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Monoclonal Antibodies to a Proenkephalin A Fusion Peptide Synthesized in *Escherichia coli* Recognize Novel Proenkephalin A Precursor Forms*

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Monoclonal antibodies have been generated to a chimeric peptide comprised of *Escherichia coli* β-galactosidase fused to the amino acid sequence 69–207 of human preproenkephalin A. Two monoclonal antibodies, PE-1 and PE-2, were identified by their ability to recognize the same segment of proenkephalin A fused to the cII gene product of the *E. coli* bacteriophage λ. The binding domains of PE-1 and PE-2 have been broadly located, with respect to the primary translation product, within the amino acid sequences 152–207 and 84–131, respectively. Immunoblot analysis of total bovine adrenomedullary chromaffin granule lysate reveals PE-1 and PE-2 immunoreactive forms of observed molecular mass 35, 33, 29, 24, 22, and 15 kDA, and an 18-kDa PE-1 immunoreactive form. Separation of granule membranes from their contents reveals differential membrane association of these high molecular weight polypeptides. There is preliminary evidence that PE-1 may be detecting a subset of polypeptides where shortening from the NH_2 terminus has occurred. We postulate that the 35-kDa form represents the intact bovine enkephalin precursor of predicted molecular mass 27.3 kDa. This experimental approach should be generally applicable to the generation of antibodies which will recognize intact peptide precursors together with their post-translational cleavage products.

Proenkephalin A is one of the three opioid precursor molecules and gives rise to the well known opioid pentapeptides Met- and Leu-enkephalin. The primary structure of preproenkephalin A has been deduced in a number of mammalian species from the nucleotide sequence of its cloned cDNAs (Comb *et al.*, 1982; Legon *et al.*, 1982; Noda *et al.*, 1982; Yoshikawa *et al.*, 1984). The predicted molecular mass of proenkephalin A is approximately 27 kDa. There is an extremely high degree of sequence homology between the proenkephalin A molecules of different mammalian species, indicating that nonenkephalin as well as enkephalin regions have been conserved during evolution and so may have intrinsic biological importance. Within the precursor the enkephalin sequences are flanked by pairs of basic residues which are potential sites of proteolytic cleavage. Thus, complete processing of the precursor molecule will yield four copies of Met-enkephalin and one copy each of Leu-enkephalin, Met-enkephalin-Arg^6-Gly^7-Leu^8 and Met-enkephalin-Arg^6-Phe^7. However, incomplete processing has the potential to yield many different extended enkephalin-containing peptides. Indeed a wide array of such peptides has been identified, many of which have been isolated and characterized, mainly from bovine adrenal medulla (for review see Udenfriend and Kilpatrick, 1983). There is evidence that some of these extended forms may be even more biologically active than the originally described pentapeptides. For example, peptide E, Bam-22P, and Bam-20P have been shown to be more potent in the guinea pig ileum bioassay than Met- or Leu-enkephalin (Kilpatrick *et al.*, 1981; Mizuno *et al.*, 1980). More recently, even larger peptides have been detected; for example, synenkephalin which does not contain an enkephalin sequence (Liston *et al.*, 1983), and peptides of predicted molecular mass *8.6* kDa,[1] *12.6* kDa (Jones *et al.*, 1982), *18.2* kDa (Kilpatrick *et al.*, 1982), and *23.3* kDa (Patey *et al.*, 1984) (Fig. 1). The biological significance of these high molecular mass polypeptides remains to be determined.

Studies focusing on the high molecular weight forms of proenkephalin have often been limited because of the fact that the majority of antibodies currently available has been raised to small synthetic enkephalin and nonenkephalin peptides. Such antibodies therefore exhibit limited immune recognition of the parent prohormone. Consequently, the detection of larger forms usually requires proteolytic liberation of the smaller peptides from their larger precursors after chromatographic separation. One possible solution would be to immunize with purified intact enkephalin precursor, but this is impractical as the prohormone has not been convincingly identified, possibly because of its instability and low abundance. In this work, therefore, we chose to synthesize proenkephalin A as a fusion protein with β-galactosidase in an *Escherichia coli* expression system. The method is particularly suitable in this context as it could be anticipated that a prohormone would be afforded protection from degradation when expressed as a fusion protein within a bacterial milieu. Furthermore, because of its conjugation to a highly immunogenic bacterial protein, it is in the form of a ready-made "hapten-carrier" complex. In this paper we demonstrate our success in utilizing this approach to raise monoclonal antibodies which may recognize the naturally occurring precursor proenkephalin A together with a wide range of intermediates.

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[1] Peptides that have been named according to their predicted molecular mass (*8.6*, *12.6*, *18.2*, and *23.3* kDa) are denoted in italics.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Production of β-Galactosidase-Preproenkephalin-A-(69–207) Fusion Protein for Immunization—A human preproenkephalin A cDNA clone (Legon *et al.*, 1982) was used to generate a 411-base pair restriction fragment encoding the amino acid sequence 69–207 of the primary gene product (Fig. 1, PPE-(69–207)). This was cloned into the pUR series of expression vectors (Rüther and Müller-Hill, 1983) as described under "Experimental Procedures." These vectors possess a series of unique restriction enzyme sites at the 3′ end of the *E. coli lac Z* gene, the expression of which is driven by its natural promoter and operator. The cloning sites are in different reading frames with respect to the *lac Z* gene in the three different vectors. The segment of preproenkephalin A cDNA was cloned in translational phase with the COOH terminus of β-galactosidase in the vector pUR 291. Recombinant plasmids were transformed into competent cells of the *E. coli* strain BMH 71-18, which lacks the endogenous *lac Z* gene but carries the *lac* i^Q mutation which confers the phenotype of overproduction of the lac repressor. Thus, despite the high copy number of the plasmid, expression of the fusion protein should be suppressed until addition of the synthetic inducer IPTG.[3]

To identify which colonies contained the insert in the correct orientation and translational phase, a random selection of transformants were cultured in L-broth in the presence of IPTG. Crude bacterial lysates were made by sonicating the cells in SDS sample buffer and electrophoresed on 7.5% denaturing polyacrylamide gels (see "Experimental Procedures"). Production of β-galactosidase-proenkephalin A fusion protein was easily identified by Coomassie staining of the gels, the hybrid polypeptide being more abundant and migrating more slowly than unfused β-galactosidase (Fig. 2, *lane 2*). Restriction endonuclease cleavage analysis of plasmid DNA from the positive colonies confirmed the presence of the enkephalin insert in the correct orientation. Bacterial lysates were then fractionated on preparative SDS-polyacrylamide gels in order to obtain sufficient fusion protein for immunization. A 100-ml bacterial culture yielded approximately 10–15 mg of fusion protein electrophoretically purified from the excised gel band (Fig. 2, *lane 4*). The lower band of the doublet represents proteolytically liberated β-galactosidase. The hybrid protein was used directly as an immunogen in rabbits and BALB/c mice.

