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Apolipoprotein E: Phospholipid Binding Studies with Synthetic Peptides Containing the Putative Receptor Binding Region†

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Received February 22, 1985; Revised Manuscript Received June 10, 1985

ABSTRACT: To define the lipid and receptor binding regions of apolipoprotein E (apoE), we have synthesized four peptides beginning at residue 169 and continuing through the putative receptor binding region and ending at residue 129 so as to include a proposed lipid binding domain. The peptides were synthesized by solid-phase techniques, cleaved with anhydrous HF, and purified by ion-exchange and semipreparative reversed-phase high-performance liquid chromatography (HPLC). The peptides had the correct amino acid composition and were greater than 99% pure by analytical reversed-phase HPLC. The circular dichroic spectrum of each peptide was recorded before and after mixing with dimyristoylphosphatidylcholine. With apoE(148-169), apoE(144-169), and apoE(139-169), no changes were observed in the ellipticity at 222 nm. However, with apoE(129-169), an increase in α-helicity to ~42% was observed. Density gradient ultracentrifugation of the lipid-peptide mixture permitted isolation of a complex with apoE(129-169) with a molar ratio of lipid to peptide of 125:1, which was stable to recentrifugation. The α-helicity of the peptide in the complex was estimated to be 56%. No complexes were isolated from the gradients of the shorter peptides. Therefore, we conclude that the amphipathic helix formed by residues 130-150 contains one of the lipid binding regions of apoE.

Apolipoprotein E (apoE)[1] is a constituent of human plasma chylomicrons, very low density lipoproteins, and high-density lipoproteins (Mahley, 1978) and exhibits sequence heterogeneity that is genetically determined (Utermann et al., 1980; Zannis & Breslow, 1981). The apoprotein is responsible for the interaction of the lipoproteins with cellular receptors for control of cholesterol metabolism [for review, see Mahley & Innerarity (1983)]. Due to the sequence heterogeneity, several apoE isoforms are defective in their ability to interact with receptors, which results in the lipoprotein disorder type III hyperlipoproteinemia (Mahley & Angelin, 1984).

The amino acid sequence of apoE and three common isoforms has been determined (Rall et al., 1982a,b, 1983). The

† This research was supported by grants from the National Institutes of Health (HL-30064 and HL-27341, SCOR on Atherosclerosis).

[1] Abbreviations: apoE, apolipoprotein E isolated from human plasma very low density lipoproteins; apoE(129-169), a synthetic peptide comprising residues 129-169 of apolipoprotein E; DMPC, dimyristoylphosphatidylcholine; CD, circular dichroism; HPLC, high-performance liquid chromatography; *t*-BOC, *tert*-butyloxycarbonyl; Tris, tris(hydroxymethyl)aminomethane; TEAP, triethylammonium phosphate; AUFS, absorbance units full scale.

0006-2960/85/0424-6984$01.50/0

lipoprotein receptor binding region has been localized to residues 140–150 by using cyanogen bromide cleavage, enzymic fragmentation (Innerarity et al., 1983), and apoE monoclonal antibodies (Weisgraber et al., 1983). The reported amino acid substitutions for Arg or Lys in the isoforms with reduced receptor binding also occur in this region. A requirement for the protein to be bound to phospholipid for receptor binding has also been reported (Pitas et al., 1980), and by CD spectroscopy, the protein has been shown to have a high helical content (65%) in the presence of phospholipid (Innerarity et al., 1984). Amphipathic helices that have been shown to be responsible for lipid binding (Morrisett et al., 1975; Sparrow & Gotto, 1982) are predicted to occur at residues 60–78, 130–150, 203–221, 226–243, and 245–266 (Rall et al., 1982a). Therefore, to more clearly define the receptor binding and lipid binding regions of apoE, we have synthesized four fragments starting at residue 169. We report here our results on the interaction of these peptides with dimyristoylphosphatidylcholine.

