Purification of recA-based Fusion Proteins by Immunoabsorbent Chromatography

CHARACTERIZATION OF A MAJOR ANTIGENIC DETERMINANT OF ESCHERICHIA COLI recA PROTEIN*

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Monoclonal antibodies to Escherichia coli recA protein were prepared, characterized, and used as affinity reagents for the purification of recA and recA:somatostatin fusion proteins. The monoclonal antibodies recognize an antigenic determinant or determinants located between amino acids 260 and 330 of recA. Addition of a fragment of the recA gene coding for these amino acids to an unrelated gene (β-galactosidase) allowed the resulting β-galactosidase fusion protein to be recognized by the recA monoclonal antibodies.

High-level accumulation of small, foreign peptides in Escherichia coli generally requires expression of the peptides as fusions to larger proteins. Consequently, the successful production of foreign oligopeptides in E. coli depends on the efficient purification and subsequent cleavage of the fusion protein. Purification schemes for fusion proteins based on affinity characteristics have been described recently. Uhlen et al. (1) have shown that fusion proteins containing the amino terminus of Staphylococcus protein A will bind to IgG-Sepharose. Sassenfeld and Brewer (2) purified a recombinant human urogastrone-polyarginine fusion protein using SP-Sephadex, and Germino and co-workers (3, 4) have purified fusion proteins containing the E. coli β-galactosidase protein using affinity columns specific for β-galactosidase.

An alternative to these procedures would be a purification scheme based on antibodies to the protein used as a carrier for the peptide product of interest. Such a scheme would allow the flexibility of being able to use any carrier for which a suitable antibody can be prepared without building additional purification elements into the fusion protein. Immunooaffinity chromatography has been utilized for the purification of a wide variety of proteins (5). Recently, monoclonal antibodies have been available for use in protein purification (6–9).

We report in this paper development of an immunooaffinity purification scheme for the isolation of recA-containing fusion proteins from extracts of E. coli. The scheme utilizes monoclonal antibodies prepared to full-length recA. We have designed the purification scheme based on information about a major antigenic determinant of recA identified by computer analysis of the recA primary sequence and the ability of monoclonal antibodies to bind to various regions of the recA molecule.

MATERIALS AND METHODS

Protein Assays—Protein concentrations were determined by the procedure of Lowry et al. (10) or Bradford (11). SDS-polyacrylamide gel electrophoresis was run as described by Laemmli (12). Western blots were prepared by a modification of the procedure described by Renart et al. (13). Somatostatin was determined by subjecting the purified fusion protein to cleavage with CNBr followed by radioimmunoassay (14).

Immunooaffinity Chromatography—Immunooaffinity reagent was prepared by coupling purified monoclonal antibodies to Bio-Rad Affi-Gel 10 (7, 9). Protein samples were prepared by sonication of E. coli cells in 50 mM potassium phosphate, pH 7.0, 10% glycerol, 1 mM EDTA, 100 mM NaCl. Insoluble material was removed by centrifugation at 12,000 × g at 4 °C for 30 min. The supernatant was loaded directly onto the affinity column in lysis buffer. Chromatography was run as described earlier for the purification of bovine somatotropin from pituitary and E. coli extracts (9).

RESULTS

Characterization of a Major Antigenic Determinant of recA—Hybridoma cell lines secreting monoclonal antibodies to recA protein were prepared and screened as described in the Miniprint. In order to characterize the hybridomas and their secreted monoclonal antibodies, ascites antibodies from seven of the cell lines were used in Western blot analyses of recA protein and several deletion derivatives of recA. Fig. 1 shows that three of the seven monoclonal antibodies tested appear to recognize recA protein. The other four antibodies apparently are specific for contaminants present in the recA preparation used to immunize the mice. Interestingly, the mouse polyclonal antibody strongly recognizes only one of the contaminating proteins, although faint bands are present which might correspond to the other contaminants. Control lanes which were not incubated with monoclonal antibodies showed no hybridization with labeled protein A or rabbit anti-mouse antibodies (data not shown).