Production of a Secondary cII-Preproenkephalin A-(69–207) Fusion Protein for Screening of Sera and Monoclonal Supernatants—In anticipation that the β-galactosidase-preproen-

[2] "Experimental Procedures" 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 included in the microfilm edition of the Journal that is available from Waverly Press.

[3] The abbreviations used are: IPTG, isopropylthiogalactoside; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; IGSS, immunogold silver staining; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; FCS, fetal calf serum.

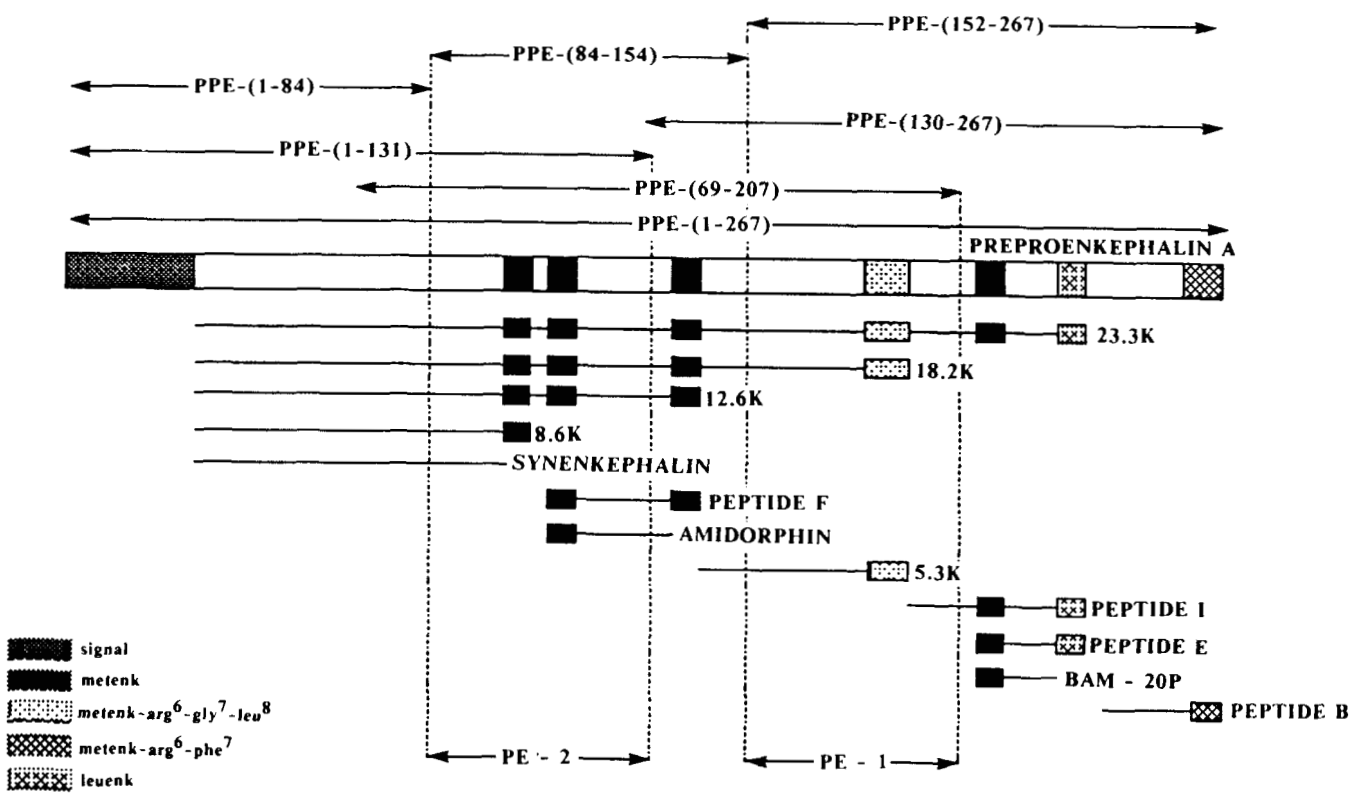

FIG. 1. **Schematic representation of the binding domains of the monoclonal antibodies PE-1 and PE-2 in relation to the primary structure of preproenkephalin A.** The *central boxed region* illustrates the intact precursor. The *lower portion* of the diagram shows some of the high molecular weight proteolytic cleavage products of proenkephalin A. The *upper section* illustrates the cDNA fragments used to generate fusion proteins for immunization or epitope mapping.

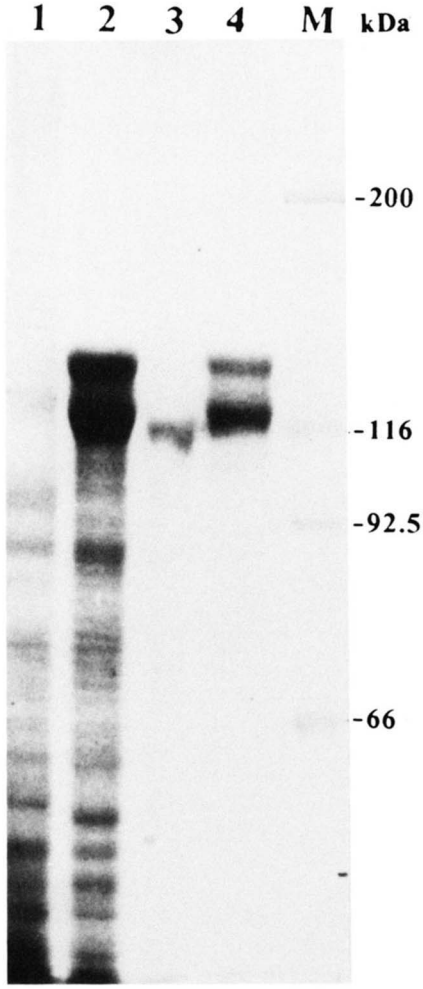

FIG. 2. **Appearance of β-galactosidase-preproenkephalin A-(69–207) fusion protein on a Coomassie-stained denaturing polyacrylamide gel.** *Lanes 1* and *2* represent total lysates of bacteria transformed with the recombinant plasmid pβ-gal-(PPE-69-207), cultured in the absence (*lane 1*) or presence of IPTG (*lane 2*). The fusion protein can be seen as the *uppermost band* in *lane 2*. The *lower band* of the doublet represents proteolytically liberated β-galactosidase. *Lane 4* shows the appearance of the fusion protein after electrophoretic purification. *Lane 3* shows unfused β-galactosidase for comparison. Positions of molecular mass markers are shown on the *extreme right*.