Materials and Methods

The solvents used in the peptide synthesis and for the HPLC were purchased from Burdick and Jackson, Muskegon, MI. The polystyrene resin was from Lab Systems, San Mateo, CA. The *t*-BOC amino acids were purchased from Bachem, Torrance, CA. Trifluoroacetic acid was from Halocarbon Corp., Hackensack, NJ, and was distilled. Diisopropylethylamine and acetic anhydride were purchased from Aldrich, Milwaukee, WI, and were distilled before use. Cesium chloride was from Alfa, Danvers, MA. Triethylammonium phosphate was purified by passage through a PrepPak C_{18} column on a Waters Prep LC500. Buffer salts were from Fisher, Pittsburgh, PA. Ultrapure Tris base was from Schwarz/Mann, Spring Valley, NJ.

Peptide Synthesis. The peptides were synthesized by solid-phase methodology (Barany & Merrified, 1980) using a Schwarz/Mann Bioresearch synthesizer modified for computer control (Edelstein et al., 1981). *t*-BOC-Gly was attached to 4 g of aminomethylpolystyrene through the methylphenylacetyl linkage (Sparrow, 1976); the loading was 0.15 mM/g of resin. The peptides were synthesized by the sequence of reactions and washes previously reported (Bhatnager et al., 1983). Resin was removed after the attachment of residues 148, 144, 139, and 129. The peptide was deprotected and cleaved from the resin (1 g) by treatment for 30 min at 0 °C with 20 mL of anhydrous HF containing 2 mL of anisole and 0.2 mL of ethanedithiol. HF was removed under vacuum, and the peptide and resin were washed with ether. The peptide was dissolved in trifluoroacetic acid. Trifluoroacetic acid was evaporated in vacuo and the peptide precipitated with ether. After centrifugation, the peptide was dissolved in 1 M Tris–6 M guanidine hydrochloride and desalted on a 5 × 50 cm column of Bio-Gel P-2 equilibrated in 0.1 M ammonium bicarbonate. The peptide-containing fractions were lyophilized.

Peptide Purification. The crude peptide was further purified by ion-exchange chromatography on a 2.6 × 26 cm column of SP-Sephadex equilibrated in 0.02 M NaH_2PO_4 and 6 M urea, pH 3.75, at 4 °C. After the peptide (~150–200 mg) was loaded in the starting buffer, a linear gradient of 0.02 M NaH_2PO_4, 6 M urea, pH 3.75, and NaCl in 0.02 M NaH_2PO_4 and 6 M urea, pH 3.75, was used to elute the peptide. The peptide was located by measuring the absorbance at 275 nm. The peptide-containing fractions were pooled and desalted on Bio-Gel P-2 equilibrated with 5% acetic acid. After lyophilization, amino acid analysis was performed on each peptide. Semipreparative reversed-phase high-pressure liquid chromatography (Hancock & Sparrow, 1984) was performed on a Spectra Physics 8000B chromatograph fitted with a Waters RCM-100 radial compression module containing a 10-μm Radial-Pak C_{18} column. The peptide (~25 mg) was injected in 0.15 M triethylammonium phosphate and 6 M guanidine hydrochloride, pH 3.0. A gradient of 0.15 M TEAP, pH 3.0, to 50% 2-propanol at 1.5 mL/min was used to elute the peptides. Analytical HPLC was performed on a 0.46 × 25 cm Vydac C_4 column on a Spectra Physics 8100 chromatograph. A gradient of 0.1 M $NH_4H_2PO_4$, pH 3.0, to 50% 2-propanol was used to elute the peptides. Disc gel electrophoresis was performed at pH 4.5 in 10% polyacrylamide gels as previously reported (Shuster, 1971). Amino acid sequencing was performed with an Applied Biosystems gas-phase sequencer.

Phospholipid Binding. Approximately 0.5 mg of peptide was lyophilized in a 13 × 100 mm screw-cap culture tube. The peptide was solubilized in 1 mL of 0.1 M NaCl and 0.01 M Na_2HPO_4, pH 7.4, and the concentration determined by recording the UV spectrum between 320 and 240 nm on a Cary 15 spectrophotometer.

