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Expression of Variant Forms of Proopiomelanocortin, the Common Precursor to Corticotropin and β-Lipotropin in the Rat Pars Intermedia†

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ABSTRACT: Proopiomelanocortin, the common glycoprotein precursor to adrenocorticotropin (ACTH) and β-lipotropin (β-LPH), is the most abundant protein synthesized in rat neurointermediate lobes. It represents 30% of the total amount of radioactive proteins obtained after a 1-h pulse incubation with [^{3}H]phenylalanine. Several forms of this protein can be separated by a high-resolution two-dimensional gel electrophoresis technique. The three most abundant species which can be reproducibly characterized by their apparent molecular weights (M_r) and isoelectric points (pI) were called form I (M_r 34 000; pI 8.2), form II (M_r 36 000; pI 8.2), and form III (M_r 35 000; pI 7.3). Additional minor forms, representing together ~30% of the total of forms I, II, and III combined, are also observed. They have very close molecular weights but differ by their isoelectric points. When glycosylation is prevented by tunicamycin, forms I and II are replaced by a new molecule with the same pI of 8.2 but a slightly lower M_r (32 000). This form is referred to as form T_1. Similarly, form III is replaced by form T_2 (M_r 33 000; pI 7.3). Forms T_1 and T_2 are supposed to be nonglycosylated peptides. They were further characterized by microsequencing and peptide mapping. They both have the same N-terminal amino acid sequence with leucine residues in positions 3 and 11, and they both contain identical [^{3}H]phenylalanine-labeled tryptic fragments, two of them corresponding to the sequences 1–8 of ACTH and 61–69 of β-LPH. However, a limited digestion with the *Staphylococcus aureus* (V8 strain) protease generates a collection of peptides different for each form. These results suggest the presence of at least two different gene products corresponding to the major forms of proopiomelanocortin in the rat pars intermedia.

In the rat pars intermedia, β-endorphin and α-melanotropin (α-MSH)[1] are synthesized as part of a large common precursor (Crine et al., 1978, 1979; Mains & Eipper, 1979; Crine et al., 1980) named proopiomelanocortin (Chrétien et al., 1979; Crine et al., 1979). The existence of a similar precursor has also been extensively documented in the mouse pituitary tumor cell line (Roberts et al., 1978; Eipper & Mains, 1978a) and in the anterior lobe of the pituitary (pars distalis) (Taii et al., 1979). The two lobes of the pituitary (pars intermedia and pars distalis) contain, however, very different collections of final maturation products derived from the precursor: adrenocorticortopin (ACTH) and β-lipotropin (β-LPH) are major end products of the maturation process in the anterior lobe while α-MSH and β-endorphin occur in substantial amounts in the pars intermedia [for a recent review, see Krieger et al. (1980)], suggesting that the processing of the common precursor varies according to the tissue. The reason for this in totally unknown. One hypothesis states that there could be tissue-specific differences in the proteolytic enzymes responsible for cleaving the precursor at various sites during the processing. Unfortunately, the nature and specificity of these processing enzymes are still poorly documented and this hypothesis rests on very few experimental observations. Alternatively, since proopiomelanocortin is a glycosylated peptide (Eipper et al., 1976; Roberts et al., 1978), it has been proposed that glycosylation of critically positioned amino acid residues in the polypeptide backbone could direct the cleavage of specific sequences through conformational effects.

Careful analysis of the precursor by sodium dodecyl sulfate ($NaDodSO_4$) polyacrylamide gel electrophoresis revealed that a least two forms of this glycoprotein are synthesized by pars intermedia cells (Loh, 1979; Crine et al., 1979) or AtT-20 tumor cells (Roberts et al., 1978; Eipper & Mains, 1978a). It was proposed that these peptides represent the product of differential glycosylation of a single polypeptide chain (Roberts

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[1] Abbreviations used: α-MSH, α-melanotropin; ACTH, adrenocorticopin, adrenocroticotropic hormone; LPH, lipotropic hormone; $NaDodSO_4$, sodium dodecyl sulfate; Cl_3AcOH, trichloroacetic acid; MEM, minimum essential medium; Me_2SO, dimethyl sulfoxide; LC, liquid chromatography; pI, isoelectric point.

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et al., 1978) and that each of these forms could give rise to different maturation products. In order to obtain a better characterization of the different forms of proopiomelanocortin, we separated the different peptides synthesized in rat neurointermediate lobes in the presence or in the absence of tunicamycin, using the two-dimensional gel electrophoresis technique of O'Farrell (1975). We show here that the heterogeneity caused by differential glycosylation of a single polypeptide cannot account for the existence of all the precursor forms observed in the rat pars intermedia and that there are significant differences in the amino acid sequence of at least two of these peptides.