kephalin A fusion protein would elicit a pronounced anti-β-galactosidase response another gene fusion was made which would direct the synthesis of an identical enkephalin sequence fused to a different prokaryotic gene product. This could therefore be used directly to test sera and hybridoma culture medium for anti-proenkephalin activity without the need for a double screening procedure. The proenkephalin sequence was subcloned from pUR291 into plcII (Nagai and Thøgersen, 1984) to position it at the 3′ end of a sequence encoding the NH_2-terminal 31 amino acids of the phage λ *cII* gene product, under control of the λ P_L promoter (see "Experimental Procedures"). The recombinant plasmid was transformed into the lysogenic *E. coli* strain QY-13, in which the prophage carries the mutation cI^{857} which encodes a temperature-sensitive repressor. Thus, when the bacteria are grown at 30 °C, the repressor is fully active and transcription is prevented. However, when the temperature is raised to 42 °C, the repressor becomes unstable and expression of the fusion protein is allowed. When lysates of these cells are subjected to SDS-PAGE, the cII-preproenkephalin A-(69–207) fusion protein can be seen on a Coomassie-stained gel as a plasmid-specified 23-kDa protein present in cultures grown at 42 °C but not in cultures grown at 30 °C (see Fig. 3, *panel a*). The identity of this fusion protein was confirmed by Western blotting of these bacterial lysates. The blots were incubated with a rabbit polyclonal antiserum (R139) which had been raised to the β-galactosidase-preproenkephalin A-(69–207) fusion protein. Prior to immunostaining, the rabbit antiserum was preabsorbed using a crude *E. coli* lysate coupled to a cyanogen bromide-activated Sepharose column to remove antibodies to endogenous *E. coli* antigens as far as possible. This antiserum identifies the cII-preproenkephalin A-(69–207) fusion protein whereas the preimmune serum does not (Fig. 3, *panel b*). The remaining strips in this blot demonstrate the results of immunostaining identical material using antisera raised against the pentapeptide Met-enkephalin and the octapeptide Met-enkephalin-Arg^6-Gly^7-Leu^8. These sequences are present in the cDNA segment in three and one copies, respectively. Both antisera recognize the 23-kDa peptide confirming its identity as the cII-preproenkephalin A fusion protein.

Generation of Monoclonal Antibodies, PE-1 and PE-2—Sera from BALB/c mice immunized with the β-galactosidase-preproenkephalin A-(69–207) hybrid polypeptide were tested for antiproenkephalin activity by immunoblotting against bacterial lysate expressing the cII-preproenkephalin A-(69–207) fusion peptide. The sera were again preabsorbed using *E. coli* lysate immobilized on a Sepharose column. A clear anti-proenkephalin response was observed after a total of six injections of immunogen.

The spleens of the two mice with the strongest peripheral response were used to generate hybridoma clones. Clones were screened by testing supernatants from the microtiter wells against nitrocellulose strips of immunoblotted cII-preproenkephalin A-(69–207) prepared from Western blots of total bacterial lysate as described under "Experimental Procedures." Out of nine original positive clones, two were characterized and designated PE-1 and PE-2.

Characterization and Preliminary Epitope Mapping of PE-1 and PE-2—In order to map the binding regions of PE-1 and PE-2 on the primary structure of the precursor, a series of β-galactosidase-preproenkephalin A fusion peptides incorporating overlapping regions of sequence was constructed. For this part of the study a cDNA clone extending over the entire protein coding region, amino acids 1–267, was used (Comb *et al.*, 1982). The cDNA was cleaved at a variety of restriction endonuclease sites to generate a series of cDNA fragments which were cloned into the pUR vectors (see "Experimental Procedures"). The products of ligation were transformed into *E. coli* strain TG2 which is *lac* Z^-, *lac* i^Q. Recombinants were identified by colony hybridization using a radiolabeled preproenkephalin cDNA probe, and the orientation of the insert was confirmed by restriction endonuclease digests of purified plasmids. The ability of the constructs to direct the synthesis of fusion proteins was confirmed by analyzing total bacterial lysates by SDS-PAGE as described under "Experimental Procedures." The following hybrid polypeptides were generated in this way: β-galactosidase-preproenkephalin A-(1–267), -(1–131), -(130–267), -(1–84), -(84–154), -(152–267) (Fig. 1).

Fig. 4 illustrates the results of immunoblot analysis of these fusion peptides. It can be seen that PE-1 recognizes β-galactosidase-preproenkephalin A-(1–267) and -(130–267), together with the original immunogen, -(69–207). PE-1 also binds β-galactosidase-preproenkephalin A-(152–267) (not shown). PE-2 recognizes β-galactosidase-preproenkephalin A-(1–267), -(1–131), -(84–154), and the original immunogen -(69–207). β-Galactosidase-preproenkephalin A-(1–84) is rec-