A dispersion of dimyristoylphosphatidylcholine (DMPC) was prepared by sonicating 30 mg of DMPC in 2.8 mL of 0.1 M NaCl with a probe sonicator. The dispersion was maintained at 4 °C under nitrogen during the 15 min required to clarify the solution. The dispersion was centrifuged for 30 min at 14000 rpm in a Beckman J-21 centrifuge to remove any titanium. A microphosphorus analysis (Bartlett, 1959) was performed to determine the concentration and the sample counted in a scintillation counter. The specific activity was found to be 50000 cpm/mg of DMPC.

The DMPC and peptide were mixed in appropriate quantities to obtain molar ratios of lipid to peptide of 40:1 and 100:1. The screw-cap tubes were placed in a 24 °C bath for 24 h. The CD spectrum were recorded before and after the addition of DMPC as well as after the incubation. The volume of the peptide–lipid mixture was adjusted to 1.5 mL with 0.01 M Na_2HPO_4 and 0.10 M NaCl, pH 7.4. A solution (2.5 mL) containing 0.01 M Na_2HPO_4, 0.10 M NaCl, and 1.31 M CsCl, pH 7.4, was placed in the bottom of a 5-mL polyallomer tube and the peptide–lipid solution carefully added to the top. The tube containing the peptide–lipid mixture was rinsed with 1 mL of the CsCl solution and the rinse solution added to the top of the centrifuge tube. The tubes were placed in the buckets of a Beckman SW 50.1 rotor and centrifuged at 45000 rpm at 20 °C for 72 h. The gradients were fractionated from the top of the tube into 250-μL aliquots with a peristaltic pump, Densiflow, and a drop-counting fraction collector. Each tube was analyzed for peptide by the micro-Lowry procedure (Lowry et al., 1951) using the peptide for a standard curve and for lipid by counting a 25-μL aliquot and back-calculating from the specific activity of the phospholipid. The density of each fraction was determined from the refractive index. The results were plotted in Figures 2 and 3 as mg/mL peptide and lipid.

Circular Dichroism. The circular dichroic spectrum was recorded with a Cary 61 spectrometer with an E. G. and G. Princeton Applied Research lock-in amplifier. The instrument was calibrated with D-(−)-pantoyl lactone. The peptide concentration was 0.5 mg/mL, and the cell path length was 0.05 cm. The spectrum was recorded between 250 and 200 nm. The helicity was estimated by the equation α-helix = $[\theta]_{222}$ + 3000/39000 × 100 (Morrisett et al., 1973).

Results

Peptide Purification and Characterization. The peptides

Table I: Amino Acid Composition of Purified Synthetic Peptides

amino acid[a]	peptide 148–169	144–169	139–169	129–169
aspartic acid	2.77 (3)[c]	2.96 (3)	2.98 (3)	3.15 (3)
threonine[b]	(0)	(0)	(0)	0.81 (1)
serine[b]	(0)	(0)	0.66 (1)	2.01 (2)
glutamic acid	3.17 (3)	3.07 (3)	3.08 (3)	5.04 (5)
glycine	2.10 (2)	2.00 (2)	1.78 (2)	1.72 (2)
alanine	3.99 (4)	3.98 (4)	4.02 (4)	5.00 (5)
valine	1.00 (1)	1.06 (1)	0.96 (1)	2.29 (2)
leucine	4.00 (4)	5.09 (5)	6.00 (6)	7.99 (8)
tyrosine	1.00 (1)	0.93 (1)	0.88 (1)	0.93 (1)
lysine	1.01 (1)	1.99 (2)	2.89 (3)	3.12 (3)
histidine	(0)	(0)	0.88 (1)	1.02 (1)
arginine	2.92 (3)	4.91 (5)	5.48 (6)	7.99 (8)

[a] Amino acid analyses were performed on a Beckman 119 analyzer after hydrolysis for 24 h in 6 N HCl at 110 °C. [b] Uncorrected for destruction. [c] The numbers in parentheses are the theoretical values.

