Journal of Supramolecular Structure
and Cellular Biochemistry 15:15–27 (1981)
Control of Cellular Division and Development: Part A: 339–351

Proteolytic Domains of the Epidermal Growth Factor Receptor of Human Placenta

Edward J. O'Keefe, Teresa K. Battin, and Vann Bennett

Department of Dermatology, University of North Carolina, Chapel Hill, North Carolina 27514 (E.J.O'K., T.K.B.) and Department of Cell Biology and Anatomy, The Johns Hopkins School of Medicine, Baltimore, Maryland 21205 (V.B.)

Microsomal membranes from human placenta, which bind 5–20 pmol of ^{125}I-epidermal growth factor (EGF) per mg protein, have been affinity-labeled with ^{125}I-EGF either spontaneously or with dimethylsuberimidate. Coomassie blue staining patterns on SDS polyacrylamide gels are minimally altered, and the EGF-receptor complex appears as a specifically labeled band of 180,000 daltons which is not removed by urea, neutral buffers, or chaotropic salts but is partially extracted by mild detergents. Limited proteolysis by alpha chymotrypsin and several other serine proteases yields labeled fragments of 170,000, 130,000, 85,000, and 48,000 daltons. More facile cleavage by papain or bromelain rapidly degrades the hormone-receptor complex to smaller labeled fragments of about 35,000 and 25,000 daltons. These fragments retain the binding site for EGF, are capable of binding EGF, and remain associated with the membrane. Alpha chymotryptic digestion of receptor solubilized by detergents yields the same fragments obtained with intact vesicles, suggesting that the fragments may represent intrinsic proteolytic domains of the receptor.

Key words: epidermal growth factor, epidermal growth factor receptor, integral membrane proteins, hormone receptor, limited proteolysis

The membrane receptor for epidermal growth factor (EGF) has been identified in microsomal preparations from human placenta [1] and cultured cells [2, 3] by affinity labeling using photoactivated bifunctional cross-linking reagents. Using these methods and labeling the receptor with ^{125}I-labeled EGF, a single band of M_r 190,000 was observed in autoradiograms by Das and Fox [2] and by Baker et al [3] in fibroblasts, while two closely spaced bands of approximately 180,000 and 160,000 daltons were described by Hock et al in human placenta [1]. A single band of 150,000–170,000 daltons was described in A-431 epidermoid carcinoma cells by Wrann et al [4]. Reducing agents did not affect the migration of the receptor on SDS gels, and the labeled band was immunoprecipitable by anti-EGF IgG, indicating that labeled EGF remains associated with this 180,000-dalton protein [5].

Received May 8, 1980; accepted September 17, 1980.

0275-3723/81/1501-0015$04.00

Incubation of cells containing receptor previously labeled with ^{125}I-labeled EGF results in the production of three fragments of 59,000–70,000 daltons, 38,000–50,000 daltons, and 30,000–37,000 daltons in Swiss 3T3 cells and human fibroblasts, but not in A-431 cells, which have 10- to 20-fold more receptors than do fibroblasts [2–5]. Inhibitors of lysosomal enzymatic activity result in the production of a 130,000-dalton protein from the originally labeled 190,000-dalton band and prevent formation of the three lower molecular weight bands; this 130,000-dalton band is seen following tryptic digestion of labeled cells [2]. In other experiments using cells treated with proteases after brief exposure to label, ^{125}I-labeled EGF was found to be cleaved from the receptor [3].

These experiments indicate that the EGF receptor is probably composed of a single polypeptide that is cleaved in intact fibroblasts by lysosomal hydrolases to yield discrete fragments. It has been suggested that one or more of these fragments is mitogenic and mediates the action of EGF in cells [2]. It is not known whether similar fragments can be generated by controlled proteolysis of the receptor in membrane preparations or whether such fragments would be water-soluble or would remain associated with or buried in the membrane. In order to study such fragments generated by controlled proteolysis, we have used human placental microsomal membranes affinity labeled with ^{125}I-labeled EGF and then exposed to proteases. These studies demonstrate that similar discrete fragments are produced by enzymes of differing specificities although with different facility, and that the EGF binding site on the receptor is extremely resistant to proteolytic digestion. The smallest fragment visualized, of approximately 25,000 daltons, remains associated with the membrane and contains the EGF binding site. The fragments generated may depend on the intrinsic tertiary structure of the receptor, since identical fragments are produced after solubilization in detergents. The relationship of the fragments generated in intact cells to those obtained by limited proteolysis is unknown.