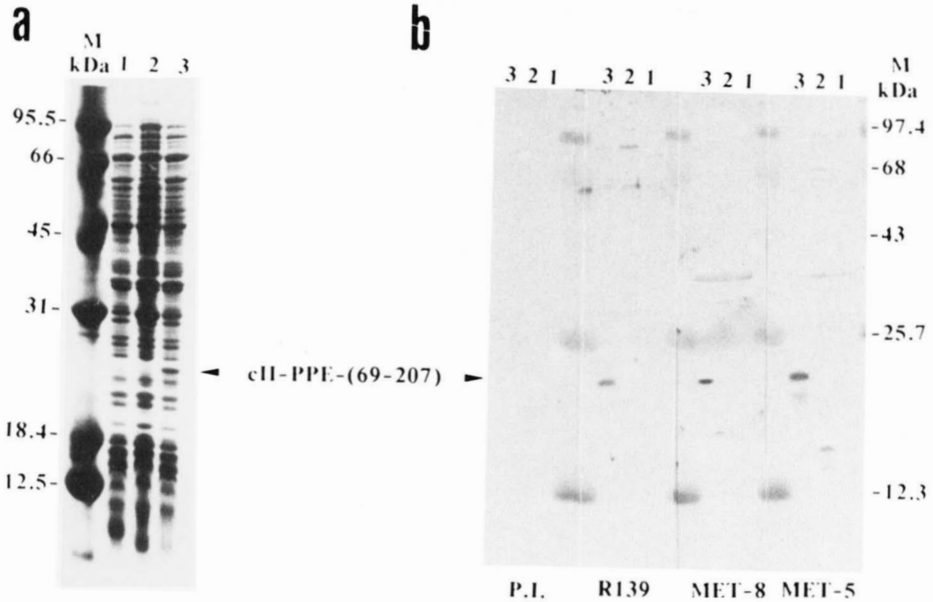

FIG. 3. **Appearance of cII-preproenkephalin A-(69–207) fusion protein.** *Panel a*, appearance on a 10% Coomassie-stained SDS-polyacrylamide gel. *Lane M*, molecular mass standards. *Lane 1*, total lysate of host bacteria transformed with the parent vector pLcII, cultured at 42 °C. *Lanes 2* and *3*, total lysates of bacteria transformed with the recombinant plasmid pcII-PPE-(69–207), cultured at 30 °C (*lane 2*) or 42 °C (*lane 3*, fusion protein visible at ~23 kDa). *Panel b*, immunoblot analysis of identical material (*lanes 1*, *2*, and *3* as for *panel a*). Nitrocellulose strips were immunostained with different polyclonal antisera using a peroxidase detection method. Antisera were as follows: *P.I.*, a rabbit preimmune serum as a negative control; *R139*, a rabbit polyclonal antiserum obtained after immunization with the β-galactosidase-preproenkephalin A-(69–207) fusion protein; *MET-8*, a polyclonal antiserum to the octapeptide Met-enkephalin-Arg^6-Gly^7-Leu^8; *MET-5*, a polyclonal antiserum to the pentapeptide Met-enkephalin. All polyclonal antisera had been preabsorbed using a crude *E. coli* lysate coupled to a CNBr-activated Sepharose column.

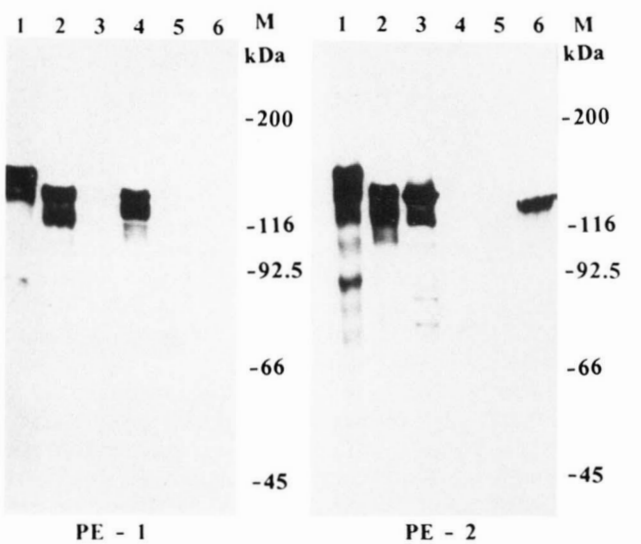

FIG. 4. **Preliminary epitope mapping of PE-1 and PE-2 by immunoblot analysis of a series of β-galactosidase-preproenkephalin A fusion proteins (see also Fig. 1).** Total bacterial lysates were subjected to Western blotting followed by immunostaining with either PE-1 (*left-hand strip*) or PE-2 (*right-hand strip*). In each case, *lanes 1–6* represent β-galactosidase fusion proteins in the following order: β-gal-PPE-(1–267), -(69–207), -(1–131), -(130–267), -(1–84), and -(84–154).

ognized by neither antibody. Thus the binding domains of PE-1 and PE-2 can be broadly located, with respect to the primary translation product, within the amino acid sequences 152–207 and 84–131, respectively (Fig. 1).

Subclass determination of the hybridoma clones was carried out by incubation of monoclonal supernatants with purified fusion protein spotted onto nitrocellulose followed by immunoperoxidase detection using subclass-specific second antibodies. PE-1 and PE-2 were both found to be of subclass IgG_1.

Demonstration That PE-1 and PE-2 Recognize Naturally Occurring High Molecular Weight Proenkephalin A-derived Peptides—In order to determine whether the monoclonal antibodies would recognize naturally occurring proenkephalin A-derived peptides, we prepared bovine adrenomedullary chromaffin granule lysates as described under "Experimental Procedures." In the first instance, acidic lysates were made by sonication of isolated granules in 0.1 M HCl plus protease inhibitors in order to limit proteolytic processing. The samples were then subjected to SDS-PAGE using a 12% separating gel followed by immunoblot using a peroxidase detection method. This revealed that PE-1 and PE-2 recognize forms of observed molecular mass 35, 33, 29, 24, 22, and 15 kDa. An 18-kDa form is recognized by PE-1 alone, which may therefore represent an NH_2-terminally shortened form (Fig. 5). The 24-, 22-, and 15-kDa forms also recognized by PE-1, which apparently comigrate with PE-2 immunoreactive forms, may represent other NH_2-terminally shortened forms (see "Discussion"). To our knowledge these PE-1 immunoreactive forms have not been described previously nor have the 35- and 33-kDa forms. Both antibodies also recognize bovine proenkephalin A-derived peptides in chromaffin granule lysates after chromatographic separation and assay of fractions in an enzyme-linked immunosorbent assay system. These data are described in detail elsewhere.[4]

The Distribution of PE-1 and PE-2 Immunoreactive Peptides within Chromaffin Granules—Studies by others have suggested that high molecular weight proenkephalin A-de-

[4] Jackson, S., Spruce, B. A., Glynn, B., Glover, D. M., and Lowry, P. J., *J. Mol. Endocrinol.*, in press.

