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Production of specific antibodies against protein A fusion proteins

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The gene for Staphylococcal protein A was fused to the coding sequence of bacterial β-galactosidase, alkaline phosphatase and human insulin-like growth factor I (IGF-I). The fusion proteins, expressed in bacteria, were purified by affinity chromatography on IgG-Sepharose and antibodies were raised in rabbits. All three fusion proteins elicited specific antibodies against both the inserted protein sequences and the protein A moiety. In the case of IGF-I, the protein A moiety in the fusion protein may act as an adjuvant since native IGF-I alone is a poor immunogen. The results suggest that the protein A fusion system can be used for efficient antibody production against peptides or proteins expressed from cloned or synthetic genes. To facilitate such gene fusions a set of optimized vectors have been constructed.

Key words: antibody production/Staphylococcal protein A/fusion proteins

Introduction

Antibodies against proteins or peptides are important tools to study protein structure and function. Synthetic peptides have recently been used to raise antibodies against unknown proteins by deducing the amino acid sequence from the DNA sequences, taking into account the structural properties of the peptide residues (Sutcliffe *et al.*, 1980; Lerner *et al.*, 1981; Hopp and Woods, 1981). Small peptides, although invariably antigenic, are not always immunogenic and therefore require attachment to a carrier molecule to induce an immune response in recipient animals (Arnon *et al.*, 1971). These conjugates are not well defined with respect to coupling sites and ratio peptide versus carrier. Therefore considerable heterogeneity may arise in a population of coupled molecules.

An alternative approach to obtain specific antibodies is described in this paper. A defined fusion between the gene encoding Staphylococcal protein A (SpA) and the gene of interest was produced and the purified fusion protein subsequently used for immunization. Similar studies have previously been carried out with fusion proteins consisting of β-galactosidase and different inserts (Reed, 1982; Ullman, 1984; Schoner *et al.*, 1985) and with the vaccinia virus system (Perkus *et al.*, 1985). Fusion with the protein A moiety, however, has several advantages. First and most important, the purification of the fusion protein was facilitated by IgG affinity chromatography giving >95% yields and a high degree of purification (Nilsson *et al.*, 1985a). Second, the fusion protein could be directed to different compartments of the recombinant host cell by varying the leader sequences (Nilsson *et al.*, 1985b). Third, the problem of solubilizing the 'inclusion bodies', often obtained using the β-galactosidase fusion system (Schoner *et al.*, 1985), was avoided as the gene fusion product usually did not precipitate in the host cell. Fourth, protection of the desired product against host specific proteolysis was achieved (Nilsson *et al.*, 1985a). Finally, the protein A moiety served as a carrier protein during immunization and due to its repetitive structure probably enhanced the immune response.

The protein A fusion system was used to investigate the immunogenic properties of bacterial β-galactosidase, alkaline phosphatase and human insulin-like factor I (IGF-I), representing different macromolecules with regard to origin, function, structure and size. β-Galactosidase is a large enzyme consisting of four identical subunits with a total mol. wt of 460 kd (Ullman, 1984), alkaline phosphatase is a dimer with a total mol. wt of 96 kd (Hoffman and Wright, 1985) and IGF-I is a monomer, containing disulfide bridges, with a mol. wt of 7.7 kd (Rinderknecht and Humbel, 1978). After immunization of rabbits all three fusion proteins gave rise to specific antibodies against both the inserted protein sequences and the protein A moiety.

To facilitate expression a new set of gene fusion vectors based on the protein A gene was constructed by moving the fusion point in the protein A gene to conserve all five IgG-binding domains (Moks *et al.*, 1986), providing vectors with the M13 mp8 multilinker in all three reading frames.

