

pET-prof, a plasmid for high-level expression of recombinant peptides fused to a birch profilin-derived hexadecapeptide tag: A system for the detection and presentation of recombinant antigens

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Abstract

We have previously identified a birch pollen profilin hexadecapeptide (Bp36/51), which was recognized by a monoclonal antibody (moAb 4A6) with high affinity. Here, we report the construction of a T7 RNA polymerase-driven high-level plasmid expression system, pET-prof, capable of producing proteins and peptides containing the Bp36/51 birch profilin-derived peptide fused to their N-terminus. As examples, the cDNAs coding for two major timothy grass (*Phleum pratense*) pollen allergens, Phl p 2 and Phl p 6, as well as for an alder (*Alnus glutinosa*) pollen allergen, Aln g 4, were overexpressed in *Escherichia coli* as Bp36/51-tagged proteins. All three recombinant allergens were readily detected in nitrocellulose-blotted *E. coli* extracts by the Bp36/51-specific moAb 4A6. We demonstrate comparable IgE recognition of Bp36/51-tagged and untagged recombinant allergens by immunoblotting. A sandwich ELISA was developed using plate-bound moAb 4A6 to immobilize and present Bp36/51-tagged recombinant allergens to IgE antibodies of allergic patients. Using immunoelectronmicroscopy, we demonstrate that even under harsh fixation conditions, tagged allergens can be localized simultaneously in situ by moAb 4A6 and allergen-specific antisera. We suggest the use of the pET-prof system for the high-level expression of Bp36/51-tagged polypeptides that can be rapidly detected in total protein extracts, immunolocalized in situ, immobilized and presented to other antigen-specific antibodies (e.g. IgE), even when they occur in minute concentrations. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

The production of recombinant proteins containing affinity tags in heterologous hosts has become a frequently used procedure for the rapid purification of recombinant proteins (Nilsson et al., 1996). It is based on the fusion of cDNAs coding for an affinity tag to

the cloning site of an expression vector to yield fusion proteins that can be detected and/or purified via tag-specific ligands or antibodies. The frequently used affinity tag systems comprise *Schistosoma* glutathione S-transferase (GST) (Smith and Johnson, 1988), *Escherichia coli* maltose-binding protein (MBP) (Guan et al., 1988), *Staphylococcus* protein A (Nilsson and Abrahmsen, 1990), polyhistidine (Van Dyke et al., 1992), calmodulin-binding peptide (Zheng et al., 1997), *Bacillus circulans* chitin-binding domain (CBD) (Chong et al., 1997), T7-, HSV-, FLAG-, S-tag systems (Nilsson et al., 1996) and cellulose-binding domains (Greenwood et al., 1992). Most of the tag systems published so far are designed for the expression and

Abbreviations: BCA, bicinchoninic acid; cDNA, complementary DNA; ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; moAb, monoclonal antibody; OD, optical density; PBS, phosphate-buffered saline; RAST, radioallergosorbent test.

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subsequent purification of tagged recombinant proteins or for the analysis of in-vivo gene expression.

Diagnostic procedures for immunologically mediated diseases (e.g. Type I allergy, autoimmune diseases, celiac disease) as well as basic immunological research (detection of antigen/antibody interactions, epitope mapping studies, T-cell research, studies on antigen presentation/processing, etc.) require, to an increasing extent, the production of defined recombinant antigens. Immunological screening assays do not necessarily require large amounts of purified authentic antigens but rather can be performed with small amounts of fusion proteins that can be easily immobilized and presented without compromising their antigenic properties. Moreover, the tag itself should not interact with structures of the human body, nor should it be a target for already established immune responses or give rise to new immune responses. Tag systems suitable for immunological research, epitope mapping and production of recombinant antigens should permit the rapid detection and quantification of tagged recombinant peptides and allow the tagged polypeptide to be presented to specific antibodies or immune cells in various immunological assays (e.g. ELISA, Westernblot, immunohistochemistry, cellular proliferation assays).

Using a mouse monoclonal antibody, 4A6, we have previously defined a peptide epitope on birch profilin (Wiedemann et al., 1996). Birch profilin represents an actin-binding protein and cross-reactive plant allergen (Valenta et al., 1991). Although recognized by moAb 4A6, the peptide epitope was bound neither by IgE nor by IgG antibodies of profilin allergic patients. Notably, moAb 4A6 bound the peptide epitope and the complete protein with a comparable high affinity (Wiedemann et al., 1996) and, despite the structural similarities of eukaryotic profilins from various sources (Fedorov et al., 1997), selectively recognized profilins of trees belonging to the order *Fagales* (e.g. birch, alder). A birch profilin-derived hexapeptide could be defined as a minimal epitope for moAb 4A6, which was not recognized by moAb 4A6 when Gln-47 was replaced by acidic amino acids (e.g. Glu, Asp). In an attempt independent from that reported by Rüdiger et al. (1997), we describe the construction of a T7 RNA polymerase-driven plasmid expression system, pET-prof, which produces recombinant antigens containing the 4A6-defined birch profilin epitope (BP36/51) fused to their N-terminal portions. The cDNAs coding for three relevant pollen allergens that do not share epitopes with birch profilin, i.e. the major timothy grass pollen allergens, Phl p 2 (Dolecek et al., 1993) and Phl p 6 (Petersen et al., 1995), and the 2 EF-hand calcium-binding alder pollen allergen, Aln g 4 (Hayek et al., 1998), were expressed using pET-prof as Bp36/51-tagged recombinant proteins. We studied the ability of moAb 4A6 to detect tagged allergens in *E. coli* extracts by immunoblotting

and immunogold electron microscopy. The IgE binding capacity of tagged and untagged recombinant allergens was compared by immunoblotting. We further investigated whether moAb 4A6 can be used to immobilize tagged allergens from total *E. coli* extracts and present them to IgE antibodies from allergic patients in an ELISA assay.