METHODS

Placenta Membranes

Placentas were obtained fresh after delivery, separated from the umbilical cord, and manually cut into 5–10 cubic centimeter pieces in cold Dulbecco's phosphate buffered saline to remove contained blood. The washed pieces were then transferred to 0.25 M sucrose to remove salt and then to 1 volume of 0.25 M sucrose for homogenization in a Waring blender at 4°. The suspension was made 2 mM in iodoacetate and 2 mM in EDTA, and 200 μg per ml phenymethylsulfonyl fluoride was added immediately prior to homogenization. The material was then further homogenized for 60 seconds at full speed with a Polytron homogenizer and centrifuged at 4° in a Sorvall GSA rotor at 2,400 rpm for 10 minutes. The sedimented material was again homogenized with the Polytron and centrifuged as before. The sedimented material was again homogenized with the Polytron, sedimented, and the supernatants were pooled and centrifuged at 10,000g for 30 minutes and the pellet was discarded. The supernatant was then centrifuged at 40,000g for 4 hours to obtain the microsomal fraction. To purify this material further, the pellet was resuspended in 35% (w/v) sucrose and centrifuged at 40,000g for 1 hour, and the supernatant diluted with 1 mM EDTA to a final concentration of 0.25 M sucrose and centrifuged at 40,000g for 4 hours. The pellet was resuspended in 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA, or in 25 mM Tris buffer, pH 7.4, at a concentration of 20 mg per ml and frozen at −70° and is referred to hereafter as "membranes."

^{125}I-Labeled EGF

EGF was purified to homogeneity by the method of Savage and Cohen [6] and labeled by the method of Hunter and Greenwood [7] to a specific activity of 50–175 uCi per μg. The labeled material was purified on a Sephadex G-25 column equilibrated with 0.1 M sodium phosphate buffer containing 0.1% bovine serum albumin. Following reduction of Chloramine T with sodium metabisulfate, 5 mM tyrosine was added 60 seconds prior to chromatography to prevent iodination of albumin.

SDS Gel Electrophoresis

Electrophoresis was performed according to the method of Fairbanks [8]. Gels contained 6% acrylamide and 0.45% bis acrylamide and were polymerized with 0.03% TEMED and 0.2% ammonium persulfate. Samples were added to electrophoresis buffer to achieve a final concentration of 1% SDS, 10 mM Tris-HCl, 10 mM EDTA, pH 8.0, 5% sucrose, 40 mM dithiothreitol, and 0.1% bromophenol blue. Samples containing 40–70 μg protein were added to 5-mm wells and run on 3-mm thick gels in a Hoefer slab gel apparatus at 20 volts constant voltage. Running buffer contained 40 mM Tris, 20 mM sodium acetate, 2 mM EDTA, 0.2% SDS, and was adjusted to pH 7.4 with acetic acid. Standards used were human erythrocyte membranes, phosphorylase, beta galactosidase, and bovine serum albumin. Autoradiograms were produced by exposing the dried gels to Kodak X-O-Mat XR-1 film at –70° in the presence of Dupont Cronex lightning-plus intensifying screens.

Affinity Labeling of the EGF Receptor

^{125}I-labeled urogastrone or murine EGF (5×10^{-8}M) was incubated for 60 minutes at 4° with placenta membranes (10 mg protein per ml) in 10 mM sodium phosphate buffer, pH 7.4, containing 1 mM EDTA. The spontaneously labeled membranes [3, 5] were diluted in 5 volume 1.0 M Tris-acetate buffer, pH 7.4, sedimented at 160,000g for 20 minutes, and resuspended in various buffers, usually 10 mM sodium phosphate, pH 7.4. For covalent labeling with dimethylsuberimidate [9], the pellet was resuspended in 0.1 M sodium borate buffer, pH 8.0, containing 0.5 mg per ml dimethylsuberimidate and incubated at 24° for 20 minutes, diluted with 5 volumes 1.0 M tris-acetate buffer, and centrifuged and resuspended as above. From 5% to 20% of radioactivity in such washed membranes is associated with the receptor on SDS polyacrylamide gels.

Binding Assays

Binding of ^{125}I-labeled EGF to placenta membranes was measured as previously described [10].

Materials

SDS gel electrophoresis reagents were from BioRad. Triton X-100 and dimethylsuberimidate were from Sigma, and Ammonyx-LO was a gift from the Onyx Chemical Company, Jersey City, New Jersey. Papain, bromelain, elastase, alpha chymotrypsin, trypsin, phosphorylase, and beta galactosidase were from Worthington; Carrier-free ^{125}I NaI was from Union Carbide. Chemicals were from Fisher or Sigma and were reagent grade or better. Urogastrone was a kind gift of Dr. H. Gregory.

Enzymatic Digestions

After labeling of the receptor in intact membranes and resuspension in appropriate buffers, various enzymes were added to the membranes and incubated as described in the figure legends. Digestions were terminated by addition of electrophoresis buffer, heating to

70° for 2 minutes, and incubation at 37° for 20 minutes. In some cases, inhibitors such as phenylmethylsulfonyl fluoride or iodoacetate were added before electrophoresis buffer to inhibit serine or sulfhydryl proteases.