Results

Expression and purification of the fusion proteins

The construction of the protein A gene fusion vectors used in this study have been described earlier (Nilson *et al.*, 1985a, b). A schematic drawing of the protein A gene containing the signal sequence (S), the IgG-binding regions (IGB) and the cell-wall binding region (CWB) is shown in Figure 1A and the different gene fusions in Figure 1B, C and D. The fusion proteins containing β-galactosidase (*lacZ*) and alkaline phosphatase (*phoA*) were expressed in *Escherichia coli* giving accumulation of intracellular or periplasmatic fusion products respectively. The fusion protein containing IGF-I was expressed in *Staphylococcus aureus* and the product was purified from the growth medium. Since β-galactosidase is an intracellular enzyme the signal sequence of the protein A gene was omitted from this construct. The constructs with IGF-I and alkaline phosphatase, on the other hand, contained the signal sequence to achieve secretion (Figure 1). The hybrid proteins were purified by IgG-Sepharose affinity chromatography (Uhlén *et al.*, 1983) and the products were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Figure 2). The slowest migrating band corresponds to a full-length fusion product, which is 31 kd for protein A itself (lane 1), 38 kd for the IGF-I fusion (lane 2), 75 kd for the alkaline phosphatase fusion (lane 3) and 147 kd for the β-galactosidase fusion (lane 4). N-terminal sequencing of the purified bands from the gel confirmed the expected N terminus

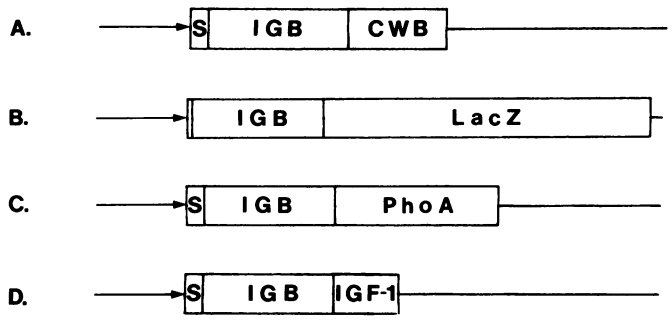

Fig. 1. Schematic drawings of the different gene constructs used in the expression of proteins for immunization. (**A**) Gene for native protein A, with the coding sequence for the signal peptide (S), the IgG-binding region (IGB) and the cell wall binding region (CWB) indicated. (**B**) Protein A−*lacZ* gene fusion for expression of protein A−β-galactosidase. (**C**) Protein A−*phoA* gene fusion for expression of protein A−alkaline phosphatase. (**D**) Protein A−IGF-I gene fusion.

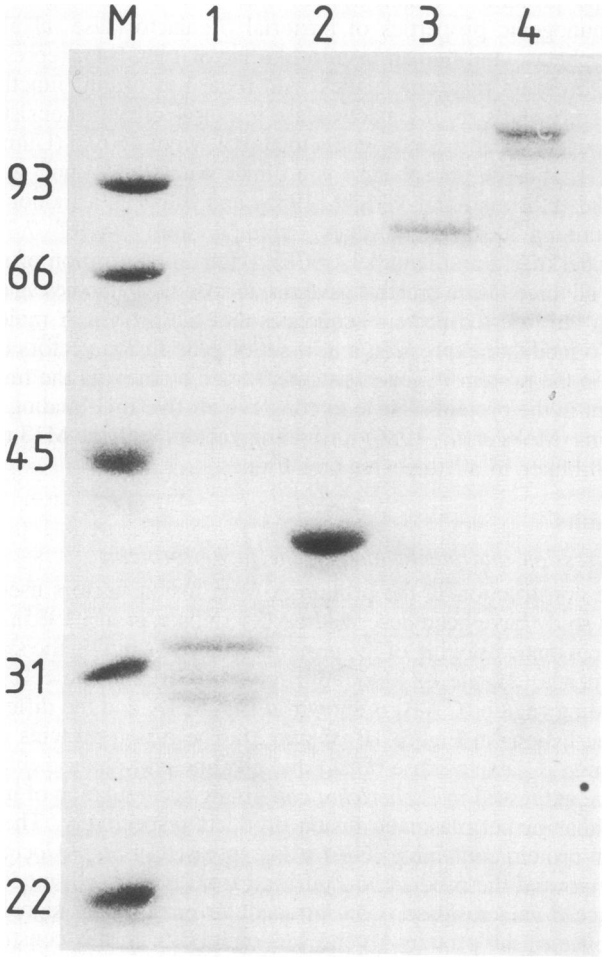

Fig. 2. SDS−polyacrylamide gel electrophoresis of the hybrid proteins. **Lane 1**, truncated portion of protein A expressed from the gene fusion vector pRIT4 (B); **lane 2**, protein A−IGF-I; **lane 3**, protein A−alkaline phosphatase; **lane 4** protein A−β-galactosidase; **lane M**, marker proteins with the size shown as mol. wt × 10^{-3}.