Experimental Procedures

Incorporation of labeled amino acids into rat pituitary cells during incubation of whole rat neurointermediate lobes was as described (Crine et al., 1980) except that the minimum essential medium (MEM; Gibco Select-Amine Kit) replaced the Krebs–Ringer buffer previously used (Crine et al., 1979). For pulse labeling incubations, the radioactive amino acids (New England Nuclear) were added to the MEM reconstituted without the corresponding nonlabeled amino acid. After the incubation, the tissue pieces were collected by low-speed centrifugation, washed in cold phosphate-buffered saline (Gibco), and extracted by three cycles of freezing and thawing in a solution of 9.5 M urea, 2% (v/v) Nonidet P-40 (Bethesda Research Laboratories), 2% (v/v) ampholines (pH range 3–10; Bio-Rad), 5% (v/v) β-mercaptoethanol, 2 mM phenylmethanesulfonyl fluoride, and 2 mM nonlabeled amino acid. The samples were centrifuged at 12800*g* for 5 min in an Eppendorf microcentrifuge, and the supernatant was stored frozen at −20 °C until used. Total trichloroacetic acid (Cl_3AcOH) precipitable counts were determined according to the procedure of Ivarie & O'Farrell (1978).

For prevention of glycosylation of proteins, rat neurointermediate lobes were preincubated for 16 h in MEM containing 5 μg/mL tunicamycin (a generous gift from Eli Lilly). The final tunicamycin concentration in the incubation medium was achieved by a 100-fold dilution of a stock solution of tunicamycin in dimethyl sulfoxide (Me_2SO). Therefore in control experiments, the tissue pieces were incubated in a medium without tunicamycin but containing 1% Me_2SO. After the preincubation period, the tissue pieces were pulse incubated for 60 min in MEM supplemented with the appropriate radioactive amino acid and 5 μg/mL tunicamycin. Preliminary experiments had shown that the preincubation in MEM containing 1% Me_2SO did not affect the rate of protein synthesis in neurointermediate lobes under these conditions. Similarly, no significant difference could be observed between tunicamycin-treated and control samples as far as the overall incorporation of radioactive amino acid into proteins is concerned.

Two-Dimensional Gel Electrophoresis of Radioactive Proteins. The radioactive proteins were resolved with the two-dimensional gel electrophoresis system of O'Farrell (1975) with minor modifications. In the first dimension, the proteins were separated according to their isoelectric points in cylindrical polyacrylamide gels (1.5 × 120 mm) by using Bio-Rad wide-range (3–10) purified ampholytes.

For the second dimension, linear gradient gels from 10% to 15% acrylamide in the discontinuous buffer system of Laemmli (1970) were cast in the Bio-Rad Model 220 slab gel electrophoresis cell with 0.75-mm spacers. Electrophoresis was at 20 mA/gel until the bromophenol blue had reached the bottom of the gel. After being fixed and stained the gels containing tritium-labeled proteins were soaked for 30 min in a commercially available autoradiography enhancer (New England Nuclear, En^{3}Hance) and then washed extensively in distilled water for 1 h. The gels were then dried under reduced pressure at 60 °C, and the radioactive proteins were detected by fluorography with Kodak X-OMat R films. [^{35}S]-Methionine-labeled proteins were detected by conventional autoradiography of fixed and stained gels prepared as described above but without the autoradiography enhancer.

Protease Digestion of Labeled Peptides. Exhaustive trypsin digestion of radioactive protein spots in the gel and analysis of the tryptic fragments by high-performance liquid chromatography was as described (Crine et al., 1980).

Partial digestion of radioactive proteins with *Staphylococcus aureus* (V8 strain) protease (agenerous gift from Dr. G. Drapeau, Université de Montréal) was performed directly on gel pieces obtained from a two-dimensional slab gel. The conditions were those of Cleveland et al. (1977). Labeled digestion products were separated on a 10–20% acrylamide gradient gel (1.5 mm thick; 30 cm long) and visualized by autoradiography.

Sequencing of Radioactive Peptides. [^{3}H]Leucine-labeled protein spots from the two-dimensional gels were cut off, and the radioactive peptides were recovered by electroelution according to Lazarides (1976). They were then sequenced as described previously (Gossard et al., 1980; Crine et al., 1980).

Results

Multiple Forms of Proopiomelanocortin in the Rat Pars Intermedia. In a typical experiment rat posterior lobes were incubated for 1 h in a medium containing [^{3}H]phenylalanine (500 μCi/mL, 110 Ci/mmol), and the radioactive proteins were extracted by boiling for 5 min in 0.0625 M Tris-HCl, pH 6.8, 2% (w/v) $NaDodSO_4$, 5% (v/v) β-mercaptoethanol, 2 mM phenylalanine, and 2 mM phenylmethanesulfonyl fluoride (Crine et al., 1979). An aliquot containing $\sim 3 \times 10^5$ cpm (as measured by Cl_3AcOH precipitation) was analyzed by $NaDodSO_4$ gel electrophoresis on a 10%–15% acrylamide gradient slab gel in the discontinuous buffer system described by Laemmli (1970). Fluorography of the dried gel for a period of 6 h revealed two main bands migrating with apparent molecular weights (M_r) of 34 000 and 36 000 as observed previously (Crine et al., 1979) (not shown). Exposures of 24 h or more yielded a single broad band covering the molecular weight region from 34 000 to 38 000 (Figure 1 insert). Scanning of the fluorogram with an integrating microdensitometer allowed us to estimate that these proteins accounted for ~30% of the radioactivity recovered on the gel. The portion of the gel corresponding to peptides from 33 000 to 38 000 (insert of Figure 1) was cut out of the dried slab and rehydrated, and the radioactive peptides were digested with trypsin as described previously (Crine et al., 1980). When the tryptic fragments were analyzed by high-pressure LC, two major [^{3}H]phenylalanine-labeled peptides coeluted with authentic ACTH (1–8) and β-LPH (61–69) (Figure 1). Quantitative analysis of the results obtained from the high-pressure LC chromatogram (Table I) allowed us to conclude that at least 90% of the proteins submitted to trypsin digestion contain the ACTH (1–8) and β-LPH (61–69) sequences. This result proved that virtually all the proteins migrating with apparent molecular weights of 33 000–38 000 are related to the proopiomelanocortin molecule.