RESULTS AND DISCUSSION

In binding studies with ^{125}I-labeled EGF the placenta membrane preparation yields a curvilinear Scatchard plot indicating the presence of high-affinity binding sites ($K_D = 1 \times 10^{-9}$M) and a capacity of approximately 6 pmols of ^{125}I-labeled EGF bound per mg protein (Fig. 1a) [10]. For a protein of M_r 180,000 this indicates that the receptor may comprise 0.2% of the protein of the microsomal membrane preparation. Transmission electron microscopy of the preparation reveals vesicles and sheets with the appearance of membranes. Gaps are visible in the vesicles indicating that they are probably not sealed and suggesting that enzymatic digestion probably affects both cytoplasmic and external sides of the plasma membrane. Membranes incubated with ^{125}I-labeled EGF in saturating concentrations and then washed and electrophoresed in 6% polyacrymamide gels in SDS show either a single band on autoradiograms or two closely spaced bands of about 180,000 and 170,000 daltons. If the membranes are homogenized in the presence of inhibitors of proteolysis (see Methods), a single band is labeled reproducibly, whereas the label on membranes prepared without inhibitors or from frozen placenta shows two bands and occasionally a third band of 130,000 daltons. The same lower molecular weight bands are produced in membranes previously incubated at 24° or 37° for 15–60 minutes. These bands are labeled specifically, since saturating concentrations of native EGF prevent labeling of all bands equally (Fig. 1b). The label seen on autoradiograms coincides with a visible band on Coomassie blue-stained gels, and the label as well as this band, which comigrates with band 2.3 of erythrocyte membranes, is especially heavy in membranes prepared in the presence of inhibitors with attention to maintenance of low temperatures.

The labeled material is not extractable from membranes by neutral buffers such as 10 mM sodium phosphate/1 mM EDTA, 50 mM EDTA, 1 M KCl, or by chaotropic salts such as 1 M KBr, KSCN, or Tris-acetate, and it resists extraction by 8 M urea or 0.2 M acetic acid. Although some proteins are removed by this washing and extraction, Scatchard plots indicate that substantial enhancement of binding capacity per mg protein is not achieved by these procedures, and some degradation of the 180,000-dalton band to lower molecular weight bands is apparent after exposure to urea. Binding affinity is altered by 0.2 M acetic acid, which reduces high-affinity binding without substantially altering binding capacity (data not shown).

The affinity label produced by incubating ^{125}I-labeled EGF alone [3, 5] with placenta membranes is identical on SDS gels to that produced in the presence of low concentrations of the bifunctional crosslinker, dimethylsuberimidate, but is less heavily labeled. A time- and concentration-dependent enhancement of the labeled 180,000-dalton band is seen using 0.1–2.0 mg per ml of cross-linker for 10–90 minutes at 24°, although at higher concentrations and longer incubation times extensive cross-linking of many membrane proteins is seen and labeled material fails to enter 6% acrylamide gels. At 0.5 mg dimethylsuberimidate per ml for 15–30 minutes at 24°, there is minimal or undetectable alteration of staining patterns and a two- to tenfold enhancement of labeling, which is confined to the bands labeled in the absence of dimethylsuberimidate. Satisfactory labeling is seen with either murine or human EGF (urogastrone) using dimethylsuberimidate, although murine EGF has only one available amino group [11], whereas urogastrone has in addition two