of protein A in each case (not shown). Faster migrating bands probably represent breakdown products in the C terminal end of the fusion proteins. This is supported by Western blot analysis using labelled human IgG which identified all bands (not shown). The single step affinity purification therefore seems to ensure an extensive purification suitable for immunization, although a small fraction of the protein consists of degraded fusion proteins.

The overall yield of fusion product from the different constructs varied considerably. The intracellular production in *Escherichia coli* of β-galactosidase fusion was 300 mg/l culture, the periplasmatic production in *E. coli* of alkaline phosphatase fusion was 50 mg/l and the extracellular production in *S. aureus* of IGF-I fusion was 5 mg/l. Although this represents a 60-fold difference in expression a one litre culture provides sufficient material for immunization and analysis.

Antibodies against the fusion proteins

The purified gene products were used to immunize rabbits by multiple site injections. After booster injections, the serum was collected and the IgG fractions recovered. Sera from all rabbits were initially screened for antibodies against β-galactosidase, alkaline phosphatase and IGF-I by immunodiffusion. They were all positive after the second booster injection (not shown). To demonstrate specificity in a more sensitive assay, we utilized the electroblotting technique transferring β-galactosidase, alkaline phosphatase and IGF-I to strips of nitrocellulose after SDS−PAGE. The strips were probed with the antibodies raised against the different fusion products. The Western blot analyses (Figure 3) demonstrate that the antibodies recognize the expected proteins and do not cross react with any of the other proteins included on the same strip.

Antibodies against protein A

The binding of protein A to the Fc portion of IgG must be blocked in order to demonstrate specific antibodies against SpA. Human IgG was used for this purpose because it has the same affinity for protein A as rabbit IgG (Lindmark *et al.*, 1983). By preincubating the nitrocellulose paper with human IgG and maintaining it in the reagent buffers during subsequent incubations (Figure 4, sheets 1 and 3) the Fc binding was abolished. In contrast, IgG from immunized animals bound to SpA regardless of whether the nitrocellulose sheet had been preincubated with human IgG or not. This is demonstrated in Figure 4 (Sheets 2 and 4) using IgG from a rabbit immunized with SpA-β-galactosidase. The same result was obtained using antisera against the alkaline phosphatase and the IGF-I fusion proteins (not shown). The dot blot analysis therefore demonstrates that the protein A part in the fusion proteins is itself immunogenic and gives rise to specific antibodies. It is obvious that at least some antigenic epitopes must be distinct from the Fc-binding domains of the native protein A molecule and thus cannot be competed out with human IgG.

Improved vector constructions

We have previously described several protein A gene fusion vectors for expression of foreign genes (Nilsson *et al.*, 1985b). Although these vectors have been used to express several gene products, they suffer from two drawbacks. Firstly, only one reading frame is available in the mp8 linker, and secondly the *Sau*3AI site used for gene fusion (Figure 5A) gives a protein fused in the middle of one of the IgG-binding domains of the protein A molecule which may affect the folding of the inserted product and might give a more unstable product. A fusion point at the end of the IgG-binding domains was therefore introduced using the *Bst*NI site located 18 bp downstream from region C (Figure 5A). This part of the protein A molecule, region X, has an extended tertiary structure (Guss *et al.*, 1984) making it suitable as a fusion point.