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1 Portions of this paper (including part of "Materials and Methods," Figs. 4–6, Table 1, and additional references) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9500 Rockville Pike, Bethesda, MD 20814. Request Document No. 85-M-46, cite the authors, and include a check or money order for $2.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

2 The abbreviations used are: SDS, sodium dodecyl sulfate; recA(90):somatostatin, protein with amino acids 1–329 of recA fused (at the carboxyl terminus) to somatostatin; recA(70), protein containing amino acids 1–260 of recA fused at the carboxyl terminus to a 14-amino acid oligopeptide (not somatostatin); recA(90), protein with amino acids 1–329 of recA fused at the carboxyl terminus to a 14-amino acid oligopeptide (not somatostatin); recA(del), recA protein with amino acids 50–259 deleted; PAGE, polyacrylamide gel electrophoresis; ELISA, enzyme-linked immunosorbent assay.
**Western blot analysis of anti-recA monoclonal antibody binding to recA and recA deletion proteins.** E. coli extracts containing recA protein and the recA deletions recA(90), recA(70), and recA(del) were prepared as described in the Miniprint and run on 12.5% SDS-polyacrylamide gels under reducing conditions. Full-length recA protein was loaded after the gel run had begun in order to differentiate it from recA(90) protein. Following electrophoresis, one edge of the gel was removed and stained with Coomassie Brilliant Blue R. The remainder of the gel was subjected to Western blot procedures as described. The blotted gel was cut into strips, and each strip was incubated with a different anti-recA monoclonal or polyclonal antibody preparation followed by 125I-protein A or 125I-labeled rabbit anti-mouse antibody. A shows the autoradiogram of the resulting blot for seven monoclonal antibody preparations (F43-B1-C3, F40-G4-C3, F47-H5-A1, F45-F1-A4, F47-B1-B5, F47-F6-C2, and F47-E5-D3) and one murine polyclonal antibody preparation. Roman numerals indicate the positions on the gel of the four recA constructs diagrammed in B. These positions were determined from wells in which the different recA deletion proteins were loaded independently (data not shown). B depicts the structures of the various recA genes used to produce the variant recA proteins with which to challenge anti-recA antibodies.

The data presented in Fig. 1 indicate that all three of the monoclonal antibodies specific for recA protein also bind to deletion derivatives of recA containing the first 329 amino acids or amino acids 1-49 fused to 259–353. None of the three bind to a deletion of recA with only the amino-terminal 259 amino acids, although the polyclonal antibody preparation does bind strongly to this recA(70) protein. In addition, the recA monoclonal antibodies did not bind to a fragment of recA containing amino acids 1–94, although polyclonal antibody preparations bound this small fragment weakly (data not shown). Apparently, amino acids 260–329 of the recA protein make up at least a portion of a major antigenic determinant of recA. In a computer analysis of the recA primary sequence, using the Hopp and Woods (15) program, this region of recA is predicted to be highly hydrophilic, consistent with its identity as an antigenic determinant.

In order to test if this highly hydrophilic region of the recA protein is sufficient to act as an antigenic determinant, the fragment of the recA gene coding for amino acids 260–353 was fused to the gene for β-galactosidase as described in the Miniprint. The resulting fusion protein was tested in Western blot analysis with one of the recA-specific monoclonal antibodies, F47-E6-C2. As Fig. 2 shows, the recA antibody bound to recA protein and to a protein of the size expected for the β-galactosidase-recA fusion protein, but not the β-galactosidase. The band at the molecular weight of recA in the lane with the β-galactosidase-recA fusion protein (lane IIIB) is due to recA present in the crude cell lysate loaded onto the gel. When the same Western blot was washed extensively in 5 mm granidine thiocyanate, 50 mM Tris, pH 7.5, 50 mM dithiothreitol and hybridized with a mouse anti-β-galactosidase polyclonal antibody preparation, a protein of the size expected for the β-galactosidase-recA fusion was again detected (lane IIIB). The numerous small cross-reactive bands in the same lane (lane IIIB) are apparently proteolytically derived fragments of the fusion protein which occur in this strain. At least some of these fragments do appear to also carry the recA determinant (lane IIIB).