2. Materials and methods

2.1. Bacterial strains, plasmids, antibodies, recombinant allergens, and human sera

Competent *E. coli* BL21 (DE3) cells and plasmid pET-17b were purchased from Novagen (Madison, WI). Mouse monoclonal anti-birch profilin antibody 4A6 (Wiedemann et al., 1996) and a mouse monoclonal anti-Bet v 1 antibody of the same isotype (IgG1), Bip 1, (Laffer et al., 1996) were purified by affinity chromatography to protein G Sepharose (Pharmacia, Uppsala, Sweden). The protein concentrations of purified moAb 4A6 and moAb Bip 1 were determined by Micro BCA Protein Assay (Pierce, Rockford, IL). Recombinant pollen allergens without tag (rPhl p 2, rPhl p 6, rAln g 4) were purified as described previously (Vrtala et al., 1996; Hayek et al., 1998). Pollen allergic patients were diagnosed, based on a positive case history indicative of pollen allergy, positive skin prick reactivity to pollen extracts and the measurement of specific serum IgE antibodies by CAP RAST (Pharmacia, Uppsala, Sweden). The IgE reactivity profile to recombinant grass and tree pollen allergens was determined for each serum used in this study as described previously (Niederberger et al., 1998). Moreover, the sensitization to rPhl p 2, rPhl p 6 and rAln g 4 was determined by skin-prick testing as described previously (Hayek et al., 1998). Sera from non-allergic individuals were used for control purposes.

2.2. Construction of plasmid pET-prof

Synthetic 5'-phosphorylated oligonucleotides (#1: 5' TAT GGC CCA GAG CTC TTC CTT CCC ACA GTT TAA GCC TCA GGA AAT CAC TGG T 3'; #2: 5' GAT CAC CAG TGA TTT CCT GAG GCT TAA ACT GTG GGA AGG AAG AGC TCT GGG CCA 3') that anneal to a double-stranded DNA coding for the birch profilin Bp36/51 hexadecapeptide (Wiedemann et al., 1996) and contain overhanging ends suitable for insertion in the *Nde*I and *Bam*HI site of plasmid pET-17b were purchased from MWG-Biotech (Ebersberg, Germany). Five micrograms of each oligonucleotide were annealed, and the resulting double-stranded DNA was ligated into the *Nde*I/*Bam*HI-digested plasmid pET-17b and transformed into *E. coli* BL21 (DE3).

Clones expressing Bp36/51 were identified by colony screening using moAb 4A6 and an alkaline phosphatase-conjugated rabbit anti-mouse antiserum (Jackson, Szabo, Vienna, Austria) as described previously (Harlow and Lane, 1988). Plasmid DNA was isolated from moAb 4A6-reactive clones, and the insertion of the Bp36/51-encoding DNA was verified by Southern blot hybridization using the ^{32}P - γ -ATP-labeled oligonucleotide #1 as a probe (Sambrook et al., 1989). Plasmid DNA from a clone that was positive by both antibody screening and hybridization was sequenced to verify the correct insertion of exactly one copy of the Bp36/51-encoding DNA (Sanger et al., 1977).

2.3. Expression of Bp36/51-fused recombinant pollen allergens Phl p 2, Phl p 6 and Aln g 4

The cDNAs coding for the mature Phl p 2, Phl p 6 and Aln g 4 allergens were PCR-amplified with a PCR-Core kit (Perkin Elmer Applied Biosystems, Foster City, CA) using DNA from IgE-reactive lambda gt11 phage as templates and the following oligonucleotide primers (MWG, Ebersberg, Germany): Phl p 2 fwd: 5' CCG GAA TTC GTC CCG AAG GTG ACG TTC ACG 3'; Phl p 2 rev: 5' CCG GAA TTC CTA ACT AAC TAC TCT TCT GGC GCG TAG GT 3'; Phl p 6 fwd: 5' CCG GAA TTC GGG AAG GCC ACG ACC GAG GAG 3'; Phl p 6 rev: 5' CCG GAA TTC CTA ACT AAC TAC GCG CCG GGC TTG ACA GC 3'; Aln g 4 fwd: 5' CCG GAA TTC GCT GAC GAT CAT CCA CAG GAC 3'; Aln g 4 rev: 5' CCG GAA TTC CTA ACT AAT TAG AAT ATC TTG GCA ACA TCG 3'. *EcoRI* sites (underlined) were incorporated into the primers to allow in-frame insertion of the allergen-encoding cDNAs into plasmid pET-prof. PCR products were digested with *EcoRI*, gel-purified and subcloned into the *EcoRI* site of pET-prof. The DNA sequence of each construct was verified by sequencing (Sanger et al., 1977). In order to achieve expression of recombinant Bp36/51-tagged allergens, plasmids were transformed into *E. coli* BL21 (DE3). Transformed *E. coli* cells were grown in LB medium containing 100 mg/l ampicillin at 37°C to an optical density (600 nm) of 0.4, and protein expression was induced by addition of isopropyl- β -D-thiogalactoside (IPTG) to a final concentration of 0.5 mM for 4 h. *E. coli* cells were harvested by centrifugation at 4500 rpm (Hettich Rotanta TRC) at 4°C for 10 min. Total *E. coli* extracts were obtained by resuspending the cells in sample buffer (Laemmli, 1970) and boiling for 5 min. The soluble *E. coli* protein fractions were obtained by homogenizing cells in phosphate-buffered saline (PBS) with an ultra-turrax T25 (Janke & Kunkel, Staufen, Germany) and removing insoluble materials by centrifugation (18 000 rpm, 20 min, Sorvall RC5C; SM-24 rotor).