Three different linkers were synthesized by the phosphoamidite method (Elmblad *et al.*, 1984) and used to convert the *Bst*NI site to an *Eco*RI-site so that all three reading frames were ob-

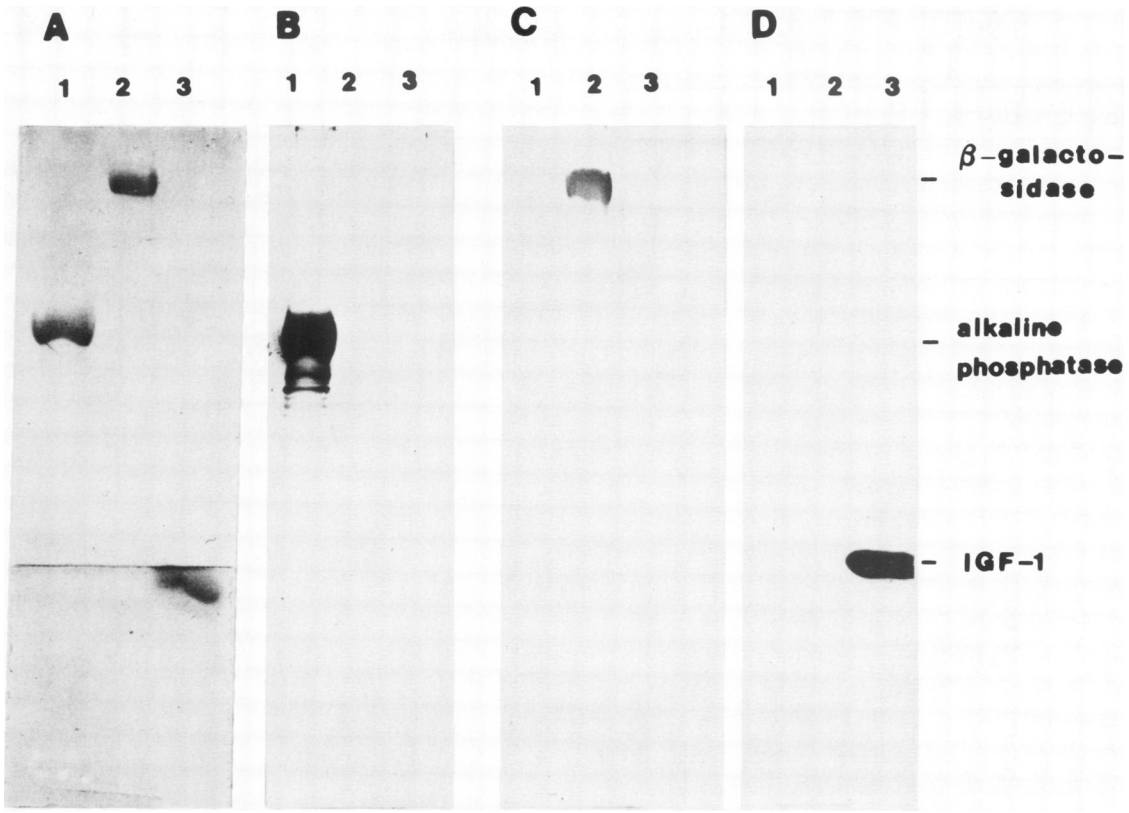

Fig. 3. Immunoblot analysis of the antibodies. Four sets of alkaline phosphatase (**lane 1**), β-galactosidase (**lane 2**) and IGF-I (**lane 3**) were separated by a 10 to 20% step gradient SDS–polyacrylamide gel electrophoresis. Three sets (**B, C** and **D**) were transferred to nitrocellulose filters. **A**, Coomassie blue stained gel (the interface between the different gel concentrations can be observed); **B**, probed with antibodies to the SpA–alkaline phosphatase; **C**, probed with antibodies to SpA–β-galactosidase; and **D**, probed with antibodies to the SpA–IGF-I gene fusion. Antibodies bound to the strips were detected by the peroxidase-anti-peroxidase method.