**Affinity Purification of RecA:Somatostatin Proteins**—Imunoaffinity chromatography has been used to purify recA(90):somatostatin and the protein containing amino acids 1–49 and 260–353 of recA (recA(del)) as well as recA protein. All of these proteins are soluble products in E. coli, eliminating the need to solubilize the protein with SDS or a similar agent prior to purification. Fig. 3A shows the SDS-polyacrylamide gel electrophoresis analysis of the affinity-purified proteins. All the proteins appear to be greater than 90% pure following a single-step purification through the affinity column. The identity of the purified protein bands as recA derivatives was confirmed by Western blot analysis (Fig. 3B). All three of the purified proteins bind to murine anti-recA polyclonal antibodies. The band at the same molecular weight as recA in the purified recA(del) protein lane represents recA protein present in the host cell used to prepare this deletion protein. The
FIG. 2. The binding of F47-E6-C2 monoclonal antibody to a β-galactosidase:recA fusion protein. Lysates of cells uninduced and induced for the production of β-galactosidase:recA fusion protein (see "Materials and Methods" and Fig. 1) were analyzed on SDS-polyacrylamide gels. Purified β-galactosidase (Sigma) and recA were run in separate lanes on the same gel. Duplicate gels were prepared; one gel was stained with Coomassie Brilliant Blue R (panel A) and the other gel was subjected to Western blot procedures. The Western blot was incubated with anti-recA antibody (F47-E6-C2) and 125I-protein A. Following autoradiography (panel B), the gel was washed with 5 M guanidine thiocyanate, 50 mM Tris, pH 7.5, 50 mM dithiothreitol and rehybridized with mouse polyclonal antibodies to P-galactosidase and 125I-protein A (panel C). Lanes 1, lysate of uninduced cells producing β-galactosidase:recA fusion protein; lanes 2, lysate of same cells following induction; lanes 3, pure β-galactosidase; lanes 4, pure recA.

FIG. 3. SDS-polyacrylamide gel electrophoresis and Western analyses of recA deletion proteins purified by immunoaffinity chromatography. Protein samples were reduced and subjected to electrophoresis on 7.5-15% gradient SDS gels. A, a Coomassie Brilliant Blue-stained gel containing: lane 1, molecular weight markers (66,000, bovine serum albumin; 45,000, ovalbumin; 24,000, trypsinogen, phenylmethylsulfonyl fluoride-treated; 18,400, β-lactoglobulin; 14,300, lysozyme); lanes 2, 4, and 6, crude extracts of cells containing recA protein, recA(90):somatostatin fusion protein and recA(del) protein, respectively; lanes 3, 5, and 7, immunoaffinity-purified recA protein, recA(90):somatostatin fusion protein, and recA(del) protein, respectively, lane 8, purified recA standard. B, Western blot analysis of a duplicate polyacrylamide gel. The blot was hybridized to a mouse anti-recA polyclonal antibody preparation. The positions of the molecular weight markers and the gel lanes are indicated.

bands of molecular weight greater than 66,000 in lanes 2, 4, and 6 represent a protein contaminant present in the recA used as antigen to prepare polyclonal antibodies. As can be seen on the stained gel and the Western blot, even a protein which is not abundant in the lysate (the recA(del) protein) can be purified by the immunoaffinity chromatography.