In the two-dimensional electrofocusing/electrophoresis system (Figure 2a), these proteins could be further resolved into several components migrating with very close M_r's but with distinct isoelectric points. The major forms focused at pH 8.2 (forms I and II) and 7.3 (form III) while minor forms

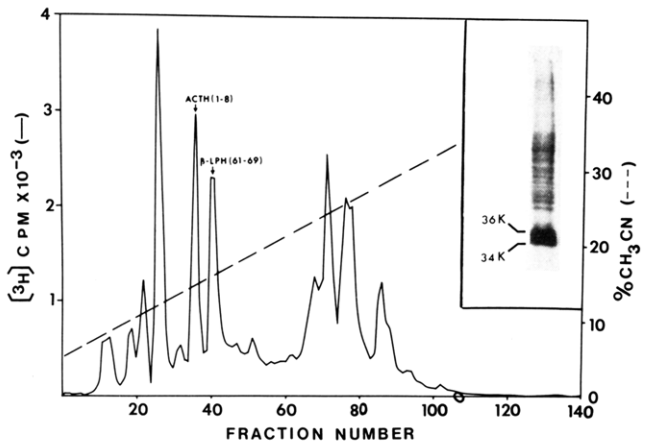

FIGURE 1: Characterization of proopiomelanocortin in the rat pars intermedia by high-pressure liquid chromatography of its tryptic fragments. Rat neurointermediate lobes were incubated for 1 h in MEM containing [^{3}H]phenylalanine, and the radioactive proteins were extracted and analyzed by $NaDodSO_4$ gel electrophoresis on a 10–15% acrylamide gradient. Fluorography was for 24 h (insert). Part of the gel containing the proteins migrating with apparent molecular weights between 33 000 and 38 000 was cut off, and the proteins were digested with trypsin. An aliquot of the tryptic digest containing 70 170 cpm was oxidized with performic acid and analyzed by high-pressure LC on a μBondapak C_{18} column by using a reverse-phase mode. The aqueous buffer was 0.02 M triethylammonium phosphate (pH 3.0). A linear gradient of 5–10% acetonitrile (CH_3CN) in 90 mL was used. Fractions of 0.4 mL were collected and assayed for radioactivity. Recovery of the radioactive peptides from the column was 95%. Five to ten micrograms of oxidized synthetic ACTH (1–8) and β-LPH (61–69) were run as markers on the high-pressure LC column together with the radioactive peptides.

Table I: Analysis of the Trypsin Digestion Products of Proopiomelanocortin[a]

fractions	radioact (cpm × 10^{-3}) recovered	radioact (cpm × 10^{-3}) expected	yield (%)
total put on the slab gel	300		
after trypsin digestion of the 33 000–38 000 region of the gel	70.17	90[b]	78
after high-pressure LC	68.15	70.17[c]	95
peak ACTH (1–8)	6.85	7.6[d]	90
peak β-LPH (61–69)	6.80	7.6[d]	89

[a] Proteins synthesized in rat neurointermediate lobes during a 1 h pulse with [^{3}H]phenylalanine were analyzed by slab gel electrophoresis (Figure 1 insert). The 33 000–38 000 region of the gel was cut off, and the proteins were digested with trypsin and submitted to high-pressure LC analysis as shown in Figure 1. The amount of radioactivity recovered at each step of the protocol was quantitated. [b] 3×10^5 Cl_3AcOH precipitable counts were loaded in one track of the gel and submitted to electrophoresis. Scanning of the fluorogram with a Vitatron densitometer showed that 30% of all the radioactive proteins were recovered in the 33 000–38 000 region of the slab gel. A total of 9×10^4 cpm was therefore expected from this part of the gel. [c] 70 170 cpm eluted from the gel pieces after trypsin digestion was loaded on the high-pressure LC column. [d] There are nine phenylalanine residues in the murine proopiomelanocortin (Eipper & Mains, 1978a). Out of the 68 150 cpm recovered from the high-pressure LC column, 7600 should therefore constitute both the ACTH (1–8) and β-LPH (61–69) peaks, since these two tryptic fragments each contain one phenylalanine residue.

were seen with p*I* values of 7.5, 7.1, and 7.0 (Figure 2a). Since all these proteins migrate in the 34 000–38 000 region of the gel, they are thought to correspond to multiple forms of the ACTH/β-LPH precursor.

