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Carbohydrate-recognition domains as tools for rapid purification of recombinant eukaryotic proteins

Maureen E. TAYLOR and Kurt DRICKAMER
Department of Biochemistry and Molecular Biophysics, Columbia University, 630 West 168th Street, New York, NY 10032, U.S.A.

Methods have been developed for expression and purification of eukaryotic proteins by creating fusions with the carbohydrate-recognition domain (CRD) of the galactose-specific rat hepatic lectin. In order to generate the fusion proteins, vectors have been constructed so that cDNAs for passenger proteins can be inserted in any reading frame following a segment of DNA encoding the CRD. The feasibility of using this approach as an aid to protein purification has been demonstrated using human placental alkaline phosphatase. Following expression in either of two different eukaryotic expression systems, the CRD–phosphatase fusion protein can be isolated by one step of affinity chromatography on galactose-Sepharose under mild, non-denaturing conditions. Incorporation of a proteinase-sensitive linker allows cleavage of the CRD from the passenger protein. Immobilised proteinase could be rapidly separated from the cleavage products and the released, active phosphatase was purified away from the CRD by re-chromatography on galactose–Sepharose. These methods provide a means of isolating correctly folded recombinant eukaryotic proteins when cDNAs are available, but the properties of the encoded proteins are unknown.

INTRODUCTION

The potential value of gene fusions in protein purification has been recognized for several years. For example, fusion of proteins to an antigenic peptide such as β-galactosidase allows isolation of fusion products on immobilized antibody (Shuman *et al.*, 1980; Harris, 1983), while proteins created by fusion to the maltose-binding protein of *E. coli* can be isolated on sugar-containing resins (di Guan *et al.*, 1988). A major drawback to the antibody-purification approach is the need for harsh elution conditions to release the fusion product from the affinity column. The maltose-binding fusions can be purified under milder conditions, but this approach is limited to prokaryotic expression systems. Many eukaryotic proteins are not folded correctly in bacteria. In order to use fusion-protein-purification techniques to demonstrate biological activities of eukaryotic proteins which have been characterized by isolation of cDNAs, it is necessary to employ fusion domains which are active in eukaryotic expression systems and which allow purification under non-denaturing conditions.

Ca^{2+}-dependent carbohydrate recognition domains (CRDs) have been identified in a number of mammalian proteins (Drickamer, 1988*a*, 1989). These CRDs have various specificities for monosaccharides and are approx. 130 amino acids long. They can function independently of the rest of the proteins to which they are attached and can be purified by a single step of affinity chromatography on inexpensive saccharide-containing resins (Hseuh *et al.*, 1986; Drickamer, 1988*b*). Elution is readily achieved by removal of Ca^{2+} or competition with monosaccharides. It should, therefore, be possible to fuse a CRD to passenger proteins and use the sugar-binding activity of the CRD to purify the fusion proteins.

We describe the construction of eukaryotic expression vectors in which foreign proteins can be fused to the CRD of the galactose-specific rat asialoglycoprotein receptor (rat hepatic lectin, RHL). The vector consists of the coding region for the signal sequence of dog preproinsulin fused to the codons specifying the CRD of RHL. This is followed by a proteinase-sensitive linker with the sequence Ile-Glu-Gly-Arg (Nagai & Thogersen, 1984), which is specifically cleaved by factor Xa (Magnusson *et al.*, 1975) or by trypsin and allows release of the passenger protein from the CRD. We have used human placental alkaline phosphatase as a model protein to test the use of this method in two eukaryotic expression systems.

MATERIALS AND METHODS

Materials

Restriction enzymes and linkers were from New England Biolabs, and T4 DNA ligase and polynucleotide kinase were from Boehringer Mannheim. Rabbit reticulocyte lysate, dog pancreas microsomes, [^{35}S]methionine and Amplify fluorography solution were from Amersham, and G418 was from Gibco. Sepharose 6B, D-galactose, divinylsulphone and *p*-nitrophenylphosphate were from Sigma. Trypsin [tosylphenylalanylchloromethane ('TPCK')-treated] was from Worthington, and Affi-gel 10 was from Bio-Rad Laboratories.