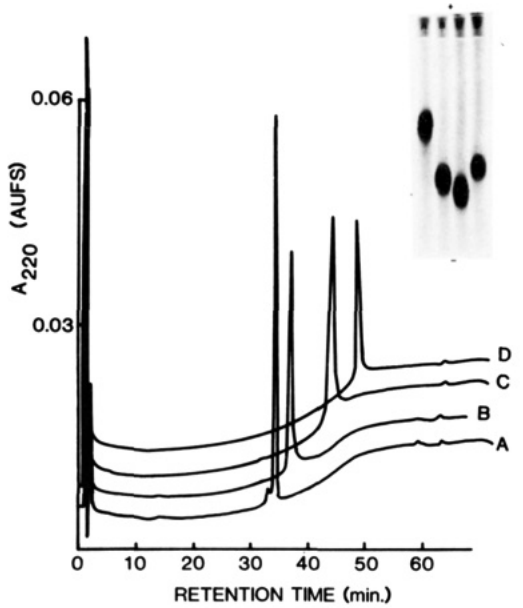

FIGURE 1: Analytical HPLC analysis of purified synthetic peptides. The peptide (~12 μg) in 6 M guanidine was injected onto a 0.46 × 25 cm Vydac C_4 column. The peptide was eluted with a 60-min linear gradient from 0.1 M $NH_4H_2PO_4$, pH 3.0, to 50% 2-propanol at a flow rate of 1.5 mL/min. Traces: A, apoE(148–169); B, apoE(144–169); C, apoE(139–169); D, apoE(129–169). The inset shows the disc gel electrophoresis at pH 4.5 of each peptide (~50 μg). Gels: A, apoE(148–169); B, apoE(144–169); C, apoE(139–169); D, apoE(129–169).

were eluted from SP-Sephadex by a linear gradient of starting buffer that contained 0.02 M NaH_2PO_4 and 6 M urea, pH 3.75, and starting buffer containing sodium chloride. A major peptide peak was observed at the following NaCl concentrations: apoE(148–169), 0.22 M; apoE(144–169), 0.36 M; apoE(139–169), 0.60 M; apoE(129–169), 0.48 M. The major peaks were pooled, desalted on Bio-Gel P-2 in 5% acetic acid, and lyophilized. Amino acid analysis of the peptides indicated that they had the expected composition (Table I). The analyses of other peaks in the chromotogram indicated they were termination products formed during the synthesis. High-performance liquid chromatography on a radially compressed C_{18} column indicated the peptides to be of greater than 95% purity. The peptides were further purified by semipreparative reversed-phase HPLC to greater than 99% purity (Figure 1). The small peak (~35 min) in the chromatogram of apoE(148–169) (Figure 1, trace A) integrated as less than 1% of the area of the major peak. Disc gel electrophoresis (Figure 1, inset) also indicated the peptides were of high purity.

Table II: Ellipticity of Synthetic Fragments of Apolipoprotein E in the Presence of DMPC

peptide	$[\theta]_{222}$ for peptide alone[a]	lipid:peptide ratio	$[\theta]_{222}$ peptide + DMPC[a]	$[\theta]_{222}$ isolated complex
148–169	−5073	40:1	−3900	none
		100:1	−2174	none
144–169	−3503	40:1	−3359	none
		100:1	−2193	none
139–169	−3231	40:1	−3864	none
		100:1	−3426	none
129–169	−10774	40:1	−13418	−18822[b]
		100:1	−16757	−18720[b]

[a] The molar ellipticity at 222 nm was calculated from $[\theta]_{222} = (MRW)\theta_{222}/(10lc)$, where c is the peptide concentration, l is the cuvette path length, MRW is the mean residue weight of peptide, and θ_{222} is the measured ellipticity angle at 222 nm. These represent the averages of three separate determinations, SD ± 200. [b] L:P ratio was ~125:1 in all isolated complexes.