2.4. SDS-PAGE and immunoblot analysis of expressed recombinant allergens; comparison of the IgE-binding capacity of tagged and untagged recombinant allergens

The protein contents of the extracts were compared by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and Coomassie Brilliant Blue staining (Coomassie Brilliant Blue R-250, Bio Rad Laboratories, Richmond, CA) (Bradford, 1976). Prestained protein standards (Amersham, Buckinghamshire, UK) were used to determine molecular weights. *E. coli* BL21 (DE3) protein extracts obtained before and after induction with IPTG as well as soluble fractions of *E. coli* BL21 (DE3) were separated by 15% SDS-PAGE (Laemmli, 1970) and blotted onto nitrocellulose (Nitrocell, Pharmacia Biotech, San Francisco, CA) as described previously (Towbin et al., 1979). The IgE-binding capacity of tagged versus untagged recombinant allergens was compared by IgE immunoblotting. Comparable amounts (5 $\mu\text{g}/\text{cm}$) of nitrocellulose-blotted tagged and untagged recombinant allergens (rPhl p 2, rPhl p 6, rAln g 4) were exposed to sera from 18 pollen allergic patients and five non-atopic individuals. Human sera were diluted 1:10 in buffer B (50 mM sodium phosphate, pH 7.5, 0.5% Tween 20, 0.5% BSA and 0.05% NaN_3) whereas mouse monoclonal anti-birch profilin antibody 4A6 was diluted to a final concentration of 1 $\mu\text{g}/\text{ml}$ in buffer A. Nitrocellulose-blotted proteins were incubated with antibodies overnight at +4°C. After washing in buffer B, bound IgE antibodies were detected with 1:10 diluted ^{125}I -labeled anti-human IgE antibodies (Pharmacia), and bound mouse antibodies were detected with a 1:500 diluted ^{125}I -labeled sheep anti-mouse antiserum (Amersham). Sheets were washed, dried and exposed to X-ray films (NEN, Boston, MA) at -70°C for 16–72 h, using intensifying screens (NEN).

2.5. Sandwich ELISA

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 100 μl /well of purified moAb 4A6 (1 $\mu\text{g}/\text{ml}$ phosphate buffered saline-PBS, pH 7.5) overnight at 4°C. The plates were washed twice with buffer C (PBS, pH 7.5, 0.05% v/v Tween 20) and blocked for 4 h at room temperature with 200 μl /well buffer C (phosphate-buffered saline, pH 7.5, 0.05% v/v Tween 20, 1% w/v HSA). Thereafter, plates were incubated with 100 μl /well of soluble *E. coli* fractions, containing recombinant Bp36/51-tagged proteins (rPhl p 2, rPhl p 6, rAln g 4) overnight at 4°C. Plates were washed five times with buffer C, incubated with 100 μl of sera (10 sera from pollen allergic patients, four sera from non-atopic individuals) diluted 1:5 in buffer B overnight at 4°C and washed again, and bound IgE antibodies were detected with 1:500 diluted alkaline phosphatase-conjugated

mouse monoclonal anti-human IgE antibodies (PharMingen, San Diego, CA). After 2 h of incubation at 4°C, plates were washed five times with Tris-buffered saline (TBS) containing 0.05% v/v Tween 20. The colour reaction was started with alkaline phosphatase substrate (Sigma, Diagnostics, St. Louis, MO). The optical density was determined at 405 nm using an ELISA reader (Dynatech, Denkendorf, Germany). The results are displayed by means of duplicate determinations with an error of less than 5%. A cut-off level representing the mean OD value obtained after testing of sera from four non-atopic individuals on the same plate was subtracted.

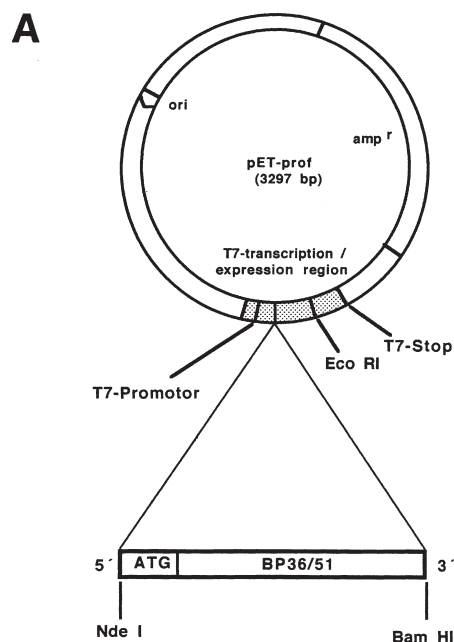
2.6. Immunogold electron microscopy

Escherichia coli BL21 (DE3) expressing Bp36/51-tagged Phl p 6 were fixed in a mixture of 4% *p*-formaldehyde and 0.5% glutaraldehyde in 0.1 M PIPES buffer, pH 7.4. They were embedded in Lowicryl K4M at –35°C using the PLT ('progressive lowering of temperature') technique. Ultrathin sections were exposed to moAb 4A6, or to a rabbit antiserum against rPhl p 6 (rαPhl p 6). For control purposes, an isotype-matched (IgG1) mouse monoclonal antibody, Bip 1, without specificity for the Bp36/51 epitope and rabbit preimmune sera were used. All antibodies and antisera used for immunohistochemistry were protein G affinity-purified, and their immunoglobulin (Ig) contents were adjusted according to spectrophotometry at 280 nm to the respective controls (preimmune Ig, isotype matched control moAb). Bound mouse monoclonal antibodies were detected with goat anti-mouse IgG, coupled to 20 nm colloidal gold particles, whereas bound rabbit antibodies were detected with goat anti-rabbit antibodies coupled to 10 nm colloidal gold particles (Plano, Wetzlar, Germany). After immunolabelling, the sections were stained with uranyl acetate and lead citrate and analysed in a Hitachi H500 transmission electron microscope operated at 75 kV. Photographs were taken using Agfa-Gevaert 23D56 films.