Inhibition of Glycosylation by Tunicamycin. Since the ACTH/β-LPH precursor has already been proved to be a glycoprotein (Eipper et al., 1976; Roberts et al., 1978), the

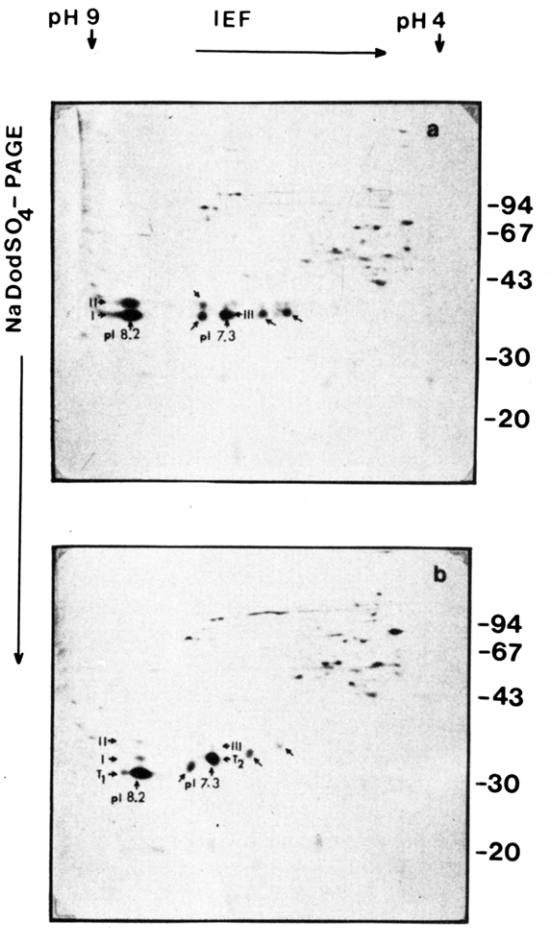

FIGURE 2: Analysis of glycosylated or nonglycosylated forms of proopiomelanocortin by two-dimensional gel electrophoresis. Rat neurointermediate lobes were preincubated for 16 h in MEM containing (a) 1% Me_2SO (control) or (b) Me_2SO and 5 μg/mL tunicamycin. They were then pulse incubated for 1 h in MEM containing [^{3}H]phenylalanine (840 μCi/mL, 100 Ci/mmol), and the labeled proteins were extracted in O'Farrell's (1975) "lysis buffer", as described under Experimental Procedures. Aliquots containing 1.6×10^5 cpm (control) or 1.9×10^5 cpm (tunicamycin treated) were analyzed by two-dimensional gel electrophoresis. Isoelectric points (p*I*) were measured on a blank gel run in parallel as described by O'Farrell (1975). Molecular weight standard proteins (Pharmacia) were included in the $NaDodSO_4$ slab gel electrophoresis dimension so as to provide a calibration scale for molecular weight determination. Fluorography was for 18 h, with films prefogged to an absorbance of 0.18.

apparent molecular weight of which is sensitive to tunicamycin action (Crine et al., 1979; Loh, 1979), it is conceivable that part of the heterogeneity observed on the two-dimensional gels could be due to glycosylation. For investigation of this possibility, glycosylation of asparagine residues in the rat pars intermedia proteins was prevented by tunicamycin, an antibiotic blocking the synthesis of the dolichol-linked oligosaccharide intermediate involved in the formation of carbohydrate side chains attached to asparagine residues of glycoproteins (Takatsuki et al., 1975; Lehle & Tanner, 1976). Preliminary experiments had shown that tunicamycin action was maximum when added at a concentration of 5 μg/mL in the incubation medium at least 8 h before performing the pulse labeling experiment. Therefore the neurointermediate lobes were routinely preincubated with tunicamycin (5 μg/mL) for 16 h. Under these conditions, new forms of the ACTH/β-LPH precursor were obtained (forms T_1 and T_2) (Figure 2b). Form T_1 (M_r 32 000) had the same p*I* as forms I and II but migrated slightly faster during the $NaDodSO_4$–polyacrylamide gel electrophoresis. Similarly, form T_2 (M_r 33 000) was slightly