recA(90):somatostatin, purified by affinity chromatography and cleaved by cyanogen bromide, yields immunoreactive somatostatin. When crude extract containing 140 μg of somatostatin (60 mg of total protein in a volume of 10 ml) was loaded onto the column, about 70 μg of somatostatin bound to the column and were released by the high salt and low pH washes. Another 61 μg of somatostatin were recovered in the material which did not bind to the column. This latter material could be purified by a second passage through the column. Since roughly 2 mg of recA(90):somatostatin were purified by one passage through the affinity column, the capacity of the column is apparently not diminished by deleting amino acids 330-353 from the carrier protein or by adding somatostatin to the carboxyl terminus of the fusion protein.
DISCUSSION

When expressing small or labile proteins in bacteria, it is helpful to express them as fusions to a stable protein (14), preferably one whose properties allow the fusion protein to be readily purified from a cell lysate (1–4). A property which many proteins might be expected to exhibit is antigenicity. A protein may be used as an antigen to generate monoclonal antibodies which can then be used to provide a simple purification procedure (5). Such an approach allows considerable latitude in the choice of the carrier segment of a fusion protein. A rapid and extremely selective purification scheme could be readily generated for any soluble fusion protein containing a recognizable antigenic determinant. As we had determined that the recA protein held considerable advantages over other carriers for the bioproduction of somatostatin, we decided to characterize the recA polypeptide with respect to its antigenic determinants.

Inspection of the linear sequence of recA protein with the analytic method of Hopp and Woods (15) identifies a region of recA around amino acid 324 which is hydrophilic and therefore likely to be in good solvent contact. According to Hopp and Woods, hydrophilic regions are likely to be located in or adjacent to an antigenic determinant. Furthermore, the region around residue 324 of recA is predicted to be α-helical by the methods of Chou and Fasman (16). Examination of the predicted α-helical structure (amino acids 315–330) using the helical wheel projection (17) shows a strong tendency toward positioning of hydrophobic and hydrophilic side chains on two distinct faces of the helix. These structural examinations support the prediction that the area around amino acid 324 is likely to make up one of the strongest antigenic determinants of the recA molecule.

Experimental characterization of the region of recA involved in antibody binding was carried out by studying the ability of monoclonal antibodies prepared against full-length recA to bind to truncated or rearranged variants of the recA molecule. Binding was tested by Western blot procedures. Although a polyclonal antibody to recA bound to all the recA variants, only variants containing amino acids 260–330 were recognized by the monoclonal antibodies (Fig. 1). This result suggests that the region of recA composed of amino acids 260–330 comprises at least a portion of a major antigenic site on the molecule, consistent with the predictions. As a further test of the predictions, the segment of the recA gene coding for amino acids 260–353 was used to construct a chimeric gene which codes for the amino-terminal 1018 amino acids of Escherichia coli β-galactosidase fused to the carboxyl-terminal 93 amino acids of recA. Western blot analyses confirmed that the fusion protein produced from the chimeric gene bound to one of the anti-recA monoclonal antibodies (Fig. 2). These data show that the carboxyl-terminal region of recA is sufficient to form a strong, specific antigen.

Analysis of antigenic sites on the recA molecule allowed design of recA: somatostatin fusion proteins which could be easily purified by monoclonal antibody affinity chromatography. The recA immunoaffinity chromatography scheme described in this paper can be used to purify fusion proteins based on recA in one step from crude extracts. Recovery is high, and elution conditions are mild compared to the chemical cleavage and high performance liquid chromatography purification steps utilized in the further purification of small peptides. Roughly 1–2 mg of recA or recA fusion protein will bind to 1 ml of immunoaffinity reagent, and the column is reusable, making this purification approach attractive for production of somatostatin or any peptide fused to recA in E. coli.

Given the facility with which currently available genetic engineering techniques allow construction of chimeric genes and the power of antibody affinity chromatography, it is useful to predict what portions of a carrier protein will have strong antigenic determinants. As we have described, predictions which can be made from current analytical methods are in good agreement with our experimental determinations of recA-based fusion proteins. Predictions concerning the structure of fusion proteins based on other carriers can be readily tested by similar procedures. After preparing monoclonal antibodies, truncation or resection of the parent gene can be used to map the location of antigenic determinants. The mapping can be done quite easily using crude cell lysates. Once identified, specific antigenic regions can be used as the basis for powerful purification techniques involving the molecule on which they reside, or the sites themselves can be translocated to other chimeric proteins for the express purpose of facilitating the identification or purification of those proteins.