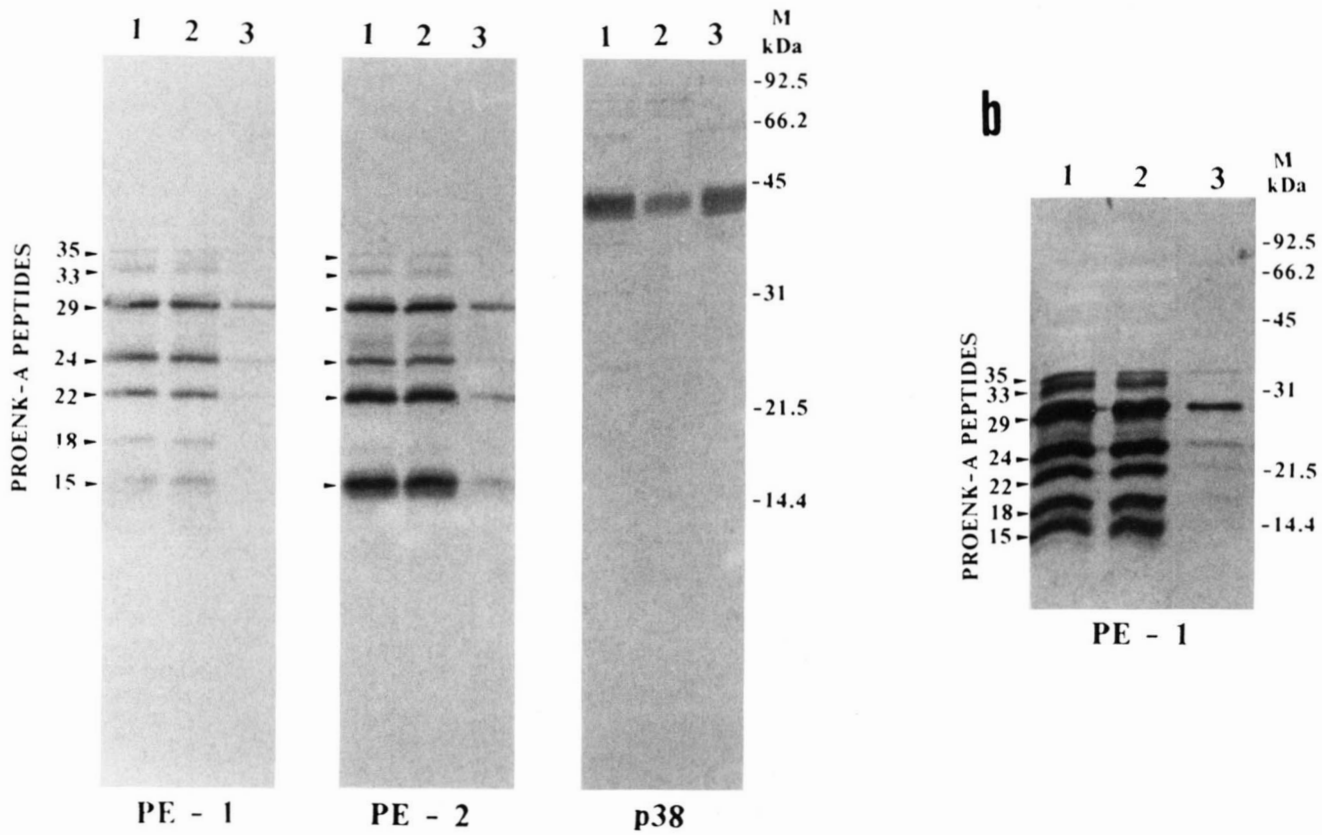

FIG. 5. **Immunoblot analysis of bovine adrenomedullary chromaffin granule lysates.** *Panel a*, peroxidase detection method used (diaminobenzidine stain). In all cases, *lane 1* represents total granule lysate, *lane 2*, the granule contents, and *lane 3*, the membrane fraction. Nitrocellulose strips were immunostained with PE-1 or PE-2 supernatants, or a polyclonal antiserum to the membrane protein p38. *Panel b*, IGSS detection method used. *Lanes 1*, *2*, and *3* as for *panel a*.

rived peptides are associated with the granule membrane (Hook and Liston, 1987; Birch *et al.*, 1986). We were therefore interested to find which of the PE-1 and PE-2 immunoreactive peptides were membrane-bound. In order to separate granule contents from membranes, total granule lysates were subjected to high speed centrifugation in an air-driven ultracentrifuge. Supernatant and pellet were carefully separated, the former representing the contents and the latter the membrane fraction. Equivalent amounts of each fraction were subjected to SDS-PAGE followed by immunoblotting using a peroxidase detection method with diaminobenzidine as substrate. All of the forms present in total lysate can be detected in the soluble fraction. In addition, the 29-, 22-, and 15-kDa PE-2 immunoreactive forms are all associated with the granule membrane, the proportion bound decreasing with diminishing molecular size. Using the same detection method, the PE-1 immunoreactive forms migrating at 29 and 24 kDa appeared to be membrane-bound (Fig. 5, *panel a*).

It was felt that the failure to detect membrane binding of the highest molecular weight forms could be a problem of detection, since they were less abundant in total lysates. We therefore employed an immunochemical detection method utilizing a gold-coupled second antibody followed by silver enhancement in order to increase sensitivity (IGSS). This revealed that the 35-kDa form recognized by PE-1 and PE-2 is indeed membrane-bound, although the 33-kDa form is not (Fig. 5, *panel b*). Furthermore, the increased sensitivity of the IGSS system revealed that the PE-1 immunoreactive 22-, 18-, and 15-kDa forms are membrane-bound and confirmed membrane association of the 29- and 24-kDa forms (see Fig. 5, *panel b*). IGSS confirmed membrane binding of the 29-, 22-, and 15-kDa forms recognized by PE-2 and the absence of membrane association of the 24-kDa PE-2 immunoreactive form (data not shown).

Using IGSS, we observed that only a minor proportion of the 35-kDa form was membrane-bound. However, immunostaining of the fractions with a polyclonal antiserum to p38, an intrinsic membrane protein, showed that p38 was clearly present in the soluble fraction, suggesting there had been incomplete separation of membrane from contents. Moreover, we were concerned that lysis of the granules under acidic conditions could have dislodged the precursor from the membrane. We therefore repeated the experiment using hypotonic lysis in 5 mM Tris succinate at pH 5.9 accompanied by freeze-thawing. Under these circumstances, however, the overall amounts of enkephalin peptides were substantially reduced and the highest molecular weight form visible in any of the fractions was the 29-kDa form, suggesting that significant proteolysis had occurred despite the presence of protease inhibitors (data not shown).

DISCUSSION

We have used a β-galactosidase-preproenkephalin A chimeric protein, synthesized in *E. coli*, to generate monoclonal

antibodies which are likely to have the capacity to recognize the naturally occurring enkephalin precursor proenkephalin A together with a range of processed products.

A number of antibodies to proenkephalin A-derived peptides are available, but these have usually been generated to small synthetic enkephalin- and nonenkephalin-containing peptides. Many of these antibodies cannot be used in direct assays for naturally occurring high molecular weight peptides, suggesting significant tertiary conformation of these precursor forms. An exception to this is an antiserum raised to a synthetic peptide corresponding to residues 95–117 of bovine proenkephalin A (Christie *et al.*, 1984; Birch and Christie, 1986). This antibody recognizes a wide range of high molecular weight intermediates and may also have the capacity to recognize the intact precursor. Another group has raised an antiserum to purified bovine adrenal medullary proenkephalin A (1–77) which has been used successfully to purify the *8.6*-, *12.6*-, *18.2*-, and *23.3*-kDa peptides (Metters and Rossier, 1987). However, the usefulness of such antibodies when employed in isolation is bound to be limited as it has become apparent that there may not be a fixed site for the first cleavage event of the intact precursor. Thus an even greater number of high molecular weight intermediates could exist than was originally envisaged. This is supported by the finding of NH_2-terminally shortened forms in which the extreme COOH terminus of proenkephalin (Met-enkephalin-Arg^6-Phe^7) is still present (Baird *et al.*, 1984). Thus, in order to gain a fully comprehensive picture of the biosynthetic pathways, there is a requirement for a comprehensive range of antibodies directed to different regions of the precursor which will recognize the uncleaved prohormone, together with processed products. This facility would be particularly important where rapid marked changes in enkephalin gene transcription are occurring, for example in the hypothalamus in response to stress (Lightman and Young, 1987). Here, accurate estimation of the primary translation product, together with processed peptides, would be crucial.