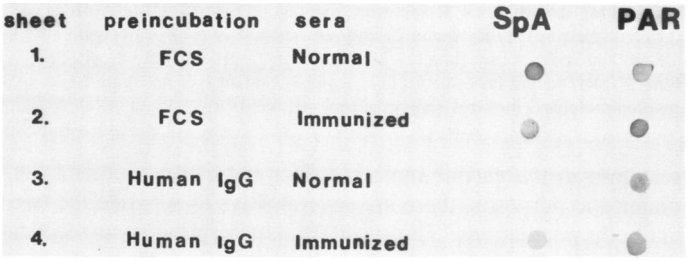

Fig. 4. Dot blot analysis of antibodies to the protein A part of the β-galactosidase fusion protein. 0.5 μg of SpA (**left**) and 5.7 μg of porcine anti-rabbit IgG (PAR) (**right**) as a positive control, was bound to nitrocellulose. The papers were prepared and processed and bound antibodies detected as described in Materials and methods. The strips were soaked either in 10% FCS (**1** and **2**) or 3.5% human IgG (**3** and **4**). Strips **1** and **3** were incubated with non-immunized rabbit IgG, and strips **2** and **4** were incubated with IgG from a rabbit immunized with the protein A–β-galactosidase fusion.

tained at the fusion point. This resulted in the vectors pRIT11, pRIT12 and pRIT13, schematically shown in Figure 5B, which are convenient for inserting coding sequences from genomic clones, cDNA clones or oligonucleotides encoding desired peptides.

The new set of vectors were evaluated by inserting the *E. coli* alkaline phosphatase gene (*phoA*) into the pRIT12 vector. The resulting plasmid pRIT17 coded for a protein A–alkaline phosphatase fusion protein similar to plasmid pRIT6 described earlier (Figure 1), but with the fusion downstream from the IgG-binding regions. *E. coli* cells containing pRIT6 and pRIT17 repectively, were grown and the periplasmic fraction collected by an osmotic shock procedure (Nosal and Heppel, 1965) and analyzed by SDS–PAGE (Figure 6, lanes 1 and 2). The desired gene fusion product constituted, in both cases, more than 80% of the total periplasmic proteins. This demonstrates the advantage of using secretion vectors. A considerable purification was obtained simply by the translocation of the gene product out of the cytoplasm of the host cells. After affinity purification a homogeneous fusion protein was obtained (lanes 3 and 4) although some degradation, especially for the pRIT6 gene product, can be observed.

Discussion

The present investigation demonstrates that the Staphylococcal protein A gene fusion system provides large amounts of a fusion protein which can be easily purified and used for immunization without chemical coupling to a carrier protein. Specific antibodies were obtained against proteins spanning over a large size range (7.7–116 kd) by immunizing rabbits with the fusion product obtained in *E. coli* or *S. aureus*.

Many properties of protein A make it an interesting fusion partner for developing antibodies against gene products. The fusion proteins can rapidly be purified by IgG affinity chromatography. For genes encoding small or normally secreted proteins the hybrid protein can be harvested from the growth medium using the appropriate protein A vector in a gram-positive host. The problem of incorrect folding often encountered when expressing peptide hormones intracellularly in bacteria, due to mixed intrachain