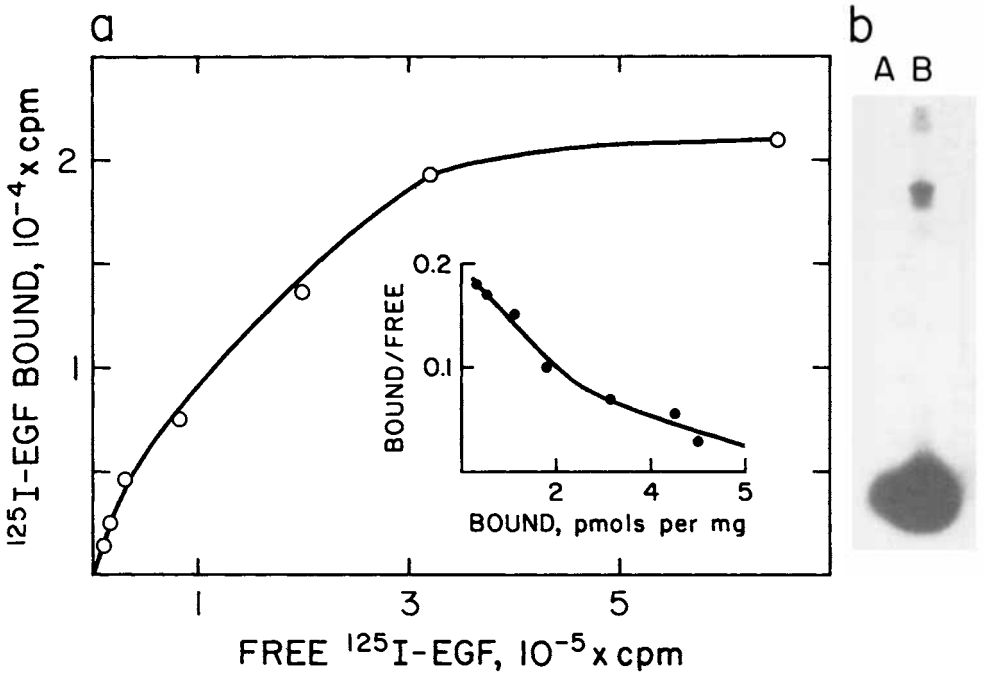

Fig. 1. Binding of ^{125}I-labeled EGF and affinity labeling of the EGF receptor in placenta membrane. a) Various concentrations of ^{125}I-labeled EGF (0.4 Ci/μmol) were incubated (30 min, 24°) with 9.2 μg of placenta membranes in 0.2 ml buffer and filtered as described [10]. Only specific binding is shown. Inset shows Scatchard plot of binding data. b) Dimethylsuberimidate cross-linking of ^{125}I-labeled EGF with (lane A) and without (lane B) 10^{-6} M native EGF added.

lysine groups [12]. ^{125}I-labeled EGF is retained in labeled membranes, since the 180,000-dalton band can be immunoprecipitated by anti-EGF antiserum and visualized on autoradiograms of SDS gels (data not shown).

Although solubilization of the labeled 180,000-dalton band is readily achieved by 0.5% Ammonyx-LO or other mild detergents with parallel partial solubilization of the Coomassie blue staining band identified with the radioactive band on autoradiograms, it is notable that complete solubilization of the labeled material is not achieved even with concentrations of detergent (eg, 10%) far above the critical micellar concentration (Fig. 2) both in placenta membranes and in KB cells extracted with Triton X-100 or Ammonyx-LO. Extraction of radioactivity reaches a plateau as determined by residual radioactivity pelleted at 160,000g for 1 hour, approximately 20% of radioactivity not removed by washing membranes in 1 M Tris-acetate remaining sedimentable after treatment at 4° with 5% (v/v) Ammonyx-LO. The Coomassie blue-stained 180,000-dalton band is apparently heterogeneous, since low ionic strength buffers may remove small amounts of material from this band that reveal no radioactive label on autoradiograms, and detergent is required to release labeled material (Fig. 2).

Proteolytic digestion of affinity-labeled membranes with chymotrypsin or trypsin results in sequential degradation of the receptor and production of a labeled 170,000-dalton band followed by 130,000 and 80,000–85,000-dalton bands on autoradiograms (Fig. 3).