Construction of plasmids

Standard recombinant DNA techniques were used in the construction of plasmids (Maniatis *et al.*, 1982). The starting plasmid pGIR-75 (Hseuh *et al.*, 1986) is a derivative of plasmid pSP64 which contains the 5′-untranslated segment of *Xenopus laevis* β-globin mRNA and the region of the dog preproinsulin gene encoding the signal sequence, B-chain, and a portion of the C-peptide (Kwok *et al.*, 1983) followed by the polylinker from pSP64. This vector was cut with *Hpa*I and *Acc*I, to remove all of the insulin-coding sequences after the first two residues of the B-chain, followed by filling of the *Acc*I site to make the vector pGIR-138, into which was cloned the portion of the RHL cDNA encoding the *C*-terminal 134 amino acids (Holland *et al.*, 1984) at the *Bam*HI site. This RHL-containing vector was modified by inserting a synthetic *Hin*dIII linker at the *Eco*RI site

Abbreviations used: RHL, rat hepatic lectin; CRD, carbohydrate-recognition domain; PLAP, placental alkaline phosphatase.

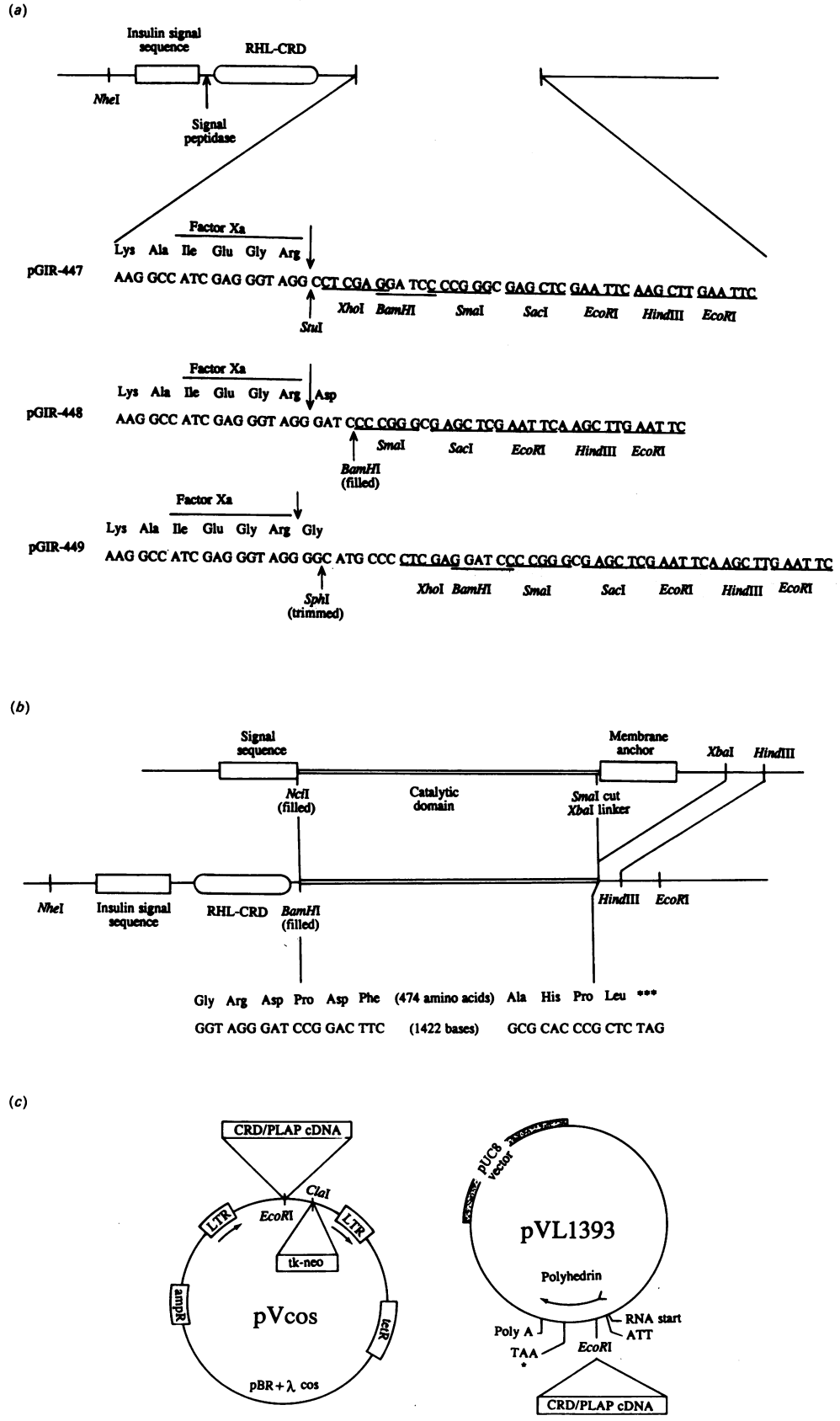


Fig. 1. Construction of expression vectors

(*a*) Structure of vectors containing the insulin-RHL CRD fusion and the protease-sensitive linker. As indicated by upward arrows, blunt ends for cloning in any of three reading frames can be created by digesting pGIR-447 with *Stu*I, cutting pGIR-448 with *Bam*HI followed by filling the 5′ overhang using the Klenow fragment of DNA polymerase I, or by cutting pGIR-449 with *Sph*I followed by trimming the 3′ overhang with T4 DNA polymerase. Restriction sites for 3′ ligation are also indicated. (*b*) Insertion of a portion of the human alkaline phosphatase cDNA into vector pGIR-448. (*c*) Transfer of RHL-PLAP fusion into pVcos for expression in rat fibroblasts and pVL1393 for expression in insect cells.

3′ to the RHL insert and a proteinase-sensitive linker encoded by a pair of synthetic oligonucleotides between the *Stu*I site within the RHL insert and the *Bam*HI site within the polylinker, to form pGIR-447 (Fig. 1*a*). Two other vectors, pGIR-448 and pGIR-449, were created by modifying the polylinker of pGIR-447 (Fig. 1*a*).

