

The gene for the Gag/p24 capsid protein of HIV-1 was amplified from a plasmid containing the proviral genome of the HIV strain HxB2 by PCR using primers 5′-TAA*GAATTC*CCTATAGTGCAG AACATCCAGG-3′ (forward, EcoRI site in italics) and 5′-GCT*TCTAG A*CAAAACTCTTGCCTTATGGC-3′ (reverse, XbaI site in italics). The amplification products were cloned in the EcoRI and XbaI sites of the modified pMALc2X vector resulting in open reading frames for fusion proteins containing the *E. coli* maltose binding protein (MBP), Gag/p24 and the C-terminal SARS-N peptide (Fig. 1). Although tandem labelling with two different fusion partners is useful in particular instances [21], the MBP fusion partner has no specific role in our analyses.

To identify minimal epitopes for the mAbs, the malE-Gag/p24-SARS-N peptide fusion genes were gradually shortened using PCR mutagenesis. For example, for C-terminal truncation of an amino acid from the original 8G7 epitope, fragments were amplified from pMALc2X-Gag/p24-8G7 (Fig. 1A) using primer 5′-TAA*GA ATTC*CCTATAGTGCAGAACATCCAGG-3′ and tag modifying reverse primer 5′-TAT*GTCGAC***CTA**GCCAGGAGTTGAATTTCTTG-3′. The amplificate was cloned into pMALc2X via the EcoRI/SalI restriction sites. N-terminal truncation of the peptides was obtained by amplifying fragments with modified forward primers, for example with the shortened 2G8 epitope primer 5′-TTG*TCTAG A*AAGAAAAAGACTGATGAAGCTC-3′ and the reverse primer 5′-CCG*AGTACT*CAACCAAGTCATTCTGAG-3′. Digested products were cloned in the XbaI and ScaI sites of pMALc2X.

Expression of tagged proteins

Escherichia coli BL21(DE3) cells were transformed with plasmids encoding the fusion proteins. Bacteria were grown in LB medium plus ampicillin (100 μg/ml) and glucose (2 mg/ml) at 37 °C and shaking of the culture at 220 rpm to an optical density of 0.5 at 600 nm. Protein expression was induced by adding IPTG to a final concentration of 0.3 mM. After 2 h at 37 °C the bacteria were harvested by centrifugation at 3000g, 4 °C, for 10 min.

SDS–PAGE and immunoblot analysis

Bacteria were resuspended in column buffer (20 mM Tris/HCl, pH 7.4; 0.2 M NaCl; 1 mM EDTA; 1 mM sodium azide) and heated to 96 °C for 10 min. The total protein amount of the bacterial lysates was determined with the Bradford assay. Lysates (1 or 2 μg of protein) were separated by SDS–PAGE. Proteins were stained with Coomassie stain (50% methanol, 0.05% Coomassie brilliant blue, 10% acetic acid). Alternatively, proteins were blotted onto nitrocellulose membranes. The membranes were incubated with blocking buffer (5% milk powder in PBS with 0.05% Tween 20 (PBST)) for one hour. Affinity purified mAbs (100 μg/ml) were diluted at a ratio of 1:500 with blocking buffer and added to the membranes over night at 4 °C. Membranes were washed with PBST and incubated for 1.5 h with an HRP-conjugated rabbit-anti-mouse secondary antibody (P0260, Dako) diluted 1:1000 in blocking buffer. Blots were again washed with PBST and developed with substrate solution (0.05% DAB in PBST; 0.1% hydrogen peroxide).

Protein purification by antibody affinity chromatography

MAbs 8G7 and 2G8 were coupled to HiTrap^TM NHS-activated HP columns (GE Healthcare) following the manufacturer's instructions. Bacteria of a 20 ml overnight culture were resuspended in 3 ml binding buffer (75 mM Tris–HCl, pH 8.0). After freezing over-night, bacteria were disrupted using the French Pressure Cell Press Sim Aminco^TM (SLM Instruments Inc., Rochester, NY, USA). Insoluble cell debris was removed by centrifugation at 9000g for 30 min. The soluble fraction (1–3 ml) was added onto 1 ml affinity columns equilibrated with 10 ml binding buffer. After washing the column with 6 ml binding buffer, the protein was eluted with 100 mM glycine/HCl, 0.5 M NaCl (pH 2.7). The acidic pH of the eluate was neutralized by addition of 1 M Tris/HCl (pH 9) and the protein solution analysed by SDS–PAGE and Western blot.