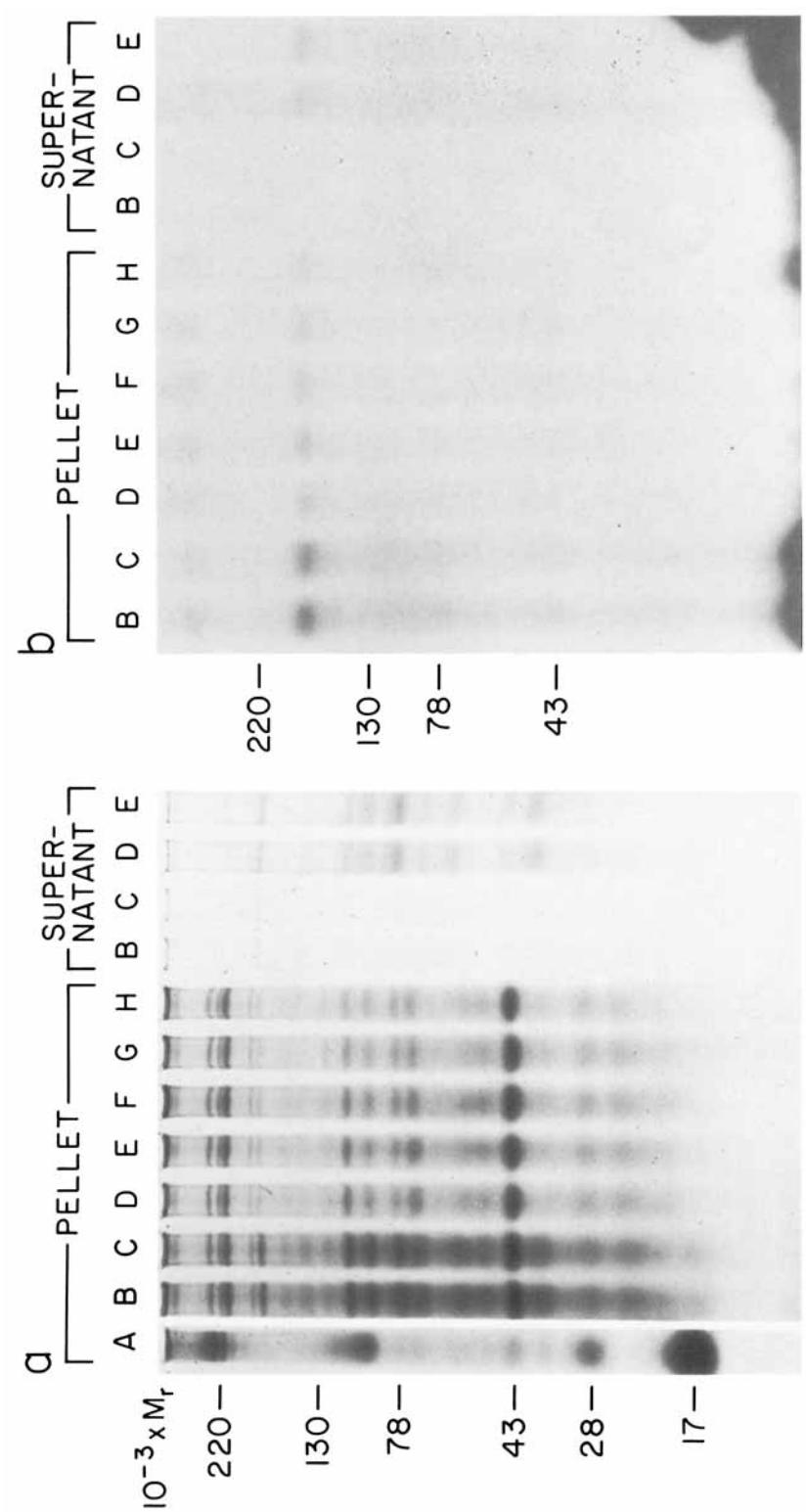

Fig 2 Solubilization of the labeled EGF receptor with Ammonyx-LO Membrane suspensions prepared from human placenta were incubated with 10 volumes of detergent diluted in 10 mM sodium phosphate buffer, pH 7 4, at 4° for 15 minutes and centrifuged at 190,000g_{av} for 40 minutes Pellets were resuspended in 10 mM sodium phosphate buffer, pH 7 4, and 40 μg electrophoresed Supernatants were added directly to electrophoresis buffer and processed as described in Methods Solubilization reached 80–90% at 2% (v/v) Ammonyx-LO under these conditions when pellets were measured for protein by the method of Lowry [15] a) Coomassie blue-stained gel, b) autoradiogram A) erythrocyte standard Ammonyx-LO concentration, percent (v/v) B) 0, C) 0 1, D) 0 5, E) 1 0, F) 2 0, G) 5 0, H) 10 0