Construction of a vector (pGIRP) for expressing a fusion of the RHL CRD and alkaline phosphatase is summarized in Fig. 1(*b*). The cDNA encoding human placental alkaline phosphatase (Millan, 1986) was cut with *Sma*I to remove the portion coding for the *C*-terminal 25 amino acids which are involved in forming a membrane anchor, and a synthetic *Xba*I linker was added to form a new termination codon. The cDNA portion encoding the signal sequence and the first eight amino acids was removed by cutting with *Nci*I. The phosphatase cDNA was then cloned into pGIR-448 which had been cut with *Bam*HI and filled (5′ end) and then digested with *Hin*dIII (3′ end).

'*In vitro*' transcription and translation

The vector pGIRP was linearized with *Eco*RI and used as a template for *in vitro* transcription (Holland & Drickamer, 1985). *In vitro* translation reactions (30 μl) containing rabbit reticulocyte lysate (10 μl), dog pancreas microsomes (10 μl), [^{35}S]methionine (60 μCi), oxidised glutathione (0.2 mM final concn.) and approximately 1 μg of mRNA were incubated at 30 °C for 1 h. After incubation, the reaction products were separated by centrifugation through sucrose followed by immunoprecipitation (Holland & Drickamer, 1985; Hseuh *et al.*, 1986).

Production of RHL-PLAP in mammalian cells

pGIRP was cut at the unique *Nhe*I site and *Eco*RI linkers were added so that the entire *Eco*RI fragment could be cloned into the expression vector pVcos (Fig. 1*c*) (Maddon *et al.*, 1985). The *neo* (neomycin-resistance) gene was cloned into this intermediate vector at the unique *Cla*I site. The final vector, pVcosP, was transfected by the calcium phosphate method (Wigler *et al.*, 1979) into rat 6 cells. After approx. 1 week, cells resistant to G418 (400 μg/ml) were grown to confluence as mixed colonies and the medium was tested for the presence of the RHL-PLAP fusion protein by assaying for phosphatase activity. For production of RHL-PLAP, transfected cells passaged on to 100-mm plates were allowed to grow to near-confluence before the medium was collected for isolation of RHL-PLAP. Fresh medium was then added to the cells and left for an additional 3–5 days before collection.