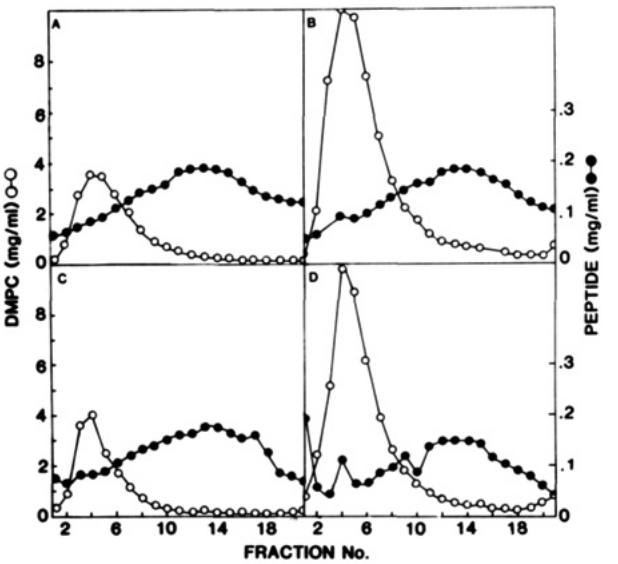

FIGURE 2: Density gradient ultracentrifugation of DMPC–synthetic apoE peptide mixtures at two lipid to peptide ratios: apoE(148–169) (A) in a 40:1 mixture and (B) in a 100:1 mixture; apoE(144–169) (C) in a 40:1 mixture and (D) in a 100:1 mixture. The peptide (●) and dimyristoylphosphatidylcholine (DMPC) (○) were plotted as mg/mL.

Amino acid sequence analysis of the purified peptides indicated the sequence was correct (data not shown).

Phospholipid Binding. The circular dichroic spectrum of the peptides alone showed very little ellipticity at 208 and 222 nm for the three shorter peptides (Table II) and was estimated to be 20–25% α-helicity. However, the $[\theta]_{222}$ for apoE(129–169) indicated that there was approximately 35% α-helicity in the absence of phospholipid. The addition of DMPC to the solutions of the three shorter peptides did not induce any significant changes in $[\theta]_{222}$; however, there was a significant increase in $[\theta]_{222}$ for apoE(129–169). The α-helicity was estimated to be 42%.

Density gradient ultracentrifugation demonstrated the absence of complex formation by the three shorter peptides (Figures 2 and 3A,B) regardless of the lipid to peptide ratio. Recentrifugation of the phospholipid and subsequent analysis for peptide by amino acid analysis of the lipid-containing fractions (data not shown) confirmed the absence of peptide in these fractions. However, apoE(129–169) did form a complex with DMPC that could be isolated by density gradient ultracentrifugation (Figure 3C,D). The circular dichroic spectrum of the isolated complex indicated significant α-helical structure in the peptide, ~56% (Table II). Recentrifugation