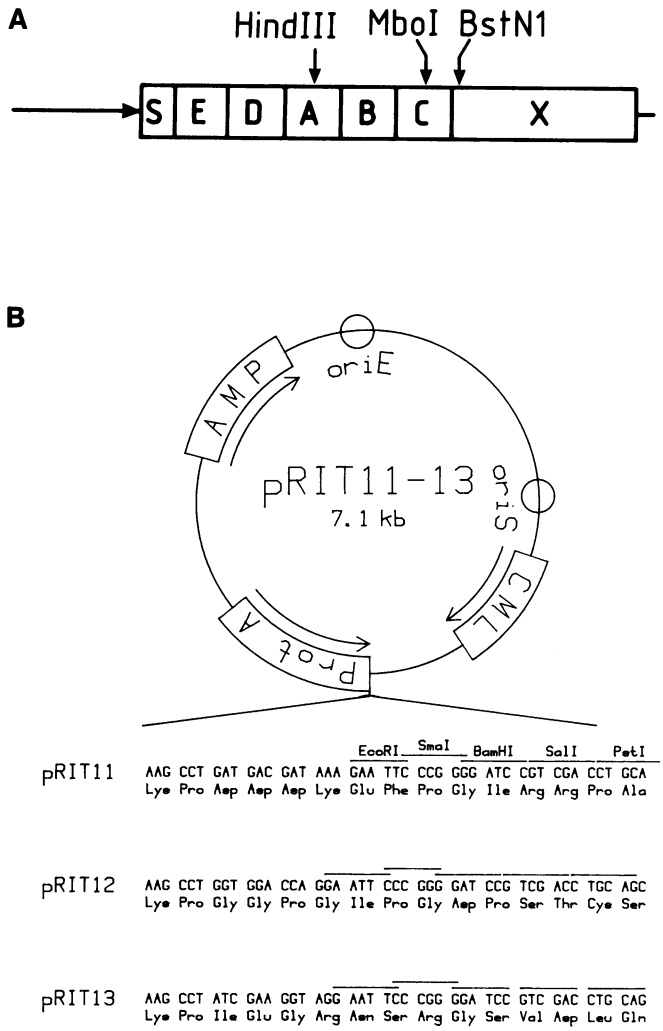

Fig. 5. The improved gene fusion vectors pRIT11, pRIT12 and pRIT13. The nucleotide sequence in the linker region of each vector is shown with the deduced amino acid sequence. Abbrevations: AMP, β-lactamase gene; ori E and ori S, origins of replication in *E. coli* and *S. aureus* respectively; CML, gene for chloramphenicol acetyl transferase; prot A, protein A gene.

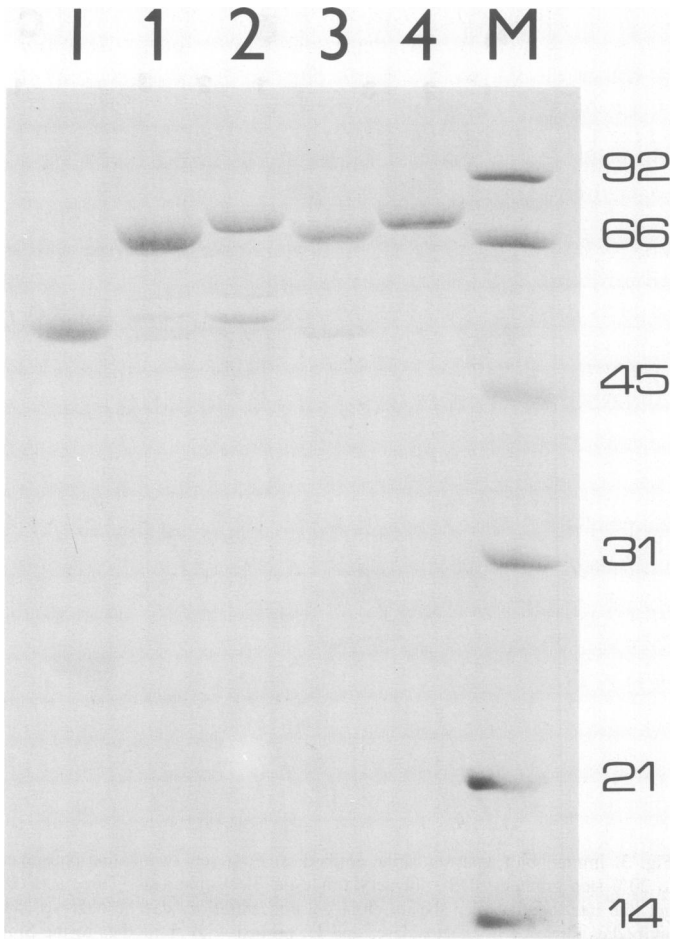

Fig. 6. SDS−polyacrylamide gel electrophoresis of the protein A−alkaline phosphatase fusion. Total periplasmic fractions of *E. coli* cells containing different plasmids (**lanes 1** and **2**) and purified fusion protein by IgG affinity chromatography (**lanes 3** and **4**). **Lane 1**, pRIT4; **lane 2**, pRIT17; **lane 3**, pRIT4; and **lane 4**, pRIT17. **Lane I**, human IgG and **lane M**, marker proteins with sizes shown as mol. wt × 10^{-3}.

disulfide bonds, is minimized in fusions with protein A since it lacks internal cysteine residues. Furthermore the IgG-binding units of protein A are independently folded globular domains (Moks *et al.*, 1986) and may therefore not interfere with the folding of the insert. By moving the fusion point downstream of the region coding for the IgG-binding domains, this aspect may have been optimized as indicated by the lack of degradation (Figure 6).