Production of RHL-PLAP in a baculovirus expression system

The procedures used for the baculovirus expression system have been described in detail by Summers & Smith (1987). The *Eco*RI insert from the eukaryotic expression vector pVcosP was inserted into the baculovirus expression vector pVL1393 (Webb & Summers, 1990) to form pVL1393P (Fig. 1*c*). Wild-type *Autographa californica* nuclear polyhedrosis-virus DNA and pVL1393P DNA were co-transfected in Sf9 cells (derived from *Spodoptera frugiperda*) and recombinant plaques were detected by hybridization with ^{32}P-labelled *Eco*RI insert from pVcosP. Following three rounds of plaque purification, one pure recombinant plaque was used to produce a stock of recombinant virus. For production of RHL-PLAP, 7.5×10^8 cells were infected with virus (multiplicity of infection approx. 10), resuspended in 250 ml of medium in a spinner flask and incubated at 27 °C for 4 days.

Isolation of RHL-PLAP fusion protein

Galactose–Sepharose was prepared by direct conjugation of galactose to Sepharose 6B with divinylsulphone (Fornstedt & Porath, 1975). Medium was centrifuged at 10000 *g* for 10 min and then mixed with an equal volume of loading buffer [20 mM-Tris/HCl (pH 7.8)/0.5 M-NaCl/25 mM-$CaCl_2$]. The medium was passed through two columns connected in series, the first containing Sepharose 6B (2–5 ml) and the second containing galactose–Sepharose 6B (2–5 ml). The pre-column of Sepharose was used to remove serum amyloid protein which binds to Sepharose in a Ca^{2+}-dependent manner (Hind *et al.*, 1985) and might be present in foetal calf serum. After the medium had passed through both columns, the galactose–Sepharose column was washed extensively with loading buffer and eluted with 20 mM-Tris/HCl (pH 7.8)/0.5 M-NaCl containing either 2 mM-EDTA or 0.2 M-galactose.

Alkaline phosphatase assays

Aliquots (2–20 μl) of medium or column fraction were added to 1 ml of 0.95 M-diethanolamine (pH 9.85)/0.28 M-NaCl/0.5 mM-$MgCl_2$, and the reaction was initiated by adding 50 μl of 0.1 M-*p*-nitrophenyl phosphate. After incubation at 30 °C for 60 min the absorbance at 405 nm was determined. A unit of alkaline phosphatase is defined as the amount of enzyme required to hydrolyse 1 nmol of substrate in 1 min.

Digestion of RHL-PLAP fusion protein with trypsin

For test digestion with soluble trypsin, RHL-PLAP isolated from the baculovirus system was incubated with various amounts of trypsin in the presence of 10 mM-$CaCl_2$ and 10 mM-$MgCl_2$ for 1 h at 37 °C. Immobilized trypsin was prepared by washing 1.5 ml of Affi-gel 10 with cold 10 mM-sodium acetate (pH 4.5) and then incubating it with 5 mg of trypsin in 0.1 M-Mops (pH 7.5) for 6 h at 4 °C. The resin was washed with 1 M-NaCl to remove unbound trypsin and stored in 1 mM-HCl/10 mM-$CaCl_2$. RHL-PLAP (1 ml final volume) dialysed against 20 mM-Tris/HCl (pH 7.8)/0.1 M-NaCl to remove galactose was incubated with immobilized trypsin (0.75 ml) in the presence of 10 mM-$CaCl_2$ and 10 mM-$MgCl_2$ for 3 h at 37 °C. After incubation, the gel was centrifuged for 2 min at 400 *g*, the supernatant was recovered, and the gel was washed with 1 ml of loading buffer. The combined supernatants were loaded on to a 1 ml column of galactose–Sepharose and the column was washed with 6 ml of loading buffer. The combined flow-through and washings were lyophilised.

