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, 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 granule membranes from their contents 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 NH2 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-Arg6-Gly7-Leu8 and Met-enkephalin-Arg6-Phe7. 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

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 iQ 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 and electrophoresed on 7.5% denaturing polyacrylamide gels (see "Experimental Procedures"). Production of β-galactosidase-preproenkephalin 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-preproenkephalin A-(69-207) fusion protein for immunization or epitope mapping.

3The 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 preproenkephalin A. The upper section illustrates the cDNA fragments used to generate fusion proteins for immunization or epitope mapping.
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-Arg⁴-Gly⁷-Leu⁹. 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 polyepitope 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 antiproenkephalin 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 Z⁰, lac Y⁰. 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 polyepitopes 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-
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3 2 I
32 I 3 2 I 3 2 I 3
z I
-97.4
-6X
-4.3
-25.7
P.I. HI39 XII.'l'-S hlI.'I'-S

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-Arg6-Gly7-Leu8; MET4, 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).

Table 1

| M kDa | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|---|---|---|---|---|---|
| 95.5  |   |   |   |   |   |   |
| 66   |   |   |   |   |   |   |
| 45   |   |   |   |   |   |   |
| 31   |   |   |   |   |   |   |
| 18.4 |   |   |   |   |   |   |
| 12.5 |   |   |   |   |   |   |

Table 2

| M kDa | 1 | 2 | 3 | 4 | 5 | 6 |
|-------|---|---|---|---|---|---|
| 200  |   |   |   |   |   |   |
| 116  |   |   |   |   |   |   |
| 92.5 |   |   |   |   |   |   |
| 66   |   |   |   |   |   |   |
| 45   |   |   |   |   |   |   |
| -200 |   |   |   |   |   |   |
| -116 |   |   |   |   |   |   |
| -92.5|   |   |   |   |   |   |
| -66  |   |   |   |   |   |   |
| -45  |   |   |   |   |   |   |

The distribution of PE-1 and PE-2 immunoreactive peptides with chromaffin granules—Studies by others have suggested that high molecular weight proenkephalin A-derived 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 IgG1.

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 NH2-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 NH2-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-
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![Diagram of 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.](image)

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.

Peptides derived from 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 antiseraum 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 antiseraum 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, 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. 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 (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

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5 S. Jackson, unpublished data.
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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 prohormones 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 dissolved 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 NH2-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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REFERENCES

Baird, A., Klepper, R., and Ling, N. (1984) Proc. Soc. Exp. Biol. Med. 175, 304-308

Bartlett, S. F., and Smith, A. D. (1974) Methods Enzymol. 31, 372-389

Beaumoni, A., Metters, K. M., Rossier, J., and Hughes, J. (1985) J. Neurochem. 44, 934-940

Birch, N. P., and Christie, D. L. (1986) J. Biol. Chem. 261, 12213-12221

Birch, N. P., Davies, A. D., and Christie, D. L. (1986) FEBS Lett. 197, 175-178

Christie, D. L., Birch, N. P., Aitken, J. F., Harding, D. R. K., and Hancock, W. S. (1984) Biochem. Biophys. Res. Commun. 120, 650-656

Comb, M., Seeburg, P. H., Adeleman, J., Eiden, L., and Herbert, E. (1982) Nature 295, 663-666

Dandekar, S., and Sabol, S. L. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 1017-1021

Evangelista, R., Ray, P., and Lewis, R. V. (1982) Biochem. Biophys. Res. Commun. 106, 895-902

Hook, V. V. H., and Liston, D. (1987) Neuropeptides 9, 263-267

Jones, B. N., Shiveley, J. E., Kilpatrick, D. L., Stern, A. S., Lewis, R. V., Kojima, K., and Udenfriend, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 2096-2100

Kennett, R. H., Denis, K. A., Tung, A. S., and Klinman, N. R. (1978) Curr. Top. Microbiol. Immunol. 81, 77-91

Kilpatrick, D. L., Taniguchi, T., Jones, B. N., Stern, A. S., Shiveley, J. E., Hullihan, J., Kimura, S., Stein, S., and Udenfriend, S. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 3265-3268

Kilpatrick, D. L., Jones, B. N., Lewis, R. V., Stern, A. S., Kojima, K., Shiveley, J. E., and Udenfriend, S. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3057-3061

Laemmli, U. K. (1970) Nature 227, 680-685

Legon, S., Glover, D. M., Hughes, J., Lowry, P. J., Rigby, P. W. J., and Watson, C. (1982) EMBO J. 10, 7905-7918

Lightman, S. L., and Young, W. S. (1987) Nature 328, 643-645

Liston, D. R., Vanderhaeghen, J.-J., and Rossier, J. (1983) Nature 302, 62-65

Loh, Y. P., and Tam, W. W. H. (1985) FEBS Lett. 184, 40-43

Lowe, A. W., Maddedu, L., and Kelly, R. B. (1988) J. Cell Biol. 106, 51-59

Metters, K. M., and Rossier, J. (1987) J. Neurochem. 49, 721-728

Mizuno, K., Minamino, N., Kangawa, K., and Matsuo, H. (1980) Biochem. Biophys. Res. Commun. 97, 1283-1290

Nagai, K., and Thibergersen, H. C. (1984) Nature 309, 810-812

Noda, M., Furutani, Y., Takahashi, H., Toyosato, M., Hiroe, T., Inayama, S., Nakashi, S., and Numa, S. (1982) Nature 295, 202-208

Noe, B. D., and Moran, M. N. (1984) J. Cell Biol. 99, 413-424

Patey, G., Liston, D., and Rossier, J. (1984) FEBS Lett. 172, 303-308

Rüther, U., and Müller-Hill, B. (1983) EMBO J. 2, 1791-1794

Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 4350-4354

Udenfriend, S., and Kilpatrick, D. L. (1983) Arch. Biochem. Biophys. 221, 308-323

Yoshikawa, K., Williams, C., and Sabol, S. L. (1984) J. Biol. Chem. 259, 14301-14306
Monoclonal Antibodies to Proenkephalin A Fusion Peptide

Experimental Procedures

Preparation of monoclonal antibodies

Details of construction are as follows: (1) The phage-angle-gal-PHE-PGF-1 (p2) was constructed by introducing a BglII fragment containing 197 proenkephalin A (Phe-A) and 34 amino acid residues of the fusion protein into phage M13. The BglII fragment was subsequently subcloned into phage phAGE-1 (p3) and phage phAGE-2 (p4). The BglII fragment was then inserted into pBR322 (p5) to generate pB322 (p6), which was transduced into a eukaryotic cell line (p7). The fusion protein was expressed in this cell line, and the antibody response was measured by ELISA. The antibody response was further analyzed by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

A fusion protein consisting of the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B was prepared by chemical synthesis. This fusion protein was then used to generate a monoclonal antibody (Mab-A) specific for the N-terminal 11 residues of Phe-A.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-B) specific for the C-terminal 51 residues of Phe-B. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-C) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-D) specific for the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-E) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-F) specific for the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-G) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-H) specific for the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-I) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-J) specific for the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-K) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-L) specific for the N-terminal 11 residues of Phe-A and the C-terminal 51 residues of Phe-B. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.

Preparation of a-fusion peptide

The fusion protein was then used to generate a monoclonal antibody (Mab-M) specific for the entire fusion protein. This antibody was further characterized by Western blotting, which revealed the presence of a single band corresponding to the fusion protein.