3. Results

3.1. Features of plasmid pET-prof

Plasmid pET-prof (3297 bp) represents a derivative of plasmid pET-17b (3306 bp) (Novagen, Madison, WI). It was constructed by replacing the *Nde*I/*Bam*H I fragment of the multiple cloning site (MCS) of pET-17b by one copy of the cDNA coding for the birch profilin hexadecapeptide Bp36/51 (Fig. 1A and B). cDNAs coding for foreign polypeptides can thus be inserted into the remaining restriction sites (*Spe*I, *Eco*RI, *Eco*RV, *Not*I, *Xho*I) of the MCS of pET-prof so that the Bp36/51-encoding cDNA is fused to their 5'-end.



B

MCS pET-17b:

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SD           Nde I   Nhe I
5' .. TAA GAA GGA GAT ATA CAT ATG GCT AGC ATG ACT GGT GGA CAG CAA
      Met Ala Ser Met Thr Gly Gly Gln Gln
      Hind III Kpn I   Sac I   Bam HI   Spe I
ATG GGT CGG GAT TCA AGC TTG GTA CCG AGC TCG GAT CCA CTA GTA ACG GGC
Met Gly Arg Asp Ser Ser Leu Val Pro Ser Ser Asp Pro Leu Val Thr Ala
Eco RI           Eco RV           Not I   Xho I
GCC AGT GTG CTG GAA TTC TGC AGA TAT CCA TCA CAC TGG CCG CCG CTC GAG
Ala Ser Val Leu Glu Phe Cys Arg Tyr Pro Ser His Trp Arg Pro Leu Glu

CAG ATC CGG CTG CTA ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA
Gln Ile Arg Leu Leu Thr Lys Pro Glu Arg Lys Leu Ser Trp Leu Leu Pro

CCG CTG AGC AAT AAC TAG .. 3'
Pro Leu Ser Asn Asn End

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MCS pET-prof:

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SD           Nde I
5' .. TAA GAA GGA GAT ATA CAT ATG GGC CAG AGC TCT TCC TTC CCA CAG
      Met Ala Gln Ser Ser Ser Phe Pro Gln
      Spe I
TTT AAG CCT CAG GAA ATC ACT GGT GAT CCA CTA GTA ACG GGC GCC AGT GTG
Phe Lys Pro Gln Glu Ile Thr Gly Asp Pro Leu Val Thr Ala Ala Ser Val
Eco RI           Eco RV           Not I   Xho I
CTG GAA TTC TGC AGA TAT CCA TCA CAC TGG CCG CCG CTC GAG CAG ATC CGG
Leu Glu Phe Cys Arg Tyr Pro Ser His Trp Arg Pro Leu Glu Gln Ile Arg

CTG CTA ACA AAG CCC GAA AGG AAG CTG AGT TGG CTG CTG CCA CCG CTG AGC
Leu Leu Thr Lys Pro Glu Arg Lys Leu Ser Trp Leu Leu Pro Pro Leu Ser

AAT AAC TAG .. 3'
Asn Asn End

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Fig. 1. (A) Construction of plasmid pET-prof. Plasmid pET-17b (3306 bp) was digested with *Nde*I and *Bam*HI to excise a portion of the multiple cloning site. This fragment was replaced by the birch profilin hexadecapeptide Bp36/51-encoding cDNA (48 bp) to yield plasmid pET-prof (3297 bp). (B) Comparison of the multiple cloning site (MCS) of pET-17b (upper part) and pET-prof (lower part). The deduced amino acid sequences are displayed below the nucleotide sequences of the MCS. The Shine Dalgarno sequence (SD) and the restriction sites that can be used for insertion of foreign cDNAs are indicated by underlining or printing in italics. The Bp36/51 amino acid sequence is underlined.

Recombinant polypeptides will thus be expressed with the Bp36/51 peptide attached to their N-terminus. All other features [ampicillin resistance gene, T7 promotor, Shine-Dalgarno (SD) sequence] of pET-prof closely resemble those of plasmid pET-17b. Target genes can therefore be cloned into pET-prof under the control of strong bacteriophage T7 transcription and translation signals, and expression can be induced by providing a source of T7 RNA in the host cell (Tabor and Richardson, 1985), which can be achieved either by selection of *E. coli* bearing a chromosomal copy of the T7 RNA polymerase gene (λ DE3 lysogen) under the control of the *lacUV5* promotor [e.g. *E. coli* BL21 (DE3)] (Studier et al., 1990) or by co-transformation with plasmids carrying the T7 RNA polymerase gene (Mertens et al., 1995).

The insertion of the Bp36/51 encoding cDNA into plasmid pET-17b was confirmed by selecting clones expressing the hexadecapeptide by immunoscreening with moAb 4A6, by hybridization of the nitrocellulose-blotted plasmid DNA with an oligonucleotide probe with specificity for this sequence and by DNA sequence analysis of the T7 transcription/expression region of pET-prof.

3.2. Expression of immunoreactive, recombinant Bp36/51-tagged antigens

In order to investigate whether cDNAs coding for foreign antigens are correctly expressed as Bp36/51-tagged recombinant proteins, we inserted the cDNAs coding for major timothy grass pollen allergens, Phl p 2 (Dolecek et al., 1993), Phl p 6 (Petersen et al., 1995) and for a calcium-binding alder pollen allergen, Aln g 4 (Hayek et al., 1998), into pET-prof. Phl p 2 and Phl p 6 represent major grass pollen allergens that are targets for IgE antibodies of more than 60% of patients suffering from grass pollen allergy. Aln g 4 and other members of the two EF-hand calcium-binding allergens occur as highly conserved cross-reactive allergens in pollens of trees, grasses and weeds. They share an average sequence identity of approximately 70% with each other and thus contain cross-reactive IgE epitopes. However, none of the three selected allergens contains cross-reactive epitopes with birch profilin. As can be seen in Fig. 2, synthesis of immunoreactive recombinant Bp36/51-tagged allergens was induced after addition of IPTG to transformed *E. coli* cells (Fig. 2: lanes +). Recombinant Bp36/51-tagged allergens were detected in the nitrocellulose-blotted *E. coli* extracts by moAb 4A6 (Fig. 2: 4A6) as well as by IgE antibodies of a pollen allergic patient (Fig. 2: IgE) but not by serum IgE of a non-allergic individual (Fig. 2: N). The high-molecular-weight moieties detected by moAb 4A6 in the induced rPhl p 2- and rPhl p 6-containing extracts (Fig. 2: Phl p 2, Phl p 6: lanes +) may represent oligomeric protein forms,

whereas the low molecular band (approximately 6 kDa) in the rPhl p 6-containing extract may result from degradation.