The repetitive globular units in protein A might also enhance the immune response, like other repetitive molecules, such as polysaccharides and flagellin (Klein, 1982). The good immune response obtained against IGF-I, normally a poor antigen in mammals (Furlanetto *et al.*, 1977; Laubli *et al.*, 1982; Baxter *et al.*, 1982), supports this hypothesis. The mitogenic effect of protein A (Kasahara *et al.*, 1980) and its role as a polyclonal B-cell activator (Sjödahl and Möller, 1979) are not fully clarified. This general effect on the immune system may, however, improve the specific immune response against the fusion proteins.

In all rabbits immunized with the fusion products, β-galactosidase, alkaline phosphatase and IGF-I, an immune response was also observed towards the protein A moiety. Although the response to the carrier molecule does not cause problems for diagnostic purposes, there are several ways to separate the two antibody populations. The most straight forward is to recover the specific antibodies over a column with purified antigens. Alternatively, the antisera can be raised in animals, such as chicken, in which no Fc binding to protein A exists (Lindmark *et al.*, 1983). The antibodies towards the protein A moiety can then be absorbed on a protein A affinity column.

Our results suggest that fusion of a cloned, or synthetic gene, to the protein A gene can be used to raise antibodies against a desired gene product. This approach may facilitate the characterization of new proteins predicted by DNA cloning techniques. It can also be used to investigate the function of specific domains of proteins by raising antibodies against them. The technique may also be used as a tool in the development of vaccines. The new set of vectors described (Figure 5) should provide suitable fusion points for most gene and cDNA products. An interesting possibility is to use the system as an alternative to synthetic peptides to generate antibodies to a defined epitope. Peptides, usually 6−20 amino acid residues long, have proved to be an excellent tool for molecular immunologists (Tainer *et al.*, 1984) and may also be used for vaccine development (Muller *et al.*, 1982). With the protein A system, a synthetic oligonucleotide coding for the

desired peptide may be inserted into the appropriate vector, and the resulting fusion protein subsequently used for immunization. Synthesis of oligonucleotides of 20–60 bases is rapid and simple, and may provide a homogeneous and exactly defined unit in the fusion protein suitable for immunization, in contrast to synthetic peptides which must be extensively purified and conjugated to a carrier protein.

Recently, we used the system to express a synthetic oligonucleotide coding for a C-terminal peptide of human IGF-I (B.Löwenadler, S.Paleus and B.Nilsson, unpublished). The gene product consisting of protein A and 14 residues of IGF-I was efficiently secreted in the *E. coli* host cells. The affinity purified fusion protein was used to successfully raise specific antibodies towards the IGF-I moiety.

Materials and methods

Bacterial strains and plasmids

E. coli HB101 (Boyer and Roulland-Dussoix, 1969) and *S. aureus* SA113 (Iordanescu, 1975) were used as bacterial hosts. The plasmids pRIT1, pRIT6 (Nilsson *et al.*, 1985b) and pUN201 (Nilsson *et al.*, 1985a) were used to express the hybrid proteins. The plasmid pNF2690 (Nilsson *et al.*, 1985b) harbouring the cI-857 gene was used to regulate the phage λ P_R promoter. Constructions were made from the plasmid pSPA12 (Uhlén *et al.*, 1983).

DNA constructions

Restriction enzymes (Boehringer Mannheim and New England Biolabs) and T4 DNA ligase (New England Biolabs) were used according to the suppliers recommendations. Transformation of competent *E. coli* was according to Morrison (1979) and transformation of *S. aureus* was as described by Götz *et al.* (1981). The synthetic oligonucleotides were prepared by the phosphoamidite method (Elmblad *et al.*, 1982) and purified by h.p.l.c. The gene coding for human IGF-I was made synthetically as described previously (Elmblad *et al.*, 1984).