The major proenkephalin A-derived peptides detected by us in bovine chromaffin granule isolates migrated on SDS-PAGE with apparent masses of 35, 33, 29, 29, 22, 18, and 15 kDa. In 1982 Dandekar and Sabol detected an enkephalin-containing precursor in the cell-free translation products of bovine adrenal medullary mRNA which migrated at 31 kDa on SDS-PAGE. Since the experiment was carried out in the absence of pancreatic microsomes the 24-residue signal sequence would be expected to contribute 2–3 kDa; therefore, bovine proenkephalin should migrate at approximately 28–29 kDa on SDS-PAGE in the absence of post-translational modifications. Beaumont *et al.* (1985) identified a proenkephalin peptide in guinea pig striatum which migrated at 31 kDa on SDS-PAGE. The presence of Met-enkephalin-Arg^6-Phe^7 immunoreactivity in the corresponding chromatographic fraction led them to postulate that this represented the intact enkephalin precursor. To date, the highest molecular weight forms reported to exist in bovine adrenal medulla are 27 kDa (Birch and Christie, 1986) and 28 kDa (Metters and Rossier, 1987), as determined by SDS-PAGE. However, cross-immunoreactivity studies indicate that these forms are likely to correspond to the *23.3*-kDa peptide rather than the intact enkephalin precursor. It seems highly likely that the 29-kDa form observed by us is in fact also this *23.3*-kDa peptide, raising the possibility that the 35/33-kDa doublet represents the intact enkephalin precursor.

The *18.2*- and *12.6*-kDa peptides have also been shown to migrate anomalously slowly on SDS-PAGE (Kilpatrick *et al.*, 1982). Comparison of our immunoblot data with those of others (Patey *et al.*, 1984; Birch *et al.*, 1986; Hook and Liston, 1987) suggests that the 22- and 15-kDa forms recognized by PE-2 correspond, respectively, to the *18.2*- and *12.6*-kDa peptides. Further studies by us suggest that this is indeed the case.[4] The 24-kDa form may represent a post-translationally modified form of the *18.2*-kDa peptide which has been observed by others to migrate as two species. We initially assumed that the 24- and 22-kDa forms recognized by PE-1 were also likely to correspond to the *18.2*-kDa peptide in modified and unmodified form. However, further studies by us indicate that the PE-1 epitope may lie outside the sequence which gives rise to the *18.2*-kDa peptide. For example, PE-1 fails to bind the purified bovine 5.3-kDa peptide in an enzyme-linked immunosorbent assay system[5] (Fig. 1). Given their apparent molecular weights, it is therefore possible that the 24- and 22-kDa PE-1 immunoreactive forms represent high molecular weight polypeptides where shortening from the NH_2 terminus of the precursor has occurred. Additionally, the PE-1 immunoreactive 15-kDa form cannot correspond to the predicted molecular mass *12.6*-kDa peptide which does not contain the PE-1 epitope (see Fig. 1) and must therefore represent a polypeptide which extends further toward the COOH terminus of the precursor. The 18-kDa form specific to PE-1 is also likely to represent another novel NH_2-terminally shortened peptide since it apparently does not contain the PE-2 epitope. However, post-translational modification within the region of the PE-2 epitope, preventing binding of PE-2, cannot at this stage be ruled out. We are currently purifying these peptides in order to define their structure.

To investigate the possibility that the 33/35-kDa doublet may indeed represent the intact enkephalin precursor, we decided to look at the association of these peptides with the granule membrane. It is known that hormone precursors are membrane-bound, which may provide a mechanism for correct sorting and orientation for proteolytic processing. Several prohormones, for example proinsulin, proglucagon, prosomatostatin, and proopiomelanocortin, have been shown to be associated with secretory granule membranes (Noe and Moran, 1984; Loh and Tam, 1985). In this study we have shown that the PE-2 immunoreactive 29-, 22-, and 15-kDa forms are all membrane-bound, the proportion bound decreasing with diminishing molecular size (Fig. 5, *panel a*). This is in keeping with the findings of others (Birch *et al.*, 1986; Hook and Liston, 1987). When the sensitivity of the immunoblot system was increased by the use of immunogold silver staining we found that the 35-kDa form was associated with the membrane, although the 33-kDa form was not. This would indicate that the 35-kDa form may represent the intact enkephalin precursor. If the 24-kDa PE-2 immunoreactive form is a modified form of the predicted molecular mass *18.2*-kDa peptide, as has been suggested by others, then its apparent failure to associate with the membrane could be related to post-translational modification. By the same argument, the 33-kDa peptide could therefore be a modified processed form, for example a modified form of the 23.3-kDa peptide, the modification possibly preventing membrane association. As already stated, we are postulating that the 24-, 22-, 18-, and 15-kDa PE-1 immunoreactive peptides may represent a subset of high molecular weight NH_2-terminally shortened polypeptides, and it was therefore of interest to us to note that all these forms appeared membrane-associated (see Fig. 5, *panel b*). This could indicate that proteolytic shortening of the membrane-bound forms is occurring from both ends of the molecule, suggesting the possibility that the region of the

[5] S. Jackson, unpublished data.

precursor which is attached to the membrane is centrally located.

It was of some concern to us to note that only a minor proportion of the 35-kDa form was membrane-bound, the majority apparently remaining in the soluble fraction. This fact may indicate that the 35-kDa form is not the intact enkephalin precursor, as the studies looking at membrane association of other prohormones found that they were approximately 80% membrane-bound (Noe and Moran, 1984; Loh and Tam, 1985). However, these studies looked at newly synthesized prohormone only and did not examine steady state levels. Another possible reason for the discrepancy is that we failed to achieve complete separation of membrane from contents. This was indicated when an antiserum to the intrinsic membrane protein p38, which has been recently demonstrated to be present in dense core granule membranes (Lowe *et al.*, 1988), revealed significant amounts of p38 in the soluble fraction. A further experimental artifact could have been introduced by our acidic lysis of the granules which may have dislodged some of the precursor forms from the granule membrane. Studies by others suggest this is a possibility (Birch *et al.*, 1986). We therefore repeated the experiment using hypotonic lysis at pH 5.9 accompanied by freeze-thawing. However, under these conditions the overall amounts of enkephalin peptides were substantially reduced and the highest molecular weight form observed was the 29-kDa peptide. This indicates that significant artifactual proteolytic processing had occurred, despite the presence of protease inhibitors and the fact that in the granule preparation we employed a final centrifugation step through 1.8 M sucrose in order to minimize lysosomal contamination (Evangelista *et al.*, 1982).