3.3. Recombinant Bp36/51-tagged allergens are expressed as soluble proteins and exhibit an IgE-binding capacity comparable to their untagged counterparts

Next, we studied whether recombinant Bp36/51-tagged allergens are expressed as soluble proteins in *E. coli*. The SDS-PAGE analysis of the soluble protein fraction gained after homogenization and high-speed centrifugation of *E. coli* extracts by Coomassie Blue staining showed that all three recombinant allergens accumulated in the soluble protein fraction (data not shown). When we compared the IgE-binding capacity of recombinant pollen allergens, rPhl p 2, rPhl p 6, rAln g 4, with their Bp36/51-tagged versions, we found that sera from all 18 pollen allergic patients tested exhibited a comparable IgE reactivity to both protein versions (Fig. 3A–C). Fifteen sera contained IgE antibodies to nitrocellulose-blotted rPhl p 2 and Bp36/51-tagged rPhl p 2 (Fig. 3A: sera 1–3, 5–7, 9–15, 17–18). Thirteen sera reacted with rPhl p 6 as well as Bp36/51-tagged rPhl p 6 (Fig. 3B: sera 1, 3–5, 7–10, 14–18) and nine sera bound to tagged as well as untagged rAln g 4 (Fig. 3C: sera 1–9). Sera that exhibited weak IgE reactivity to untagged recombinant allergens (rPhl p 6: sera 4, 8, 10, 16; rAln g 4: sera 2, 4) even appeared to react more strongly with the Bp36/51-tagged versions (Fig. 3B and C). Sera from five non-allergic individuals failed to display any IgE reactivity to the allergens (data not shown). The specificity and clinical relevance of the IgE recognition of Bp36/51-tagged recombinant allergens are supported by the fact that the IgE reactivity profile of all 18 sera with Bp36/51-tagged recombinant allergens matched the profile determined with purified untagged allergens by CAP RAST (radioallergosorbent test) and ELISA measurements as well as by skin-prick testing (see Section 2; data not shown).

3.4. Bp36/51-tagged recombinant allergens can be presented by moAb 4A6 to IgE antibodies in a sandwich ELISA

In order to establish a sandwich ELISA useful for the rapid screening of total *E. coli* extracts for a given recombinant antigen, we coated purified moAb 4A6 onto ELISA plates. ELISA plate-coated moAb 4A6 was then used to immobilize Bp36/51-tagged recombinant allergens (rPhl p 2, rPhl p 6, rAln g 4) and to present them to IgE antibodies of allergic patients (Fig. 4). The specificity of the sandwich ELISA was tested by fixing three different Bp36/51-tagged recombinant allergens out of total *E. coli* extracts and presenting them to sera from 10 pollen allergic patients with established IgE

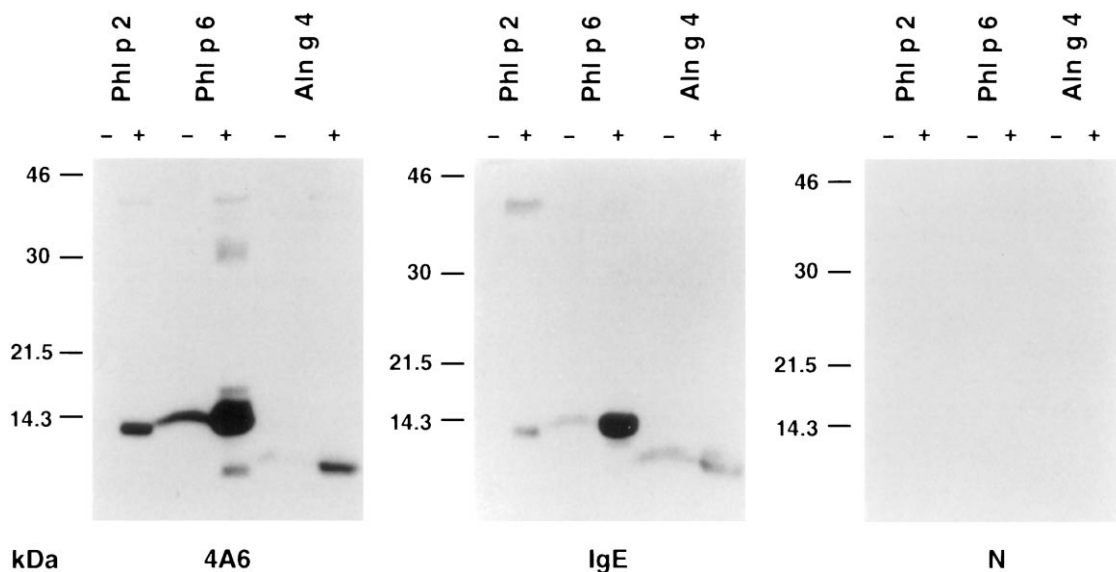


Fig. 2. *E. coli* expression and immunoreactivity of Bp36/51-fused recombinant allergens. Recombinant Bp36/51 fused allergens Phl p 2, Phl p 6 and Aln g 4 were expressed in *E. coli* BL21 (DE3). *E. coli* extracts obtained before (lane –) and after (lane +) induction of protein synthesis were blotted onto nitrocellulose. Nitrocellulose-blotted Bp36/51-fused rPhl p 2, rPhl p 6 and rAln g 4 were exposed to moAb 4A6 (4A6), serum IgE from a patient allergic to these allergens (IgE) and, for control purposes, serum from a non-allergic individual (N).