Results

Detection of proteins by immunoblot and determination of the minimal tag size

We have generated SARS peptide fusion protein expression plasmids on the basis of the pMALc2X vector using synthetic oli-
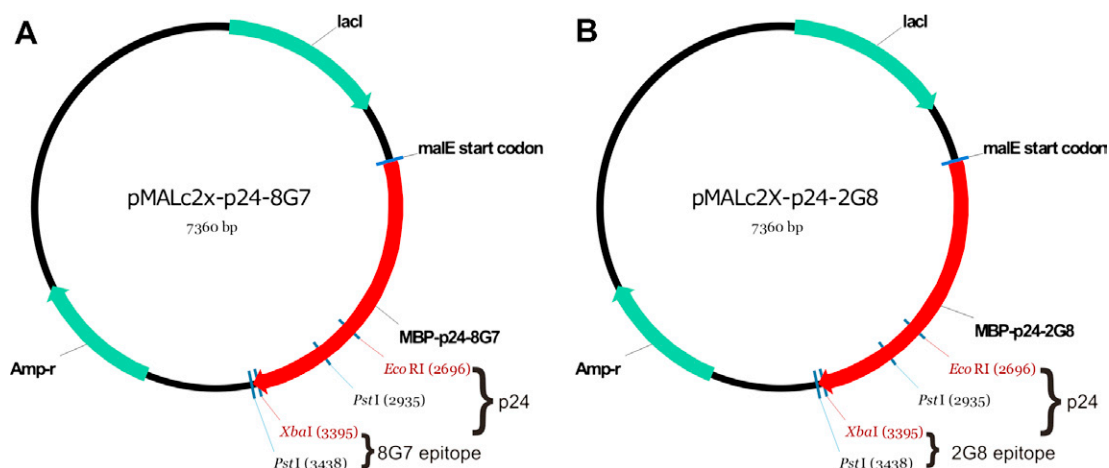

Fig. 1. Maps of prokaryotic expression plasmids with SARS peptide tags showing the relevant genetic elements. (A) Plasmid for expression of the MBP-Gag/p24 fusion protein tagged with the 8G7 epitope of SARS-N. (B) Plasmid for expression of the MBP-Gag/p24 fusion protein tagged with the 2G8 epitope of SARS-N. Maps were constructed with Vector NTI software (Invitrogen).

gonucleotides. Protein expression in bacteria was stimulated with IPTG. Bacteria were lysed and the proteins separated by SDS–PAGE. Immunoblotting of the lysates with the anti-SARS-tag antibodies 8G7 and 2G8 demonstrated binding of the antibodies to the Gag/p24 SARS peptide fusion proteins leading to bands at the expected size of 70 kDa (Fig. 2).

In order to minimally interfere with the function of the protein, the peptide tag should be small. To identify the minimum size of the tag, peptide sequences were truncated by one or more amino acids. Truncation of a single amino acid at the C-terminus only minimally affected binding of the mAb 8G7. Any further deletion regardless from which terminus of the sequence significantly reduced protein recognition. Similarly, cutting a single amino acid from the C-terminal end of the 2G8 epitope abolished recognition of the protein. Truncation from the N-terminal side of the epitope sequence likewise drastically reduced affinity of the mAb 2G8.

Thus, the optimal peptide size for use as protein tags consists of the nine amino acid sequence GNSRNSTPG for mAb 8G7 and the ten amino acid sequence KKKKTDEAQP for the mAb 2G8 (Fig. 2).

Protein purification by antibody affinity chromatography

MBP-Gag/p24 proteins fused to the optimal SARS tags were expressed in *E. coli*. MAbs 8G7 and 2G8 were immobilized on sepharose matrix columns and the soluble fraction of the bacterial lysates was added. The insoluble part was discarded. Purification was specific with both mAbs since no other proteins were detectable in the eluates (Fig. 3). The procedure was also highly efficient. For instance, the yield from 1.3 mg total protein of an *E. coli* lysate was 0.185 mg 8G7 epitope-tagged protein. Similarly, 0.48 mg of the 2G8 epitope-tagged protein was obtained from a bacterial lysate containing 3.3 mg total protein. Thus, purification of the SARS pep-
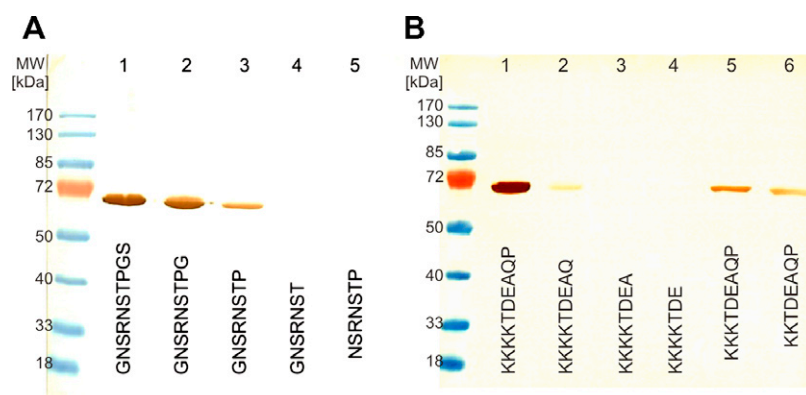