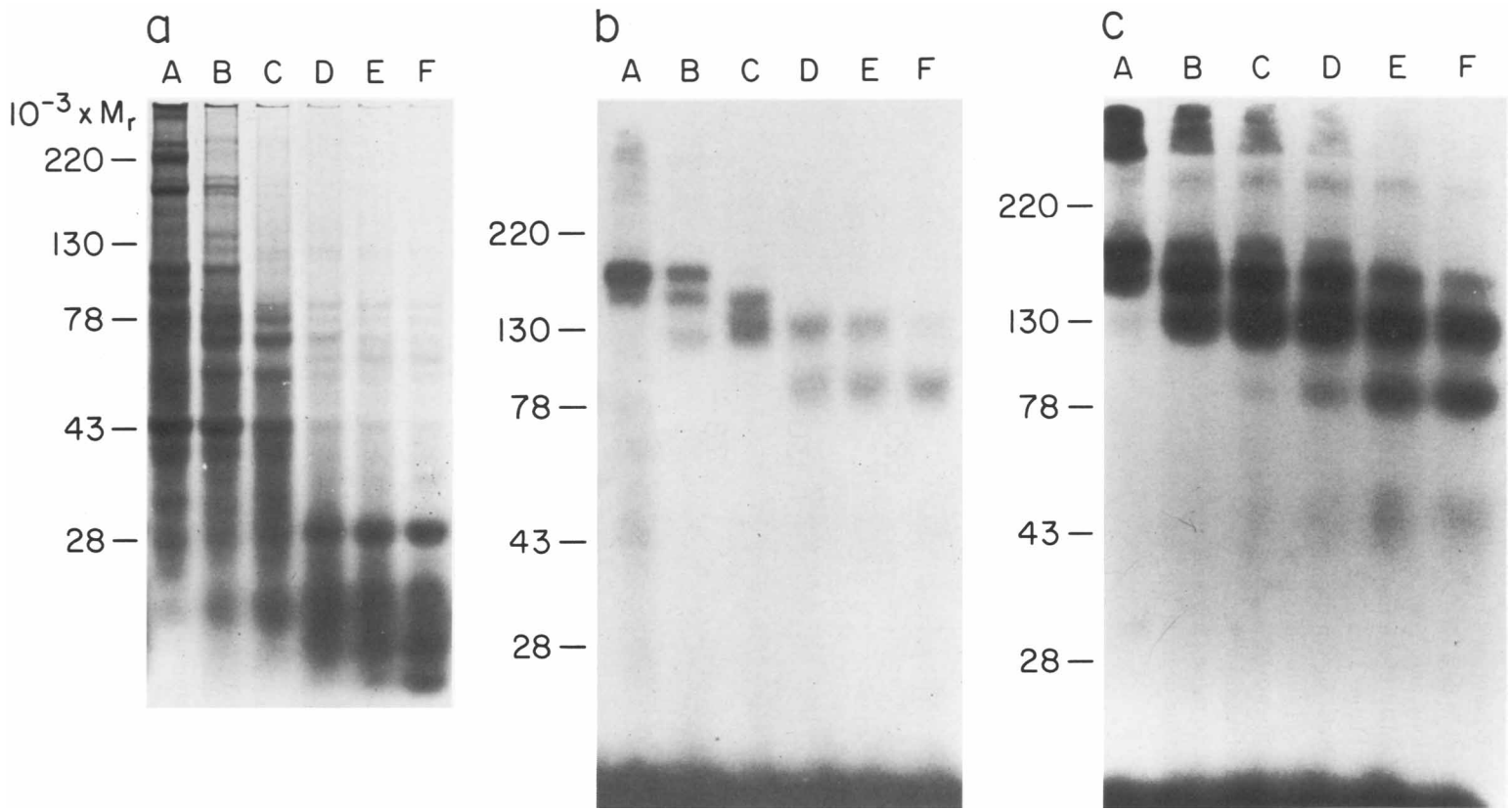

Fig 3 Digestion of labeled EGF receptor in placenta membranes by trypsin and alpha chymotrypsin Seven hundred fifty micrograms of labeled placenta membranes were incubated with enzyme for 15 minutes at 24° in 10 mM sodium phosphate buffer, pH 7 4, containing 2 mM $CaCl_2$ The reaction was terminated with 200 μg per ml phenylmethylsulfonyl fluoride and cooled to 4° Samples were dissolved in electrophoresis buffer and processed as described in Methods using 75 μg protein per lane a) and b) trypsin c) alpha chymotrypsin a) Coomassie blue-stained gel, b) and c) autoradiograms Concentration of enzyme, μg per ml A) 0, B) 20, C) 50, D) 100, E) 250, F) 500

Further degradation is difficult even in the presence of 10 mM–100 mM $CaCl_2$, and controlled proteolysis is hampered by variable degrees of degradation in different experiments. Added proteases fail to degrade further the membranes after as little as 15–30 minutes under these conditions, and repeated additions produce little additional degradation, suggesting that remaining proteins may be shielded in vesicles from further enzymatic attack.

As shown in Figure 3 the EGF receptor is among the more resistant of membrane proteins to proteolysis. Similar degradation is seen with pepsin (Fig. 4) with production of identical fragments on autoradiograms, but we have not observed degradation beyond the 130,000-dalton fragment with elastase (Fig. 4). Thrombin has minimal effects on the membranes. Digestion of membranes with sulfhydryl enzymes papain and bromelain produces fragments identical to those seen with the serine proteases and pepsin but at much lower concentrations, and the degradation proceeds readily beyond 130,000- and 80,000-dalton