smaller than form III (M_r 35000), but it had the same p*I* (7.3). Small amounts of residual forms I–III (probably normally glycosylated) were also observed. When these spots were cut off, digested in a Protosol–Econofluor mixture (1:100 v/v; New England Nuclear) at 37 °C for 16 h, and counted, it was found that they accounted for less then 5% of the radioactivity associated with T_1 or T_2. The T_1 and T_2 proteins could also be generated by digestion of regular precursor forms, obtained from cells incubated in the absence of tunicamycin, with the *Streptomyces griseus* endo-β-*N*-acetylglycosaminidase H (endo H from Miles Biochemicals) (results not shown). This enzyme cleaves *N*,*N*'-diacetylchitobiose units in asparagine-linked oligosaccharide chains (Tarentino & Maley, 1974). Since addition of carbohydrate side chains to a protein is known to increase its apparent molecular weight on NaDodSO$_4$–polyacrylamide gels, these results indicate that forms I and II of the precursor are generated by glycosylation from the T_1 peptide observed either in tunicamycin-treated cells or among endo H digestion products. Similarly, form III is thought to evolve from the glycosylation of the T_2 peptide. Moreover, we can conclude that in our incubation conditions tunicamycin had prevented glycosylation of at least 95% of either form I, II, or III. The T_1 and T_2 forms of the precursor, which are thought to correspond to the nonglycosylated peptides, have the same pI as the glycosylated forms. Therefore the carbohydrate side chains are thought to contain neutral core sugars only and are certainly free of terminal sialic acid residues. This was also confirmed by the observation that all the forms of proopiomelanocortin are resistant to neuraminidase digestion (results not shown). In conclusion, tunicamycin-sensitive glycosylation processes can explain the difference in molecular weight between the different precursor forms having identical p*I* values but cannot account for the existence of several forms differing by their isoelectric points.

Structural Analysis of the Major Forms of the Precursor Synthesized in Tunicamycin-Treated Cells. In order to elucidate the structural differences between the various forms of proopiomelanocortin characterized by distinct isoelectric points, we undertook more extensive analysis of the two major forms focusing at pH 8.2 (form T_1) and pH 7.3 (form T_2), after tunicamycin treatment. Since these forms represent nonglycosylated peptides, it was thought that structural differences in their polypeptide backbone would be easier to detect. The region of the two-dimensional gel containing these proteins labeled with [^{3}H]phenylalanine were cut off and digested with trypsin (see Experimental Procedures). The tryptic peptides were then analyzed by high-pressure LC. Figure 3 shows that in both cases very similar elution patterns were obtained. In particular, both peptides contain sequences characteristic of ACTH and β-LPH, suggesting that they are two very similar forms of proopiomelanocortin.

Since acetylation of amino-terminal residues of some of the end products resulting from the maturation of proopiomelanocortin has been proved to occur readily in rat pars intermedia (Smyth et al., 1979; Crine et al., 1979), the possibility that the presence of a more acidic form of the precursor might be due to acetylation of the N-terminal amino acid of the protein was then investigated. [^{3}H]Leucine-labeled forms of the two peptides were prepared as described under Experimental Procedures and submitted to automatic Edman degradation. Figure 4 shows that both peptides could be sequenced with the same yield and that, in both cases, leucine residues were found in positions 3 and 11, in agreement with previous results obtained from a complete mixture of all the forms of the precursor (Gossard et al., 1980). If the NH_2-terminal amino acid residue had been acetylated in the 7.3 form, the Edman degradation of this form would have been blocked. Since this

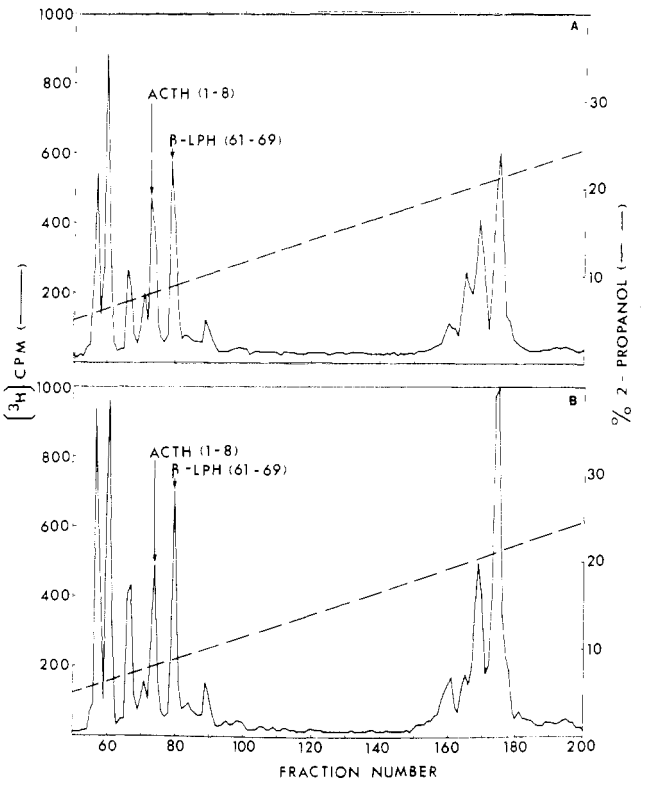

FIGURE 3: High-pressure liquid chromatography of the tryptic peptides from the two major forms of proopiomelanocortin synthesized in the presence of tunicamycin. Portions of the gel containing [^{3}H]-phenylalanine-labeled proteins synthesized in tunicamycin-pretreated neurointermediate lobes and focused with a p*I* of 8.2 (form T_1) and 7.3 (form T_2) were cut from a two-dimensional gel, digested with trypsin, and analyzed by high-pressure LC as described in the legend of Figure 1, except that 2-propanol was used as the organic phase instead of acetonitrile. (A) Form T_1 of proopiomelanocortin, 12350 cpm; (B) form T_2 of proopiomelanocortin, 19500 cpm.