In conclusion, we have used a novel approach in generating monoclonal antibodies to a complex neuropeptide precursor, preproenkephalin A. Despite the expression of the proenkephalin sequence as a fusion protein in an alien environment, the antibodies resulting from its use as an immunogen are clearly able to recognize naturally occurring proenkephalin A-derived peptides. The antibodies PE-1 and PE-2 may be recognizing the intact bovine enkephalin precursor, although further evidence for this is required. They appear also to recognize a wide range of high molecular weight intermediates, and there is preliminary evidence that a previously unidentified subset of high molecular weight NH_2-terminally shortened forms may exist. Having successfully applied this approach to one of the most complex and unstable of the neuropeptide precursors, we are confident in predicting that the method will be widely applicable to other propeptide molecules.

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SUPPLEMENT FOR

Monoclonal Antibodies to a Proenkephalin A Fusion Peptide Synthesised in E. coli Recognise Novel Proenkephalin A Precursor Forms

by **Barbara A. Spruce, Susan Jackson, Philip J. Lowry, David P. Lane, and David M. Glover**

EXPERIMENTAL PROCEDURES

Preparation of chimaeric gene constructs

Details of constructs are as follows [see also Figure 1]: the plasmid **p-beta-gal-PPE-(69-207)** was constructed by excising a 411 base pair PstI fragment, encoding amino acids 69 to 207 of human preproenkephalin A, from a cDNA clone (Legon et al,. 1982) for ligation with PstI cleaved pUR 291 (Ruther and Muller-Hill, 1983). The plasmid **p-beta-gal-PPE-(1-267)** was made by excising a 918 base pair HindII fragment, encoding the entire preproenkephalin sequence, from the plasmid pHPE-9 given to us by Dr. E. Herbert (Comb et al., 1982). The HindII fragment has 6 and 110 base pairs of cDNA corresponding to the 5' and 3' untranslated mRNA sequence. This results in an additional two amino acids (Asn and Ser) which intervene between beta-galactosidase and the natural initiation codon of preproenkephalin in the fusion protein. This fragment was blunt end ligated to linear pUR 292 that had been cleaved with SalI, flush ends being generated by filling-in using the Klenow fragment of DNA polymerase I. The plasmid **p-beta-gal-PPE-(1-131)** was constructed by subcloning a BamHI/XhoII fragment containing 400 base pairs of preproenkephalin cDNA from **p-beta-gal-PPE-(1-267)** into BamHI cleaved pUR 292. **p-beta-gal-PPE-(130-267)** was made by subcloning an XhoII/HindIII fragment containing 524 base pairs of preproenkephalin cDNA from **p-beta-gal-PPE-(1-267)** into pUR 292 that had been cleaved with BamHI and HindIII. In order to construct **p-beta-gal-PPE-(1-84)** a DdeI fragment commencing at a DdeI site in the lac Z gene and containing 260 base pairs of preproenkephalin sequence was isolated from **p-beta-gal-PPE-(1-267)**. BamHI linkers were added , excess linkers were removed by BamHI digestion, and the fragment cloned into BamHI cleaved and phosphatased pUR 290.**p-beta-gal-PPE-(84-154)** was made by adding BamHI linkers to a 213 base pair DdeI fragment and cloning into pUR 290. **p-beta-gal-PPE-(152-267)** contains a DdeI / EcoRI fragment in which the EcoRI site corresponds to that in the pUR vector polylinker. This fragment, which contains 453 base pairs of proenkephalin sequence, was end-repaired as described above, BamHI linkers were added and the fragment subcloned into pUR 292.

The plasmid **pcII-PPE-(69-207)** was constructed by subcloning a BamHI/HindIII fragment from **p-beta-gal-PPE-(69-207)** (utilising sites in the polylinker on either side of the original cloning site) into pLcII (Nagai and Thogersen, 1984) using the identical endonuclease sites at the 3' end of a portion of the cII gene encoding the N - terminal 31 amino acids.

Production of beta-galactosidase fusion proteins

1) Analytical scale

Recombinant vectors were transformed into the E. coli strains BMH 71-18 or TG2. Both strains are lac Z$^-$ but carry the mutation lac I^Q on the F' plasmid. Transformants were cultured overnight at 37^{0}C on a shaking platform, in 2ml L-broth containing 100 ug/ml ampicillin and 500uM IPTG (Sigma Chemical Co.). Cells were pelleted in an Eppendorf centrifuge, washed in ice-cold PBS[1], sonicated for 20s in SDS-PAGE sample buffer (2%SDS, 5% b-mercaptoethanol) and boiled for 5min. Samples were subjected to SDS-PAGE (Laemmli, 1970) using a 7.5% separating gel at 150V for 4 to 5h. Gels were stained in a solution containing 0.2% Coomassie blue, 10% acetic acid and 25% isopropanol, and destained using 7.5% acetic acid, 25% methanol.

2) Preparative scale

In order to produce sufficient quantities of the fusion protein beta-gal-PPE-(69-207) for immunisation, E. coli BMH 71-18 transformed with **p-beta-gal-PPE-(69-207)** was cultured overnight in 100ml of L-broth containing 100ug/ml ampicillin and 500uM IPTG. Cells were harvested at 6Krpm, 4^{0}C for 10min in a Sorvall GSA rotor. The bacterial pellet was washed with ice-cold PBS, recentrifuged,then resuspended in 20ml of SDS-PAGE sample buffer plus 1mM PMSF[1] and 2ug/ml leupeptin. The sample was subjected to several short bursts of sonication at 4^{0}C, then placed in a boiling water bath for 5min. Aliquots of lysate were stored at -20^{0}C.

Prior to SDS-PAGE, thawed samples of bacterial lysate were spun for 2min in an Eppendorf centrifuge to pellet any insoluble material. 2 to 3 ml of bacterial lysate was then loaded across the width of a 6% 3mm thick polyacrylamide separating gel overlain with a small stacking gel. Electrophoresis was carried out at 160 - 180V for 4 to 5h. The gel was placed in 0.25M KCl overnight at 4^{0}C to visualise the fusion protein band. This was excised and placed in a large dialysis bag containing SDS-PAGE running buffer (0.1% SDS, 0.025M Tris, 0.192M Glycine). The gel band was subjected to horizontal electrophoresis at 150V for 6h to electroelute the fusion protein. The current was reversed for 2min at the end of the procedure to release any fusion protein clinging to the sides of the dialysis bag. If necessary, the protein solution was concentrated in an Amikon concentrator and stored in aliquots at -20^{0}C.

Immunisation of rabbits and mice with beta-gal-PPE-(69-207)

The electroeluted fusion protein in SDS-PAGE running buffer was emulsified with Freunds adjuvant. A rabbit (R139) was injected subcutaneously with 100ug of fusion protein in complete Freunds adjuvant, and then booster injections of 50ug of fusion protein in incomplete Freunds were given at 4 to 6 weekly intervals. Balb/c mice each received intraperitoneal injections of 10 to 20 ug of fusion protein in complete, then incomplete Freunds adjuvant at intervals of 4 to 6 weeks. The pre-fusion boost was carried out in the absence of Freunds.