A new set of gene fusion vectors were constructed by inserting three synthetic linkers into the *Bst*NI site at position 1180 of the protein A gene (Uhlén *et al.*, 1984). The plasmid pSPA12 (Nilsson *et al.*, 1985a) was cleaved with *Bst*NI and *Hin*dIII, and the fragment spanning from region A to region X, was recovered from an agarose gel. The three different linkers were ligated to the fragment and each ligation mixture was used to replace the corresponding *Hin*dIII/*Eco*RI fragment of plasmid pRIT5 (Nilsson *et al.*, 1985b).

Preparation of antigen

The protein A–β-galactosidase fusion protein was purified from sonicated *E. coli* cI-857 carrying the plasmid pRIT1. Expression from the λ P_R promoter was enhanced by a temperature shift from 30 to 42°C (Zabeau and Stanley, 1982). The protein A–alkaline phosphatase fusion was expressed in *E. coli* and released from the cells by osmotic shock (Nosal and Heppel, 1965). Protein A–IGF-I was expressed in *S. aureus* and the fusion protein was recovered from the growth medium. All hybrid proteins were purified using affinity chromatography on matrix-bound IgG. After washing, the fusion proteins were eluted with 1 M acetic acid (titrated to pH 2.8 using ammonium acetate) and lyophilized.

Immunization procedure

Six New Zealand white rabbits (two for each product) were immunized by multiple site injections containing 20–200 μg of the fusion proteins in Freunds complete adjuvant. Two booster injections were given at 3 week intervals with the same amount of protein. One week after the last injection the animals were sacrificed and blood was collected by heart puncture. The IgG fraction of the antisera was prepared according to published procedures using DEAE–cellulose (Harboe and Ingild, 1973).

Dot blot analysis of antibodies to the protein A part of the fusion proteins

Commercial protein A (Pharmacia) and porcine anti-rabbit IgG (PAR, Dakopatts), were blotted on nitrocellulose papers (Millipore). The filter papers were soaked at 37°C for 1 h in either 10% FCS in PBS (Pi/NaCl) pH 7.4 or 3.5% human IgG (Kabi AB) in the same buffer. The blots were then incubated overnight at 4°C with non-immunized rabbit IgG or IgG from a rabbit immunized with a fusion product, diluted 1:100 in Pi/NaCl, 0.01% Nonidet P40 (NP40) containing either 3% BSA (Sigma) or 3.5% human IgG. The blots were washed three times for 10 min each in Pi/NaCl. Bound antibodies were visualized using the peroxidase-anti-peroxidase system (PAP) from Dakopatts, essentially as described by Glass *et al.* (1981). Porcine anti-rabbit IgG dilute 1:400 in Pi/NaCl, 0.01% NP40, 3% BSA or Pi/NaCl, 0.01% NP40, 3.5% human IgG were incubated for 1 h at 37°C with the nitrocellulose blots. The papers were rinsed in Pi/NaCl, followed by incubation for 1 h at 37°C with rabbit PAP at a dilution of 1:1600. The blots were finally rinsed in three changes of Pi/NaCl and stained for peroxidase activity in 50 mM Tris–HCl pH 7.6 containing 0.1 mg/ml 3′,3′-diaminobenzidine (Sigma) and 0.01% H_2O_2. Colour development was stopped by transferring the nitrocellulose blots to a bath with deionized water.

Characterization of antibodies by electroblotting

Purified β-galactosidase (Sigma), alkaline phosphatase (Sigma) and purified IGF-I were electrophoresed in a 10 to 20% step gradient polyacrylamide slab gel under reducing conditions using the discontinuous SDS–buffer system described by Laemmli (1970). After separation, the proteins were transferred to nitrocellulose paper according to Towbin *et al.* (1979). The electrophoretic blots were incubated with 3.5% human IgG in Pi/NaCl at 37°C for 1 h, and then cut into three strips, each containing all three electrophoresed proteins. The strips were subsequently incubated overnight at 4°C with one of the fusion protein antibodies diluted 1:100 in Pi/NaCl, 0.01% NP40, 3.5% human IgG. Detection of bound antibodies was accomplished using the PAP system, with all reagents diluted in Pi/NaCl, 0.01% NP40, 3.5% human IgG.

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