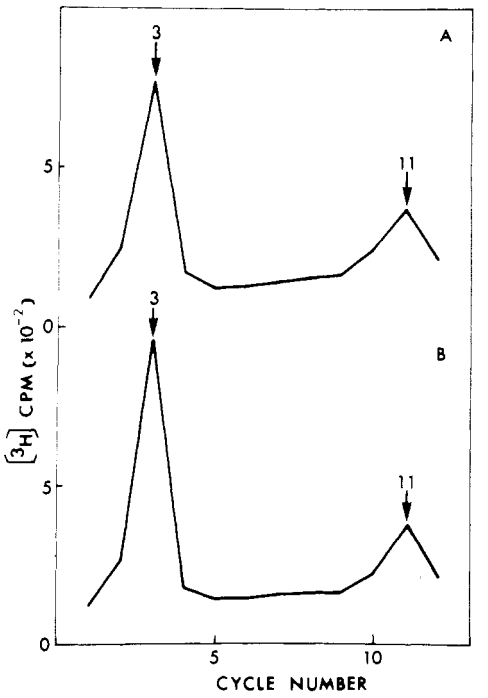

FIGURE 4: Sequencing of [^{3}H]leucine-labeled forms of proopiomelanocortin synthesized in the presence of tunicamycin. The radioactive proteins were labeled with [^{3}H]leucine from a two-dimensional gel by electroelution and sequenced as explained under Experimental Procedures. (A) Form T_1, 22000 cpm; (B) form T_2, 24000 cpm.

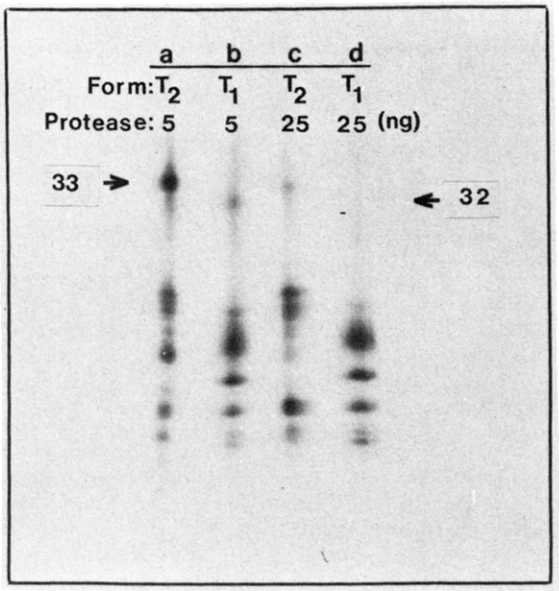

FIGURE 5: Analysis of the radioactive peptides resulting from a partial digestion of form T_1 and form T_2 labeled with [^{35}S]methionine. Portions of the two-dimensional gel containing the radioactive proteins were cut and digested with 5 ng (tracks a and b) or 25 ng (tracks c and d) of *S. aureus* (V8 strain) protease. Digestion was performed in the stacking gel of a second gel electrophoresis system as described by Cleveland et al. (1977). (Tracks a and c) form T_1; (tracks b and d) form T_2. The positions marked 32 and 33 correspond to the nondigested forms of the precursor migrating with apparent molecular weights of 32000 (form T_1) and 33000 (form T_2). Autoradiography was for 4 days.

is not the case, we conclude that acetylation does not explain the presence of the p*I* 7.3 precursor form. In addition, the p*I* 7.3 and 8.2 forms have the same partial amino acid sequence and could not have occurred through variations in the processing of the signal peptide such as that found with the growth hormone (Lingappa et al., 1977).

Another possibility is that the p*I* 7.3 form contains an additional short peptide with several dicarboxylic amino acid residues, which is absent in the p*I* 8.2 form. This would explain why the p*I* 7.3 peptide is slightly heavier and more acidic than the p*I* 8.2 form. If this hypothesis is correct, the two forms should yield different digestion products when incubated with the *S. aureus* V8 strain protease, an enzyme known to cleave peptide bonds on the carboxyl side of aspartic and glutamic acid residues (Drapeau et al., 1972). However, since these peptides nevertheless present a large degree of homology (as proved by the tryptic peptide analysis of the two [^{3}H]-phenylalanine-labeled forms) and since only segments containing essential amino acids can be labeled and therefore visualized with our methods of analysis, the presence of only a few additional small peptides would be difficult to detect after a complete digestion with a protease. We therefore decided to generate large digestion products by limited proteolysis with the *S. aureus* (V8 strain) protease, according to the procedure of Cleveland et al. (1977). Figure 5 shows that significant differences do exist between the peptide patterns generated from the p*I* 8.2 and 7.3 forms of proopiomelanocortin synthesized in the presence of tunicamycin.

Discussion

The early events of the posttranslational modification mechanisms involved in the processing of secretory glycoproteins consist of (1) the cleavage of the signal peptide (Blobel & Dobberstein, 1975) and (2) the core glycosylation of asparagine residues (Czichi & Lennarz, 1977). Both have been proposed to occur cotranslationally on the nascent polypeptide chains (Lingappa et al., 1977; Kiely et al., 1976).