RESULTS

CRD-fusion vectors

The three vectors shown in Fig. 1(*a*) are designed to facilitate introduction of passenger protein cDNAs following the preproinsulin signal sequence, the CRD of RHL, and a proteinase-sensitive linker. At the 5′ end, blunt ends can be generated in each reading frame with the introduction of at most one amino acid following the cleavage site. Several restriction sites are available for ligation of the 3′ ends of the inserted cDNAs. The polylinker would also allow other cloning strategies in which additional amino acids would be introduced at the *N*-terminus of the passenger protein. The sites available should facilitate expression of a variety of intact proteins or protein fragments. The feasibility of using this type of vector has been tested using human placental alkaline phosphatase as the passenger protein in the vector designated pGIR-448 (Fig. 1*b*).

Production of RHL-alkaline phosphatase fusion

In order to determine that the insulin signal sequence and RHL CRD function correctly in the RHL-PLAP fusion protein, the fused cDNA was translated *in vitro*. Targeting of the RHL-PLAP fusion is shown in Fig. 2(*a*). In the absence of microsomes,

protein is seen only in the supernatant fraction. In the presence of microsomes, most of the protein is associated with the pellet, indicating that the insulin signal sequence directs the fusion protein into the microsomes. The protein in the pellet is larger than that in the supernatant showing that glycosylation takes place in the microsomes. Oligosaccharides attached to the fusion protein do not interfere with chromatography on galactose–Sepharose (see below). The processed protein has a relative M_r of approx. 70000 which is expected for a fusion of PLAP (M_r = 55000) and the CRD of RHL (M_r = 15000).

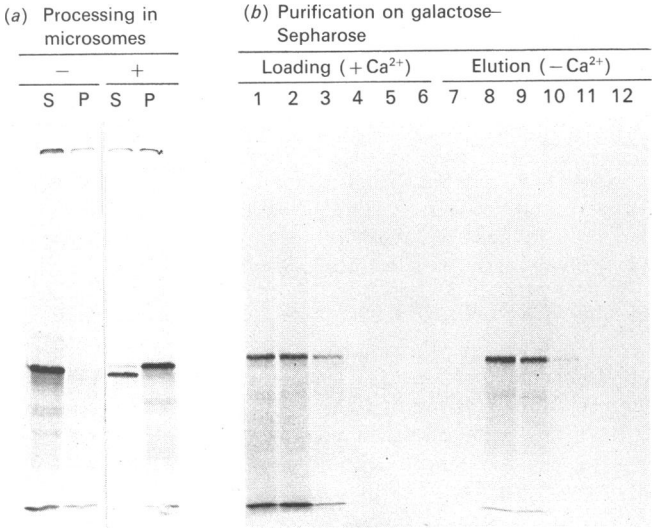


Fig. 2. Characterization of translation products of plasmid pGIRP-derived mRNA

(*a*) Processing in microsomes. mRNA was translated in a rabbit reticulocyte lysate system in the absence (–) or presence (+) of dog pancreas microsomes. Following centrifugation, supernatant (S) and pellet (P) were precipitated with antibodies raised against RHL and visualized by fluorography following SDS/PAGE in the buffer system of Laemmli (1970) on a gel containing 10% acrylamide. (*b*) Purification on galactose–Sepharose. Microsome-associated translation products were solubilized in Triton X-100 and loaded on to a 1 ml column of galactose–Sepharose. After washing with loading buffer (+Ca^{2+}) (fractions 1–6), the column was eluted with EDTA-containing buffer (–Ca^{2+}) (fractions 7–12). Each fraction was immunoprecipitated and analysed by SDS/PAGE followed by fluorography.

To test the binding activity of the fused RHL CRD, the microsome-associated protein was solubilized in detergent and passed over a column of galactose–Sepharose. Analysis of each column fraction (Fig. 2*b*) showed that, although some of the fusion protein passes through the column, a significant amount binds and can be eluted with EDTA. This result indicates that most of the fusion protein is folded correctly and that the CRD retains its binding activity.