The method for hybridoma fusion was essentially the same as that described by Kennett et al. (1978). The splenocytes of the immunised mice were suspended in serum-free medium, then fused to NS-1 myeloma cells in mid - late log growth phase using 33% polethylene glycol [Boehringer Corporation Limited] in serum free medium at room temperature. Each fusion was plated amongst 9 to 10 96-well microtitre dishes and screening commenced after approximately 10 days.

Immunoblot analysis

Proteins were separated on polyacrylamide gels, then electrophoretically transferred to nitrocellulose sheets in a Biorad Transblot apparatus according to the method of Towbin et al. (1979). Transferred proteins were transiently visualised with Ponceau stain (0.2% in 3% trichloroacetic acid). The blots were incubated overnight at 4^{0}C in blocking buffer (3% BSA[1] in PBS plus 0.05% sodium azide). Incubation with the primary antibody, either undiluted monoclonal supernatant or polyclonal antibody diluted 1/100 to 1/200 in 10% FCS[1] in was carried out in sealed polythene bags for either 2h at room temperature or overnight at 4^{0}C. The strips were washed three times in 1% Nonidet P-40 for 10 to 20min each, then rinsed several times in PBS before adding the second antibody, either peroxidase conjugated goat anti-mouse or anti-rabbit IgG (H&L) [Jackson Immunoresearch Ltd.] diluted 1/1000 in 10% FCS in PBS. Incubation times and washes were as for the primary antibody. The blots were stained using either a filtered solution of 0.05% 4-chloro-1-naphthol [Aldrich Chemical Co. Ltd.] in PBS, or 0.05mg/ml diaminobenzidene in PBS, to both of which was added 0.0002 vol of H_2O_2 (30% w/v). Immunogold silver staining (IGSS) was carried out according to the recommended protocol accompanying the **AuroProbe^{TM}BL plus-IntenSE^{TM}II** kit [Janssen Life Sciences Products].

Preparation of strips of immunoblotted cII-PPE-(69-207)

The plasmid **pcII-PPE-(69-207)** was transformed into the lysogenic E. coli strain QY-13 which carries the cI^{857} mutation on the prophage. The bacteria were grown in L-broth containing 100ug/ml ampicillin at 30^{0}C on a shaking platform to an O.D.$_{550}$ of approximately 0.6. The temperature of the cultures was then acutely raised to 42^{0}C over a bunsen flame, the flasks transferred to a 42^{0}C water bath for 15min and finally to a 42^{0}C incubator for 2h. The cells were then harvested and lysed into SDS-PAGE sample buffer. The lysate was subjected to preparative SDS-PAGE followed by Western blotting. A central strip was excised from the blot, while the remainder was stored in blocking buffer. The region of the blot containing the cII-PPE-(69-207) fusion protein was localised by immunostaining this central strip using the R139 [anti-beta-gal-PPE-(69-207)] serum which had been preabsorbed using a crude E. coli lysate coupled to a CNBr activated **sepharose column**, as described below, to remove antibodies to endogenous E. coli antigens. **The region containing cII-PPE-(69-207)** was then excised from the remainder of the blot and cut into strips which were stored in 24-well tissue culture dishes containing blocking buffer plus sodium azide at 4^{0}C until required.

Preparation of E. coli lysate coupled to cyanogen bromide activated sepharose

100ml cultures of appropriate host strains of bacteria (400 - 500ml total), transformed with plasmid vectors , were grown overnight. Cultures were pooled, the cells harvested at 4^{0}C in a Sorvall GSA rotor and resuspended in approximately 200ml of 0.1M sodium borate pH 8, 1M NaCl plus 200mg lysozyme, incubated at room temperature for 20 min. 200mg DNase 1 was added plus Triton X-100 to 0.2%. The lysate was centrifuged in a Sorvall SS34 rotor at 10Krpm, 4^{0}C for 20 min. The supernatant was coarsely filtered, and the pH adjusted to 8.5 - 9.0 with 0.1M sodium tetraborate. The supernatant was placed on ice. 5g of CNBr activated sephatose 4B (Pharmacia) beads were swollen in 10^{-3}M HCl and washed with approximately 500ml of 10^{-2}M HCl using a sintered glass funnel. The beads were rinsed with 25ml of 0.1M borate buffer pH 8 (5ml per g of dry gel), then the ligand solution was added immediately such that there was 5 - 10mg of protein per ml of gel with a ligand solution : gel volume ratio of 2 : 1. The gel suspension was mixed end over end overnight at 4^{0}C. The remaining active groups were blocked with 1M ethanolamine pH 9, then the excess protein washed away with 4 - 5 cycles of alternate 0.1M sodium borate pH 9 and 0.1M sodium citrate pH 3 washes. The gel was stored in NET buffer (150mM NaCl, 5mM EDTA, 50mM Tris pH 7.4 plus 0.05% sodium azide) at 4^{0}C. Sera for immunoblotting were preabsorbed on the column for either 2h at room temperature or overnight at 4^{0}C.

Preparation of chromaffin granule lysates

Bovine adrenal glands were obtained fresh from the slaughterhouse and transported on ice. Chromaffin granule isolation was carried out according to the method of Bartlett and Smith (1974), except that the final centrifugation step was carried out through 1.8 rather than 1.6M sucrose in order to minimise lysosomal contamination (Evangelista et al, 1982). Lysates of isolated granules were prepared in one of two ways. 1) Granules were sonicated in 0.1M HCl plus 1mM PMSF, 2ug/ml leupeptin and 0.3mg/ml iodoacetamide. In order to separate membrane from contents, the lysates were centrifuged in a Beckman air-driven ultracentrifuge. The membrane fraction was resuspended in an equivalent volume of 0.1M HCl. Immediately prior to SDS-PAGE the pH of the samples was adjusted to 7 using 1M Tris base. An equal volume of two times concentrated sample buffer was added and the samples boiled for 5min before fractionating on a 12% SDS polyacrylamide gel. 2) Granules were subjected to hypotonic lysis by the addition of approximately 3ml of 5mM Tris succinate pH 5.9 per adrenal gland, with protease inhibitors as above (TSB). Lysis was facilitated by three cycles of freeze-thaw using a dry ice-ethanol bath, thawing to 4^{0}C. Lysed granules were subjected to centrifugation in a Ti50 rotor at 40Krpm (100,000g), 4^{0}C. The supernatant was removed and frozen. The membrane pellet was washed in TSB, recentrifuged and finally taken up in a volume of TSB equal to the contents fraction. Both fractions were lyophilised then taken up in SDS sample buffer and boiled for 5min prior to SDS-PAGE.