In the case of proopiomelanocortin, Gossard et al. (1980) have shown that the labeled proteins obtained from whole rat pars intermedia cells briefly pulsed with radioactive amino acids contain labeled forms of the precursor which have already lost their signal peptide. Besides the cleavage of the signal peptide, the other early event in the processing of the proopiomelanocrotin molecule consists of the addition of several oligosaccharides to the polypeptide and generates several glycoprotein forms of the precursor. As demonstrated here, this step is sensitive to the action of tunicamycin and therefore thought to consist of the transfer of preformed core oligosaccharides from a lipid intermediate onto asparagine residues. Core oligosaccharides consist of neutral sugars only, and this type of glycosylation is known to change the apparent molecular weight of the protein in $NaDodSO_4$–polyacrylamide gels not its isoelectric point. The presence of the two different forms (I and II) characterized by an identical p*I* (8.2) but slightly different molecular weights (34000 and 36000) is therefore thought to arise through differential glycosylation of the same or very similar polypeptides, probably the nonglycosylated 32000 p*I* 8.2 form synthesized in the presence of tunicamycin. If one assumes that each carbohydrate adds 2000 to the apparent molecular weight of the precursor, one can conclude that there are two glycosylation events: the 34000 form contains one carbohydrate side chain and the 36000 form two. Glycosylation seems to occur simultaneously on the two sites as a cotranslational event since pulse incubation as short as 1 min failed to detect preferential labeling of one form vs. the other (not shown). The presence of two different glycoprotein forms could be due to random glycosylation on two acceptor sites in the same molecule, a fraction of the population being glycosylated once and the rest twice. Alternatively, in one subclass of the precursor molecules, a point mutation might generate a new site for carbohydrate addition. It is important to recall here that glycosylation occurs on an asparagine residue only if this amino acid is part of a sequence such as Asn-X-Thr or Asn-X-Ser, X being any amino acid (Neuberger & Marshall, 1969). Replacement of asparagine, serine, or threonine residues by other neutral amino acids would remain undetected with the two-dimensional electrophoresis technique used here but could lead to forms of the precursor lacking one glycosylation site. Definitive answers to that question must await more extensive results on the structure of the genes coding for rat proopiomelanocortin. Sialic acid residues are the last sugars to be added to the carbohydrate side chains as a result of the trimming and maturation process (Kornfeld et al., 1978; Hubbard & Robbins, 1979). Addition of sialic acid to glycoproteins generates new forms with more acidic p*I*. Since none of the precursor forms observed here contain sialic acid, we propose that the maturation of the precursor into smaller peptides involves glycoprotein forms in which the carbohydrate side chains have not completed their own processing yet.

Inhibition of glycosylation by tunicamycin does not prevent the synthesis of the precursor forms characterized by more acidic p*I*. Moreover, partial digestion of the two major forms synthesized in the presence of tunicamycin by the *S. aureus* protease generates different collections of peptides. These two observations are consistent with the existence of variant peptide forms of the precursor differing in their content of aspartic and glutamic acid residues. Alternatively, the p*I* 7.3 form could still contain residual charged carbohydrate side chains, resistant to both tunicamycin and neuraminidase, providing

partial protection to this form during the limited digestion experiment with the *S. aureus* protease.

Additional evidences from other laboratories, however, favor the idea of the multiple genes hypothesis. Recent characterization of mouse tumor cell β-lipotropin (Eipper & Mains, 1979) has proved that both γ-lipotropin (γ-LPH) and β-LPH are ~20 amino acid residues shorter than the corresponding LPH molecules from porcine, ovine, bovine, or human sources. Preliminary results from gene sequencing suggests that one form of rat β-LPH at least lacks the very acidic sequence 6–28 present in the bovine hormone (J. Drouin, personal communication). Kawauchi et al. (1980 a,b) have also isolated and characterized two distinct β-endorphins and two distinct β-melanotropins from salmon pituitaries which must be derived from two separate precursors. These observations combined with our results suggest that differences between various gene products in the rat pituitary might well exist in the NH_2-terminal region of the γ-LPH molecule since this region of the molecule seems to be submitted to considerable evolutionary drift. Additional mutations affecting glycosylated sequences might also have occurred elsewhere in the molecule since there is only one glycosylated form of this peptide compared to two for the p*I* 8.2 forms. Other peptides observed on two-dimensional gels with apparent molecular weights close to the two major forms but with different isoelectric points probably represent additional forms of the precursor present in lesser quantities. Their nature is presently being investigated more extensively.