reactivity profiles as well as to sera from four non-atopic individuals. The results obtained showed that five sera contained rAln g 4-specific IgE antibodies, all 10 sera reacted, albeit more weakly, with rPhl p 2, and seven sera exhibited IgE binding to rPhl p 6 (Fig. 4). None of the four sera from non-atopic individuals displayed any significant IgE reactivity (>0.1 OD) to the tagged allergens. The IgE reactivity profile determined by the catching ELISA resembled that determined by direct ELISA measurements with purified recombinant allergens as well as the clinical sensitization patterns of the patients as determined by skin-prick testing (Section 2; data not shown). MoAb 4A6 can thus be used to immobilize Bp36/51-tagged recombinant allergens directly from crude *E. coli* extracts and to present the allergens in a highly specific and sensitive manner to IgE antibodies of pollen allergic patients by ELISA

3.5. Bp36/51-tagged recombinant allergens can be simultaneously localized by moAb 4A6 and allergen-specific antibodies by immunogold electron microscopy

Monitoring the fate of tagged recombinant antigens/peptides by immunohistochemistry as well as at the ultrastructural level under denaturing fixation conditions may be relevant for many basic immunological and cell biological research questions (e.g. distribution of antigens in tissues, cells). We have therefore investigated whether Bp36/51-tagged recombinant allergens can be co-localized in situ with moAb 4A6 and specific antisera by post-embedding immunogold electron microscopy (Fig. 5A and B). As examples for the detection of tagged antigens fixed under denaturing

conditions, we investigated paraformaldehyde/glutaraldehyde-fixed ultrathin sections of *E. coli* cells containing Bp36/51-tagged recombinant Phl p 6 (Fig. 5A and B). The results obtained showed that Bp36/51-tagged rPhl p 6 was detected by moAb 4A6 (Fig. 5A; 20 nm gold particles) but not by the control mouse monoclonal antibody, Bip 1 (Fig. 5B). Allergen-specific rabbit Ig reacted with rPhl p 6 (Fig. 5A; 10 nm gold particles). Rabbit preimmune Ig failed to significantly stain an *E. coli* section containing recombinant Phl p 6 (Fig. 5B). In summary, these results show that moAb 4A6 and antigen-specific antisera allowed co-localization of Bp36/51-tagged recombinant allergens by immunogold electron microscopy, even under denaturing conditions.

4. Discussion

We report the construction of a T7 RNA polymerase-driven expression plasmid, pET-prof, which allows the *E. coli* production of foreign proteins/peptides containing a birch profilin-derived hexadecapeptide (Bp36/51) fused to their N-terminus. The described expression plasmid, in conjunction with a monoclonal antibody moAb 4A6 specific for the Bp36/51 peptide tag, represents a system for the rapid production of high levels of Bp36/51-tagged recombinant antigens to be used for basic immunological research and for diagnostic procedures. Due to the previously described high affinity of moAb 4A6 (Wiedemann et al., 1996), it was possible to immobilize oligomeric protein complexes consisting of tagged antigens, primary antibodies and alkaline-phos-