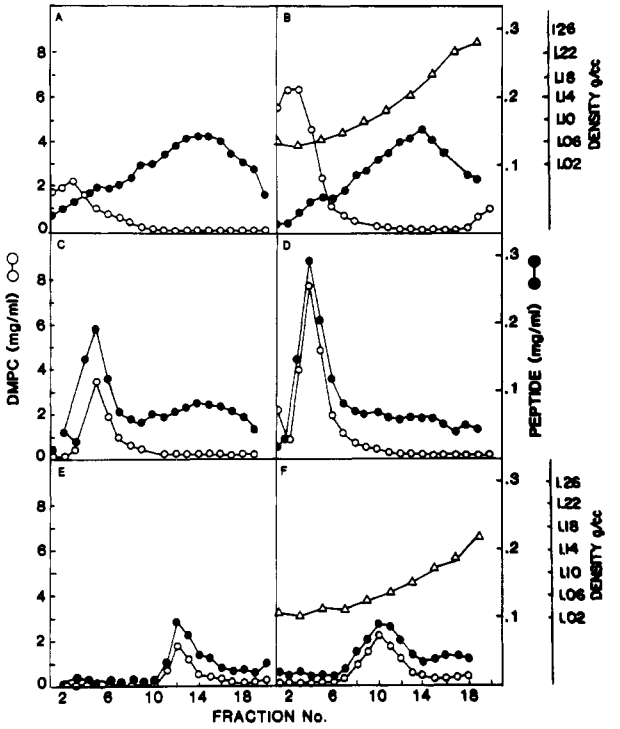

FIGURE 3: Density gradient ultracentrifugation of DMPC–synthetic apoE peptide mixtures at two lipid to peptide ratios: apoE(139–169) (A) in a 40:1 mixture and (B) in a 100:1 mixture; apoE(129–169) (C) in a 40:1 mixture and (D) in a 100:1 mixture; (E) recentrifugation of the complex from (C); (F) recentrifugation of the complex from (D). The peptide (●) and DMPC (○) were plotted as mg/mL, and the density (△) was plotted as g/cm^3.

of the complex (Figure 3E,F) permitted the reisolation of a DMPC–peptide complex with a lipid to peptide molar ratio of approximately 125:1 regardless of the starting ratio of DMPC to peptide; the ellipticity did not increase further upon reisolation.

Discussion

Apolipoprotein E has a requirement for phospholipid to be bound before interaction with the apoB–apoE receptor on human fibroblast (Innerarity & Mahley, 1978). Analysis of the amino acid sequence by the Chou–Fasman method (Chou & Fasman, 1974a,b) predicts there are 11 α-helical segments (62%), six of which may be amphipathic helices required for phospholipid binding. There are three segments (9%) of β-sheet and eight β-turns (9%). There is also a long random region between residues 164 and 202. One of the amphipathic helices is between residues 130 and 150 and contains the basic residues thought to be responsible for receptor binding from previous studies on protein fragments (Innerarity et al., 1983). Therefore, to more closely define the receptor binding region, we chose to synthesize this region of apoE beginning at residue 169 and continuing toward the amino terminal. We removed resin samples after the addition of multiple basic residues since the importance of arginine to receptor binding has been demonstrated previously (Weisgraber et al., 1978). The region between residues 129 and 139 was included since this sequence is predicted to be an α-helical region. Model building indicates that it has amphipathic character and probably contains a lipid binding domain.

The circular dichroic spectra of the shorter peptides in both the presence and absence of phospholipid indicate that ~25% of the peptide may be in an α-helical conformation (Table II). However, density gradient ultracentrifugation demonstrates that these peptides do not bind to DMPC (Figures 2 and 3A,B). The longer peptide apoE(129–169) shows conformational changes in the CD spectra in the presence of DMPC similar to those reported for other apolipoproteins (Morrisett et al., 1975; Sparrow & Gotto, 1982). A complex is isolated by density gradient ultracentrifugation from both 40:1 and 100:1 mixtures (Figure 3B,C); the estimated helical content is 56% in the isolated complex (Table II). The isolated complex had a phospholipid to peptide molar ratio of 125:1 regardless of the starting ratio. Upon recentrifugation it is isolated near the center of a more shallow gradient (Figure 3E,F) and contains the same ratio of phospholipid to peptide; there is not further increase in ellipticity. The difference in ellipticity of the mixtures and the isolated complexes can be attributed to unbound peptide that is removed during ultracentrifugation and is seen near the bottom of the gradients (Figure 3C,D). This view is supported by the fact that no further changes occur in the ellipticity or phospholipid to peptide ratio after recentrifugation.