Expression of fusion protein in eukaryotic systems

Following translation of the construct *in vitro*, the DNA coding for the RHL-PLAP fusion was transferred into a retroviral expression vector pVcos for expression in rat 6 fibroblasts, and into a baculovirus transfer vector for expression in insect cells. The results of the isolation of RHL-PLAP from the medium of transfected rat 6 cells are shown in Fig. 3. A single protein with an M_r of approx. 70000 (the expected M_r of RHL-PLAP) was eluted from galactose–Sepharose with EDTA (Fig. 3*a*). Immunoblotting with an antibody against RHL demonstrated that the protein contains the RHL CRD (Fig. 3*b*) and the phosphatase assay showed that it has phosphatase activity (Fig. 3*c*).

The fusion protein was also successfully isolated from the medium of Sf9 cells infected with the recombinant baculovirus vector (Fig. 4). It had been noted in previous experiments that storage of the fusion protein in EDTA-containing eluting buffer resulted in loss of phosphatase activity, presumably because alkaline phosphatase is a zinc-metalloenzyme (Gottlieb & Sussman, 1968). For this reason, an alternative elution method was tested in which 0.2 M-galactose was used to displace the fusion protein from the affinity column. As shown in Fig. 4, monosaccharide competition results in efficient elution, with greater retention of biological activity. Comparison of the phosphatase activity of the isolated protein with that of the medium revealed that 90% (50000 out of 56000 units) of the RHL-PLAP fusion protein was recovered by the galactose-elution method.

Cleavage of fusion proteins

The final step in testing the use of the CRD-containing expression vector was to demonstrate cleavage of the fusion protein at the peptide linker and purification of PLAP away from the CRD. Test digestions of RHL-PLAP with various amounts of soluble trypsin were carried out in the presence of 10 mM-$CaCl_2$, to stabilize the RHL CRD (Hseuh *et al.*, 1986), and 10 mM-$MgCl_2$ to stabilize PLAP. The results are shown in Fig.

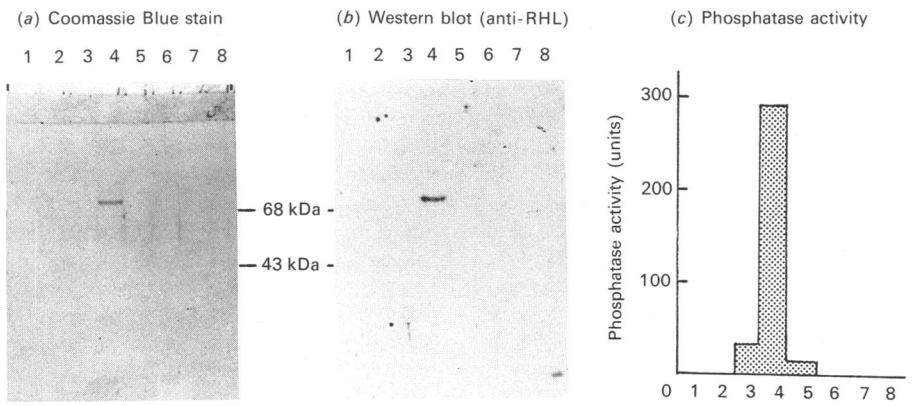


Fig. 3. Isolation of RHL-PLAP from the medium of transfected rat fibroblasts

(*a*) SDS/PAGE. RHL-PLAP was isolated from 150 ml of medium using a 2 ml column of galactose–Sepharose. Aliquots (20 μl) of each eluted fraction (1 ml) were analysed on a gel containing 10% acrylamide. The gel was stained with Coomassie Blue. (*b*) Western blot (anti-RHL). A parallel gel was transferred to nitrocellulose and the fusion protein was detected using an antibody specific for RHL followed by ^{125}I-labelled Protein A (Burnette, 1981). (*c*) Phosphatase assay. The activity shown represents the total activity of each fraction.