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Additional references are found on p. 10267.
**Supplementary Material to Purification of recA Based Fusion Proteins by Immunoaffinity Chromatography: Characterization of a Major Antigenic Determinant of E. coli recA Protein**

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### Materials

Female BALB/c mice were obtained from Charles River Breeding Labs-Cares. The tissue culture supplies were purchased from Gibco Laboratories, Grand Island, NY. The SP/2O-AgI myeloma line was obtained from the Hybridoma Center, Washington University, St. Louis, Mo. Bacterial proteins was purified from E. coli by standard techniques. Protein A was purchased from Sigma and iodinated using 125I-Boehm-Hunter reagent (New England Nuclear). Specific anti mouse antibody preparations were obtained from Litton Bionetics, and 125I-rabbit anti-mouse antibody was purchased from New England Nuclear.

### Construction of p-galactosidase:recA fusion protein

Figure 4 diagrams the construction of a plasmid used to produce a p-galactosidase:recA fusion protein. The plasmid was constructed by replacing the region ORF probe plasmid pMK257 which contains the coding sequence for the amino terminal portion of the recA gene with a fragment of plasmid pMK102 which contains the cloning sequence for the amino terminal portion of the recA gene fragment directly before the gene fusion to direct transcription of the recA gene. The new fusion gene contains the coding region for the amino terminal portion of the recA gene and a coding sequence for amino acids 260 through 353 (the carboxy terminal) of the recA protein.

### Figure 4. Construction of p-galactosidase:recA fusion protein expression vector.

Plasmid vector pMK257 contains the recA gene of E. coli from the 5' end of the ORF probe to the 3' end of the ORF probe. The plasmid was transformed into the Escherichia coli strain D5312 after the cloning vector had been digested with the restriction enzymes BamHI and SalI. Figure 3 shows the restriction map of the plasmid and the 3' end of the cloned gene fragment that was used to replace the BamHI-SalI plasmid. The cloned gene fragment was ligated into the BamHI-SalI site of plasmid pMK102 which contains the coding region for the amino terminal 260 amino acids of the p-galactosidase (gen) (9) and inserted into BamHI-digested plasmid pEM753. This plasmid was then cleaved with BamHI and a DNA fragment containing the recA promoter was inserted. This fragment is a HindIII fragment of the recA gene containing the promoter.

### Preparation of hybridoma lines

Monoclonal antibodies specific for E. coli recA were prepared as described earlier (9). Four to six day old mice were immunized with 60 μg of recA in complete Freund's adjuvant. The mice were boosted with 60 μg of recA daily for 4 days. Splenocytes were removed and prepared for fusion by standard procedures. Five hybridoma cell lines were selected using hypoxanthine:aminopterin:xanthine (HAT) medium and screened for the production of recA specific antibodies by ELISA. Each hybridoma line was analyzed by SDS-PAGE and immunoblotting. The antibodies secreted by 1 of the hybridoma lines were used to purify the recA protein antigenic determinant.

### Figure 6. Immunofluorescence purification of recA protein from a crude lysate of E. coli.

E. coli extract was prepared as described and passed through the affinity column. Figure 6A shows the profile of protein concentration on the column as determined by Bradford assay. Fractions were collected at intervals of 1 ml, 5.2 mM triton X-100; 5 M LiCl, 0.2% (v/v) triton X-100, and 5.15 ml of the crude lysate (10 μl) and fractions 10 (5 μl), 20 (5 μl), 29 (25 μl) and 45 (15 μl) were analyzed by SDS-PAGE (lanes 1-5, respectively, Figure 6B).