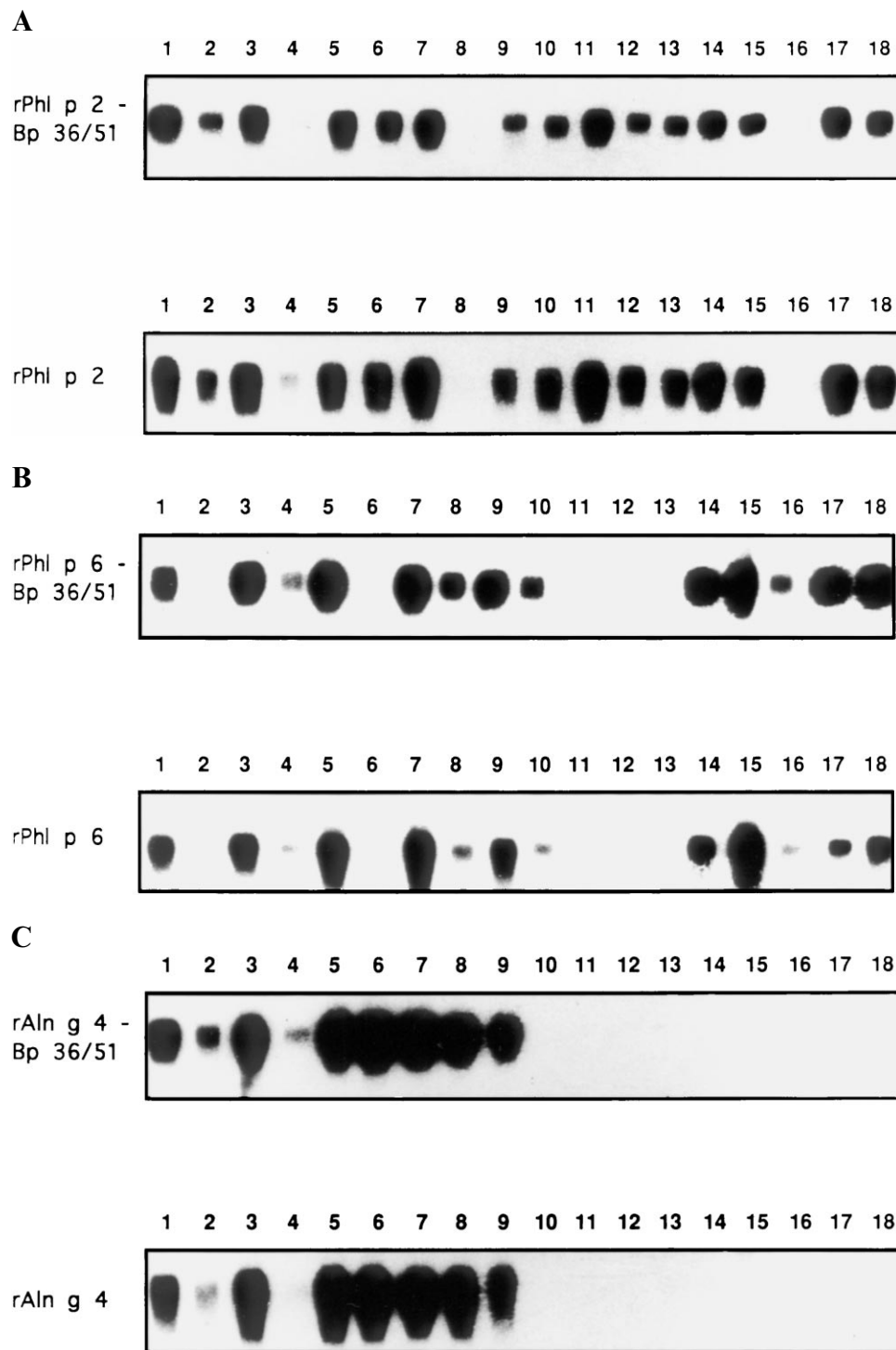


Fig. 3. Comparison of the IgE-binding capacity of recombinant allergens with that of Bp36/51-fused recombinant allergens. Nitrocellulose-blotted recombinant allergens, (A) rPhl p 2-Bp 36/51: top panel, rPhl p 2: bottom panel; (B) rPhl p 6-Bp 35/51: top panel, rPhl p 6: bottom panel; (C) rAln 4-Bp 36/51: top panel, rAln g 4: bottom panel, were exposed to sera from 18 pollen allergic patients (lanes 1–18). Bound IgE antibodies were detected with ^{125}I -labeled anti-human IgE antibodies and visualized by autoradiography.

phatase-conjugated secondary antibodies in an ELISA system. The pET-prof system may equally well be used for immunoprecipitation experiments as previously suggested (Rüdiger et al., 1997). When evaluated in a sandwich ELISA system, which was based on the immo-

bilization of tagged allergens via moAb 4A6 to ELISA plates, we found that the pET-prof system allowed sensitive and specific detection of allergen-specific IgE antibodies from allergic patients, which could not be achieved using other tag systems (e.g. hexahistidine tag)

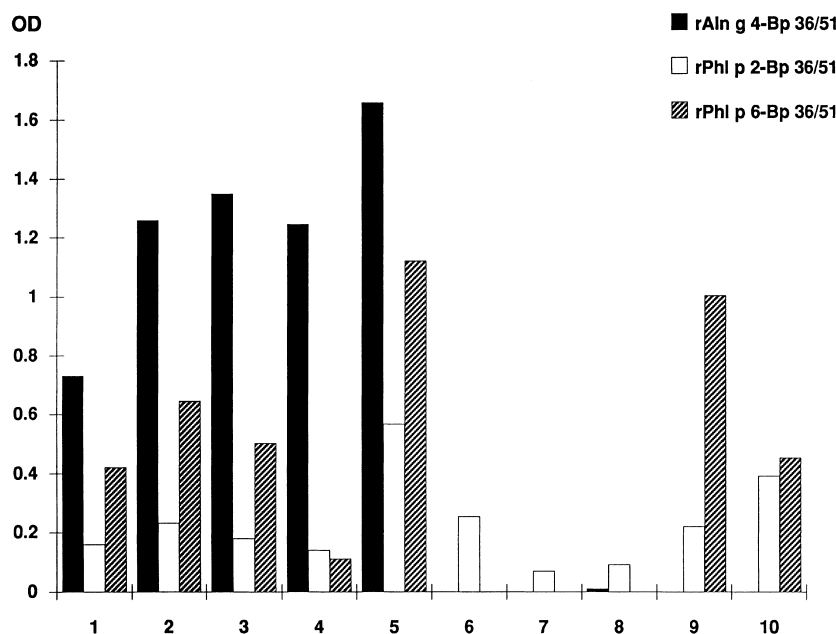


Fig. 4. Establishment of a sandwich ELISA assay for the detection of Bp36/51-tagged recombinant proteins in *E. coli* extracts. Serum IgE reactivity of 10 pollen allergic patients (1–10) to Bp36/51-tagged recombinant allergens (rAln g 4-Bp 36/51: black bars; rPhl p 2-Bp 36/51: white bars; rPhl p 6-Bp 36/51: cross-hatched bars) by sandwich ELISA. The y-axis displays the mean optical densities obtained from duplicate measurements after subtraction of a cut-off level determined from four sera from non-atopic individuals. The OD levels correspond to the amount of bound IgE antibodies.

(data not shown). The latter finding is notable, considering the fact that IgE antibodies represent the least abundant class of immunoglobulins in serum and other body fluids. The pET-prof system was thus useful in setting up a highly sensitive ELISA assay with low background levels, even at low serum dilutions, and also allowed the production of tagged recombinant allergens that closely resembled the immunological properties of the authentic antigens. This assumption is supported by experiments performed with three different recombinant allergens. The cDNAs coding for three prominent pollen allergens, the major timothy grass pollen allergens, Phl p 2 (Dolecek et al., 1993), Phl p 6 (Petersen et al., 1995), and the cross-reactive alder pollen allergen, Aln g 4 (Hayek et al., 1998), were expressed as Bp36/51-tagged recombinant proteins. Using IgE antibodies from a representative panel of allergic patients, we demonstrate that allergens with and without a tag exhibited a comparable IgE binding capacity. These results indicate that the addition of the Bp36/51 tag to the N-termini of the allergens did not significantly impair their antigenic properties.