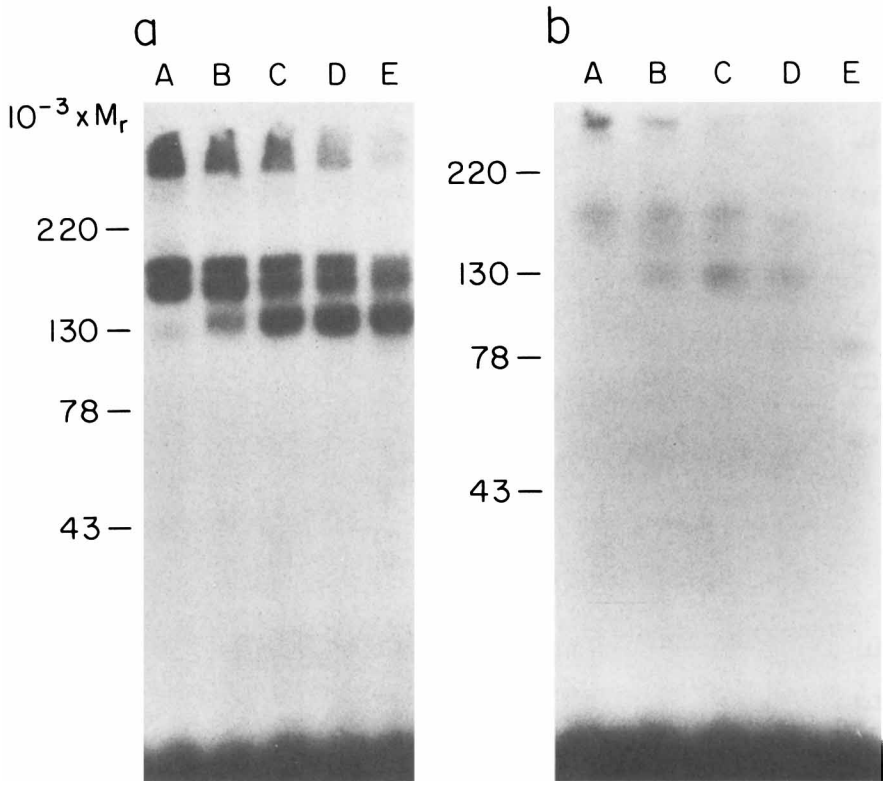

Fig 4. Digestion of labeled EGF receptor in placenta membranes by elastase and pepsin. a) Elastase Five hundred micrograms of placenta membranes labeled with ^{125}I-EGF were treated with elastase in 50 mM sodium phosphate buffer, pH 7 4, for 15 minutes at 24° in 0 125 ml reaction volume The sample was dissolved in electrophoresis buffer and heated, 60 μg of protein electrophoresed, and autoradiograms made as described in Methods. Elastase concentrations, μg per ml A) 0, B) 10, C) 50, D) 200, E) 500 b) Pepsin Labeled membranes were suspended in 1 volume of 0 5 M glycine buffer pH 2 5 and exposed to pepsin for 20 minutes at 24° Samples were added to electrophoresis buffer, heated, and 50 μg electrophoresed Pepsin, μg per ml A) 0, B) 10, C) 50, D) 250, E) 500

fragments to produce fragments of 48,000, 35,000, and 25,000 daltons (Fig. 5). These fragments are not artifacts of the labeling procedure using dimethylsuberimidate since identical results are obtained in spontaneously labeled preparations, although less radioactivity is incorporated, and identical proteolytic fragments are seen whether or not high molecular weight aggregates are produced by dimethylsuberimidate. The ability of proteases of varying specificities to produce identical sequential cleavage fragments indicates that production of these fragments is dependent on the structure of the substrate rather than on the specificity of the protease.

Prior addition of phenylmethylsulfonyl fluoride in 2:1 molar ratio to trypsin and chymotrypsin prevented digestion completely, but addition to membrane suspensions inhibited less effectively. Although this demonstrates that proteolytic action is responsible for the described degradation, digestions were usually terminated by inhibitors and prompt solubilization of membranes in SDS (electrophoresis buffer) with heating as described in the figure legends. Inhibition of pepsin with pepstatin and of sulfhydryl enzymes with iodoacetate was studied with similar results.

Although cleavage of EGF from the receptor cannot be measured accurately because of the high background of labeled, noncovalently bound, ^{125}I-labeled EGF, which migrates in the 6,000-dalton range, sufficient label remains with the fragments to permit their visualization. Bound ^{125}I-labeled EGF therefore appears to be relatively inaccessible to proteases in comparison with the major part of the receptor. Affinity labeling of membranes previously degraded by proteases results in labeling of all fragments seen with proteolysis of the undegraded receptor (data not shown); the resistance of the EGF binding site to proteolysis therefore does not depend on protection by previously bound hormone. For the same reason, the pattern of fragments produced does not depend on the presence of bound EGF but results from intrinsic proteolytic susceptibility of the receptor. The smaller fragments generated by sulfhydryl proteases are occasionally produced by trypsin and chymotrypsin, and a somewhat diffuse band is commonly seen in the 45,000–50,000-dalton range. Such heterogeneity may arise from variations in carbohydrate components of cleaved fragments.

The characteristic degradative fragments of the labeled receptor produced by various proteases could be determined in part by the disposition of various segments of the receptor in the membrane, since portions of the receptor that are not physically buried within the membrane could be more susceptible to enzymatic attack in membrane vesicles. Susceptibility to proteolysis of the solubilized labeled receptor was therefore studied. If labeled membranes are solubilized in 1% (v/v) Ammonyx-LO and centrifuged at 290,000g_{av} for 60 minutes, proteolytic treatment of the supernatant with alpha chymotrypsin yields fragments identical to those produced in intact vesicles (Fig. 6). Cleavage of the 180,000-dalton EGF-receptor complex is seen, however, at 50- to 100-fold lower enzyme concentrations. Transition to the 130,000-dalton fragment is complete with 2 μg per ml in vesicles. Disappearance of label from the region of 130,000 daltons or higher requires 100–200 μg per ml in solubilized preparations under these conditions but requires 200–500 μg per ml in vesicles. The receptor retains some tertiary structure following solubilization in 0.2% SDS, since limited proteolysis produces identical fragments under these conditions as well. Degradation by alpha chymotrypsin of the SDS-solubilized receptor which has been heated at 80° for 2 minutes does not yield the characteristic fragments, but completely degrades all labeled material, indicating that this treatment probably extensively denatures the receptor [13].