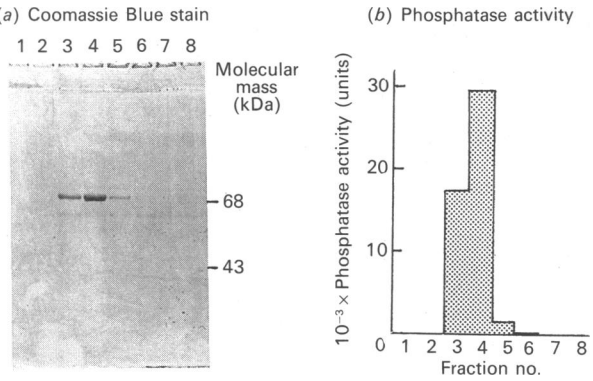


Fig. 4. Isolation of RHL-PLAP from the medium of baculovirus-infected insect cells

(*a*) SDS-PAGE and Coomassie Blue staining. RHL-PLAP was isolated from 250 ml of medium using a 5 ml column of galactose–Sepharose. Aliquots of each eluted fraction (2 ml) were analysed as in Fig. 3(*a*). (*b*) Phosphatase assay of fractions. The activity shown represents the total activity of each fraction.

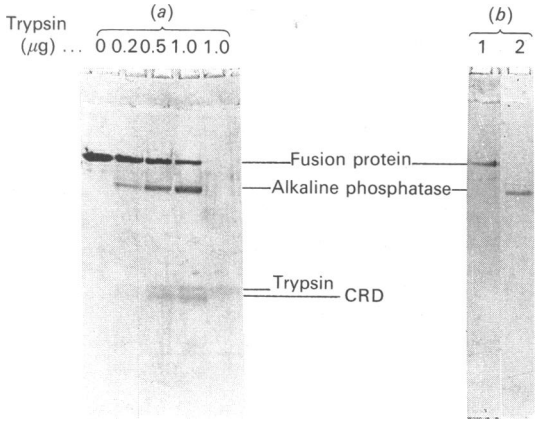
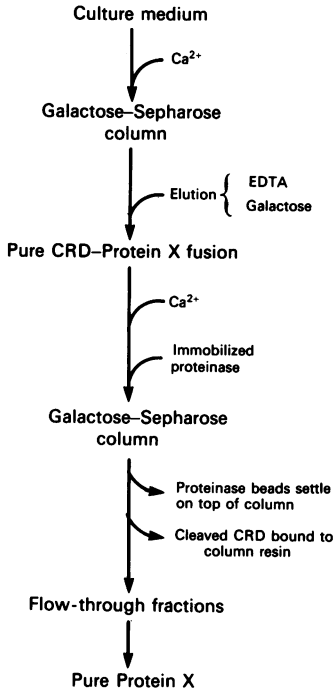


Fig. 5. Cleavage of RHL-PLAP with trypsin

(*a*) Test digestions with soluble trypsin. Aliquots of RHL-PLAP (35 μl) were incubated with 0.2–1 μg of soluble trypsin for 1 h at 37 °C, and then analysed by SDS/PAGE on a gel containing 15% acrylamide. A control incubation containing 1 μg of trypsin alone is shown in the final lane. (*b*) Re-purification of PLAP after cleavage of RHL-PLAP with immobilized trypsin. RHL-PLAP was incubated with immobilized trypsin and the reaction products were passed over a column of galactose–Sepharose. Material which passed through the column was analysed by SDS/PAGE on a gel containing 15% (w/v) acrylamide. Lane 1, RHL-PLAP before digestion with trypsin; lane 2, PLAP re-purified on galactose-Sepharose after release from RHL-PLAP by trypsin.

5(*a*). As the amount of trypsin was increased, the amount of fusion protein decreased and a new band of approx. 55000 Da, the expected size of PLAP, appeared. The RHL CRD is seen just below the major polypeptide in the trypsin preparation. Complete cleavage to PLAP and RHL CRD was achieved with 2 μg of trypsin (results not shown). After incubation of RHL-PLAP with immobilized trypsin, released PLAP could be isolated from the CRD by re-chromatography on galactose–Sepharose, since the CRD bound to the column while PLAP passed through (Fig. 5*b*). The specific activity of the final product was estimated to be 72000 units/mg. This value is comparable with, or higher than, that for commercially available phosphatase isolated from placenta by classical methods.