MoAb 4A6 was previously found to be useful in detecting Bp36/51 tagged proteins by immunofluorescence. Here, we extend this finding by demonstrating that moAb 4A6 reacted specifically with tagged allergens on ultrathin sections fixed under denaturing conditions (paraformaldehyde/glutaraldehyde fixation) for ultrastructural analyses (e.g. immunogold electron microscopy). These findings suggest that the in-situ tissue

distribution of Bp36/51-tagged peptides can be readily monitored by moAb 4A6 at the ultrastructural level. It should be equally possible to trace Bp36/51-tagged recombinant peptides after binding to cells (e.g. antigen uptake by antigen presenting cells) and at the ultrastructural level within the cells (e.g. intracellular antigen transport).

As the Bp36/51 tag is small and did not significantly impair the antigenic properties (antibody binding capacity) of the three different allergens, the pET-prof system may be used for the set up of immunological assays.

One possible research application of the pET-prof system would be the determination of B-cell and T-cell epitopes of major antigens whose DNA and amino acid sequences are known. pET-prof would allow tagged peptides to be produced that can be presented to antibodies in an ELISA system without the need to purify the recombinant epitopes. pET-prof could be used as the expression vector for the construction of epitope expression libraries from either randomly fragmented antigen-encoding DNA or defined DNA fragments generated by PCR. Antibody-reactive clones can be identified by colony screening, and tagged epitopes can be presented via moAb 4A6 by sandwich ELISA to antibodies or, perhaps, to cells (T-cells, effector cells) without the need for extensive purification. Such an epitope expression system may be superior to the use of overlapping peptides for epitope mapping (Geysen et al., 1987) at least for two reasons. First, overlapping peptides are rather expensive, and there are technical limitations

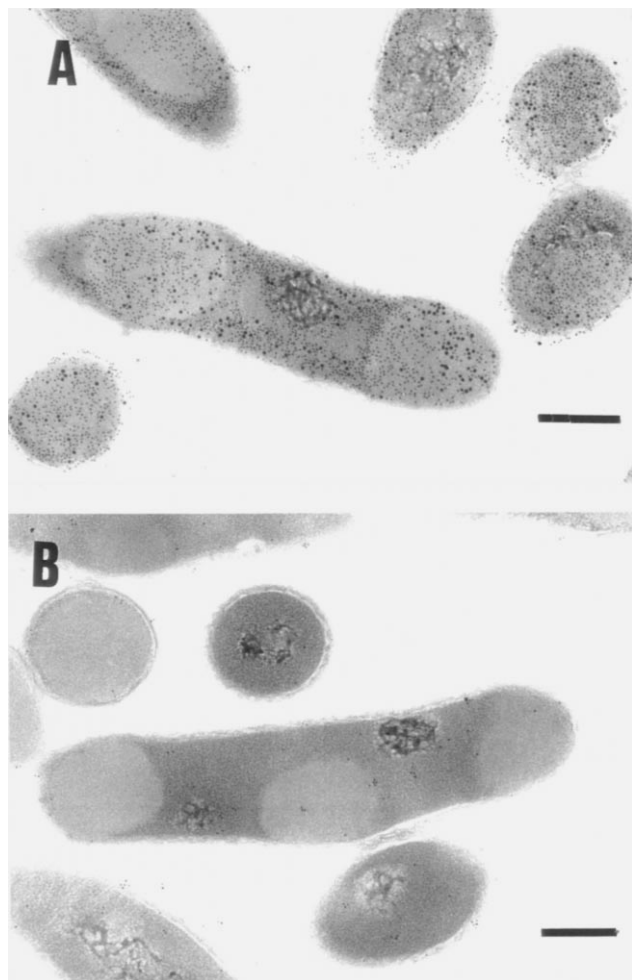


Fig. 5. Immunogold electron microscopical localization of Bp36/51-fused recombinant allergens in *E. coli*. Ultrathin sections of *E. coli* expressing recombinant Phl p 6 (A) were stained with moAb 4A6 and a specific rabbit antiserum. For control purposes, an ultrathin section of *E. coli* expressing rPhl p 6 was exposed to rabbit preimmune Ig and moAb Bip1 (B). Bound rabbit and mouse antibodies were detected with 10 nm gold-conjugated goat anti-rabbit and 20 nm gold-conjugated goat anti-mouse antibodies, respectively. The bars in the figures correspond to 0.5 μ m.

regarding their maximal possible length. Second, peptide epitope mapping was found to be less effective for the determination of B-cell epitopes than antibody-based screening of epitope expression cDNA libraries (Ball et al., 1994). The possibility of immobilizing recombinant antigens via moAb 4A6 to solid phases without impairing their immunological properties may also be useful for procedures in studying the interactions of antigens and antibodies in fluid-phase assays used for affinity/avidity measurements (O'Shannessy et al., 1993).

Finally, the pET-prof system may be also applied to optimize the production of recombinant antigens, being increasingly used for the diagnosis of immunologically mediated diseases (Type I allergy, celiac disease, autoimmune diseases, etc.) (Valenta and Kraft, 1995). Bp36/51-tagged recombinant antigens can be easily

detected and quantified in total *E. coli* extracts using moAb 4A6. It will thus be possible to use one reagent (e.g. moAb 4A6) to monitor the expression of tagged recombinant antigens and to establish purification procedures. As exemplified for three recombinant pollen allergens, it is also possible to use moAb 4A6 to immobilize recombinant antigens directly from bacterial extracts to solid phases and study their immunological properties (e.g. by sandwich ELISA).

In conclusion, we have developed a system for the high-level expression, detection and presentation of recombinant antigens/peptides based on a birch profilin-derived hexadecapeptide tag and a monoclonal antibody. This system may be useful for basic immunological research as well as for the development of reagents for immunological diagnosis.

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