Fig. 2. Detection of tagged MBP-Gag/p24 protein by Western blot and determination of minimal SARS-N epitope sequences. (A) Proteins tagged with the 8G7 epitope. (B) Proteins tagged with the 2G8 epitope. MW, molecular weight.
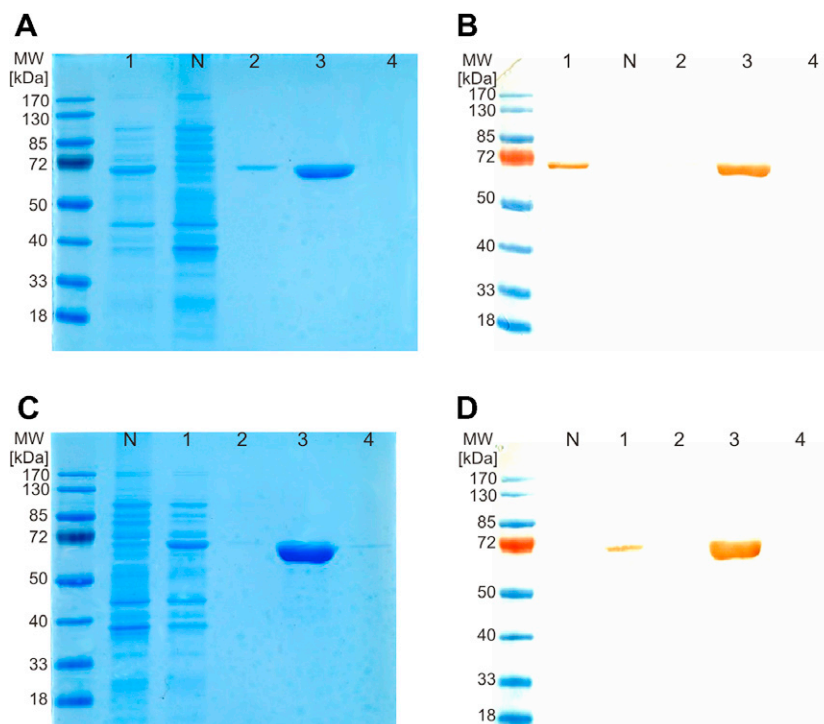

Fig. 3. Affinity chromatographic purification of SARS-N epitope-tagged MBP-Gag/p24 fusion proteins from *E. coli* lysates with SARS-N-specific antibodies. Coomassie staining (A) and Western blot (B) of the preparation steps of the MBP-Gag/p24-8G7 fusion protein. Coomassie staining (C) and Western blot (D) of the preparation of MBP-Gag/p24-2G8. N, Lysate of *E. coli* BL21(*DE3*) before induction of protein expression (2 μg of total protein). Lane 1, bacterial lysate after induction without protein purification (2 μg of total protein). Lanes 2–4, Elution fractions 1–3 of peptide-tagged MBP-Gag/p24. MW, molecular weight.

tide-tagged proteins yielded 14.2% and 14.5%, respectively, of the total protein content of the bacterial lysates.

Discussion

The work describes the generation of expression plasmids based on the pMAL vector system. Plasmids were generated with DNA oligonucleotides that span the coding sequences of peptides from the SARS-CoV nucleocapsid protein. The protocol for the generation of the expression plasmid can be applied for the creation of any other SARS tag expression plasmid.

Tagging with an oligopeptide against which a monoclonal antibody exists avoids the cost-intensive and time-consuming process of producing a specific antibody against the antigen itself. Therefore, tagging facilitates detection, purification and concentration of a desired protein. Although several peptide–antibody combinations are commercially available, none of the systems is universally applicable [12,13,15,16] and alternative systems are considered necessary [18,19].

An essential property of a tagging system is its uniqueness with respect to cross-reactive binding of the antibody to other proteins. In addition, a tag should be as small as possible to avoid interference with protein function or immunogenicity. However, very small tags increase the likelihood of cross-reactive binding of the detecting antibody. Neither of the SARS antibodies shows any reactivity in a Western blot of a gel with *E. coli* extract (Fig. 3) or with any of several mammalian cell lines [20].

Efficiency of protein purification by affinity chromatography is an important issue and depends on the antibody-peptide interaction. Low affinity of the antibody results in weak binding and poor protein yield. If affinity is extraordinarily high, protein elution from the column is inefficient [18] or only possible under harsh conditions [22]. It was shown that this problem can be overcome in some cases by generating polyol-responsive antibodies that allow gentle purification in affinity chromatography which is useful for the purification of large, labile, multiprotein complexes [23,24]. However, tagging with the SARS peptides allowed efficient enrichment and purification of the protein indicating a balanced interaction of the antibody with the antigen.

In conclusion, we have generated expression plasmids on the basis of the MBP fusion protein expression vector pMAL that contain SARS-CoV peptide sequences. Tagged fusion proteins were recognized and could be purified with SARS-N–specific mAbs. Similar expression plasmids for other purposes can easily be generated using derivatives of the described oligonucleotides. Given the absence of a universally applicable protein tag, the reagents and protocols presented here represent an alternative tagging system with high efficiency for protein purification in *E. coli*. The reagents and protocols will be made available on request.

Acknowledgments

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