These results indicate that a lipid binding region of apoE is contained between residues 129 and 169, and we conclude that it most probably resides in the amphipathic region between residues 130 and 150. In addition, the α-helicity calculated from the CD spectrum agrees well with that predicted by the Chou–Fasman analysis. When this region becomes helical, the basic residues are aligned across the face of the helix and provide a highly positively charged region that could interact with the cellular receptor. Studies are presently under way to determine the extent of interaction of these peptides with the apoB–apoE receptor on fibroblast and to also determine how substitution of Cys for Arg_{145} or Arg_{158} affects the lipid binding and/or the receptor binding as has been shown to occur for apoE-2 (Innerarity et al., 1983, 1984).

Acknowledgments

We thank Sarah Myers-Fossett and Marjorie Sampel for secretarial assistance and Sue Kelly for artwork done on the figures.

Registry No. ApoE(148–169), 88144-84-7; apoE(144–169), 88144-85-8; apoE(139–169), 88160-80-9; apoE(129–169), 88160-81-0; DMPC, 13699-48-4.

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Proteins That Mask the Nuclear Binding Sites of the Avian Oviduct Progesterone Receptor†

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Received April 24, 1985

ABSTRACT: The binding of a steroid receptor to specific nuclear sites (i.e., nuclear acceptor sites) represents the immediate event preceding the steroid regulation of gene transcription. How the same steroid receptor regulates different genes in different tissues is unknown. Since a major fraction of the nuclear acceptor sites for a variety of steroid receptors has been reported to be masked in the chromatins of a variety of tissues, the differential expression of the nuclear acceptor sites may explain this regulation of different genes. In the avian oviduct, the removal of a subfraction of chromosomal non-histone proteins, termed CP-2, results in the unmasking of the nuclear acceptor sites for the progesterone receptor (PR). Further, the extent of masking of these nuclear acceptor sites for PR has been reported to vary during cytodifferentiation of the avian oviduct. This paper describes a method for the reconstitution of the masking of PR nuclear acceptor sites in the avian oviduct chromatin using a partially purified chromosomal protein fraction (CP-2b). The reannealling of the CP-2b fraction to unmasked avian oviduct chromatin (termed nucleoacidic protein or NAP) results in the "remasking" of about the same number of nuclear acceptor sites for PR as found in intact chromatin. Because some of the PR acceptor sites on the NAP cannot be remasked, these sites either must be protected from masking or not be recognized by the masking proteins. The masking activity apparently involves only protein(s) because the unmasking of acceptor sites can be achieved with protease but not ribonuclease activities and because the dissociated masking activity is destroyed only by proteases. The masking appears to be reversible because the reconstituted masked sites can again be unmasked. Preliminary purification and characterization of the masking activity in fraction CP-2b by molecular sieve chromatography indicate a heterogeneity of size with the activity eluting in a molecular weight range of from 60 000 to >150 000. Whether the masking proteins prevent the binding of the progesterone receptor by directly binding the acceptor sites or by binding neighboring domains to condense the chromatin is unknown. It is speculated that the masking of acceptor sites may be responsible in part for determining the tissue-specific gene expression induced by steroids and/or may play a role in the unresponsiveness of certain human tumors containing steroid receptors.

Steroid hormones are best known for their effects on gene transcription and the resulting modification of the metabolism of many cellular components in their target cells (O'Malley & Means, 1974). Of the many events which must occur prior to hormone response, the nuclear event immediately preceding the alteration of gene activity is the binding of steroid–receptor complexes to the nuclear acceptor sites. In most instances, these acceptor sites have been localized on isolated chromatin (Thrall et al., 1978; Spelsberg, 1982).

Early studies in this laboratory demonstrated that the majority of the nuclear binding sites for the avian oviduct progesterone receptor are "masked". Thus, in intact avian oviduct

† This work was supported by National Institutes of Health Grants HD140-P1 and HD16705 and by the Mayo Foundation. These studies were published in a thesis by G.M.D. as part of her requirements of a Ph.D. from The University of Minnesota and its affiliate, the Mayo Graduate School.
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0006-2960/85/0424-6988$01.50/0