Scheme 1. General strategy for purification of proteins produced using CRD-containing vectors

DISCUSSION

We have described the construction and use of a eukaryotic expression vector in which foreign proteins can be fused to the CRD of the rat hepatic lectin, and the binding activity of the CRD used to purify the fusion protein. Human placental alkaline phosphatase was inserted into this vector, and the RHL-PLAP fusion protein was successfully isolated from the medium of transfected fibroblasts or insect cells by one step of affinity chromatography. The galactose-specific CRD does not interact with the normal carbohydrate constituents of secretory proteins. This eliminates the possibility of interference from oligosaccharides attached to the passenger portion of the fusion protein.

Alkaline phosphatase retains its enzymic activity while fused to the RHL CRD. We have also expressed a fusion of the RHL CRD and human growth factor 5 (Zhan *et al.*, 1988) and found that the fusion protein retains the transforming activity of the growth factor (M. Goldfarb & M. E. Taylor, unpublished work). It is likely that other proteins would also retain their activity while fused to the CRD, so for many purposes it would not be necessary to remove the CRD from the fused protein. In these cases, one step of chromatography is sufficient to achieve purification.

In situations where removal of the CRD is desirable, the proteinase-sensitive linker between the CRD and the fused protein allows release of the passenger protein. Our results demonstrate that PLAP can be released from the RHL-PLAP fusion protein by cleavage of the peptide linker with soluble or immobilized trypsin. In the latter case, PLAP can be separated from the proteinase and purified away from the CRD by re-chromatography on galactose–Sepharose. Owing to its ready availability and stability, trypsin is the proteinase of choice for proteins which are resistant to digestion in their native state. In cases where trypsin cannot be used, the blood coagulation factor Xa provides a more specific alternative. The sequence of the peptide linker, Ile-Glu-Gly-Arg, is specifically cleaved by Factor

Xa. Since this sequence is very rare in proteins, it is expected that cleavage would in most cases be uniquely at the linker site. Pilot studies indicate that cleavage of the PLAP-RHL fusion can be achieved with commercially available preparations of Factor Xa (M. E. Taylor, unpublished work).

In addition to allowing alternative methods of cleavage, the present approach to fusion-protein purification also offers flexibility in the method of elution employed to release the CRD-passenger fusion protein from the affinity resin. Since either EDTA or galactose can be used to effect elution, the likelihood that biologically active protein can be isolated is maximized. For example, the present results indicate that metalloproteins such as PLAP can be purified more effectively using the galactose competition scheme. The fact that the CRD moiety is functional in both the mammalian and insect cells provides an added dimension of flexibility, since folding of some passenger proteins may be better in one environment or the other. It is also possible to select an expression system which is optimal for producing desired quantities of individual passenger proteins.

The general strategy for isolating and cleaving CRD-fusions in two simple chromatography steps is outlined in Scheme 1. We have created three versions of the pSP64 vector containing the insulin-RHL fusion and the proteinase-sensitive linker, allowing fusion in each of the three reading frames (Fig. 1*a*). Any protein for which a cDNA is available can be fused into one of the vectors and expressed in either of the eukaryotic systems described.

A major potential use of the fusion approach described here is for evaluating biological activities of novel proteins which cannot be isolated in sufficient quantities for direct study. In many cases, cDNAs for such proteins can be obtained. Although expression of these cDNAs provides a means of obtaining larger amounts of the proteins of interest, the need to develop suitable purification schemes remains. This can be a difficult and time-consuming process, since the starting point for purification is usually a complex mixture, often containing large quantities of serum proteins and other contaminants from the medium used for cell growth. The methods described here have the potential to provide a generally applicable purification scheme even when little or nothing is known about the properties of the protein of interest. In addition to studies of enzymic, hormonal or other biological activities, the ability to purify native proteins could facilitate generation of antibodies which recognize and precipitate native proteins.

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