The proopiomelanocortin molecule provides a unique model for studying the maturation mechanism of multihormonal protein precursors. The amino acid sequence of this protein is punctuated by a serie of basic amino acid pairs which have been proposed to act as signals recognized by the trypsin-like proteases thought to be involved in the processing of several precursor molecules (Chrétien & Li, 1967; Steiner et al., 1974). However, all these basic amino acid pairs in the proopiomelanocortin molecule do not seem to play the same role in the maturation mechanism. β-LPH, ACTH, and the N-terminal peptide, for instance, are generated as stable end products of the processing of the precursor and still contain one or more of these double amino acid sequences (Crine et al., 1980). Finally, the maturation of the precursor differs according to the tissue where it takes place. This is best exemplified by the existence of distinct families of the various maturation end products found in the pars intermedia and the pars distalis of several species (Eipper & Mains, 1978b; Liotta et al., 1978; Lissitzky et al., 1978). There are also some indications that the mechanism controlling the maturation of the precursor in the pituitary may change during the development of the foetus (Silman et al., 1978).

The conclusions reported in this paper stress the possible involvement of two potentially important mechanisms controlling the processing of proopiomelanocortin. Glycosylation, already proposed by Loh & Gainer (1979), might be essential for proper processing to occur. Alternatively, the presence of several forms of the precursor characterized by different amino acid sequences suggests that the maturation of the precursor into different sets of end products might also be controlled at the level of gene expression. According to this new hypothesis, slightly different proteins would be made in different tissues, each with its own amino acid sequence dictating which processing pathway is to be followed.

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Interaction of the Chick Oviduct Progesterone Receptor with Deoxyribonucleic Acid†

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ABSTRACT: The purified DNA binding component (receptor A) of the chick oviduct progesterone receptor has been analyzed for its ability to bind to the cloned ovalbumin gene and to plasmid DNA of various structural compositions. The rapid equilibrium filter adsorption assay of Riggs et al. [Riggs, A. D., Suzuki, H., & Bourgeois, S. (1970) *J. Mol. Biol. 48*, 67] has been used to demonstrate high affinity binding of the protein to DNA (K_{diss} = 10^{-10} M at 50 mM KCl, pH 7.2). Studies of association rates are consistent with equilibrium measurements ($t_{1/2}$ = 40–80 min). Association of purified receptor with DNA and the kinetics of the interaction have been verified independently by velocity sedimentation techniques. Direct binding assays were performed with the ovalbumin structural gene (cDNA), the entire natural ovalbumin gene containing seven intervening sequences, and various ovalbumin gene fragments coding for the 5′ end of the nuclear precursor RNA, intron–exon junctions, and the 3′-noncoding region of the gene. No DNA-sequence specificity was identified for the binding of the receptor protein to any region of ovalbumin gene DNA. In contrast, the structural integrity of the DNA template greatly affected receptor binding. The poorest affinity was to supercoiled DNA and to blunt end, linear duplex gene fragments. The receptor bound saturably to DNA containing limited nicks but became nonsaturable as nicks were increased. Binding of the protein to double-stranded DNA increased susceptibility of the DNA to digestion by the enzyme S_1, a single strand specific nuclease. On the basis of preferential receptor binding to single-stranded DNA, a possible mechanism involving DNA helix destabilization is discussed.

Our laboratory has been studying the progesterone receptor of chick oviduct [for a review, see Vedeckis et al. (1978)]. This protein is a potential gene regulatory factor in oviduct cells (O'Malley et al., 1972; Buller et al., 1975) on the basis of its appearance in oviduct nuclei in vivo and in vitro following administration of the hormone (O'Malley et al., 1970). In experiments performed in vitro, receptor–hormone complexes bind with high affinity to oviduct chromatin (Spelsberg et al., 1971). Progesterone induces the specific egg-white protein avidin (O'Malley, 1967), and, when administered to estrogenized immature chicks, progesterone will also induce ovalbumin (Palmiter, 1972). These hormonal events involve rapid increases in the rate of synthesis of ovalbumin mRNA and its accumulation (Swaneck et al., 1979a,b; Harris et al., 1975). The close temporal coupling between receptor occupancy in the nucleus and the induction of mRNA (Tsai et al., 1975) has led to the hypothesis that the receptors may act directly as inducers of specific gene transcription (O'Malley et al., 1972; Palmiter, 1972). One test of this idea would be to examine the interaction of the progesterone receptor protein with the DNA it potentially regulates. We have previously reported the purification of this protein and measurements of its interaction with DNA–cellulose and with labeled chick embryo fibroblast DNA in solution (Coty et al., 1979). Due to the lack of DNA fragments of discrete composition, it was not possible to determine kinetic parameters of this interaction. These characteristics are of particular interest for steroid receptor studies in view of reports in the literature using crude receptor preparations which suggested that receptor–DNA binding was weak and transitory (Yamamoto & Alberts, 1976) and preferential for double-stranded DNA (Kallos & Hollander, 1978). In the present communication, we have investigated the interaction of the DNA-binding component (receptor A)[1] of the progesterone receptor with cloned fragments of the chicken ovalbumin gene (Gannon et al., 1978; Dugaiczyk et al., 1978, 1979; Roop et al., 1978, and 1980)

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[1] Abbreviations used: receptor A and A protein, purified DNA binding component of the chick oviduct progesterone receptor; pOV4.5, pOV2.4, pOV1.8, and pOV9.2, cloned fragments of the chick ovalbumin gene in the bacterial plasmid pBR322 (refer to Figure 5); dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HDP, helix destabilizing protein; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; bp, base pairs.

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