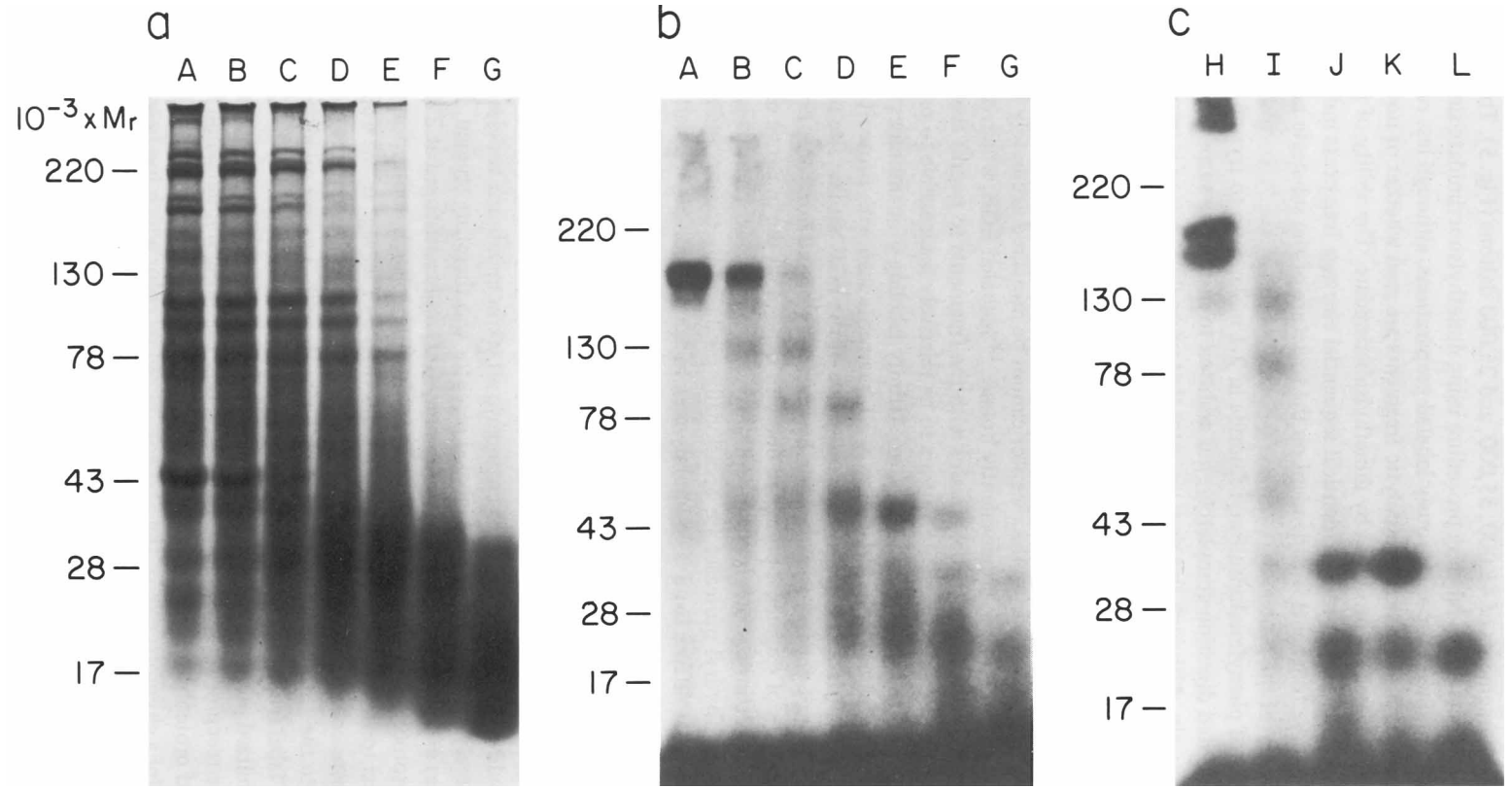

Fig 5 Digestion of labeled EGF receptor in placenta membranes by papain and bromelain Four hundred micrograms of labeled placenta membranes were incubated for 15 minutes at 24° in 0 75 ml buffer with enzyme which had been preincubated for 10 minutes with 1 mM EDTA and 5 mM cysteine in 0 1 M sodium phosphate buffer, pH 7 4 The reaction was terminated with iodoacetimide (2 mM final concentration) and the sample was dissolved in electrophoresis buffer and processed as described in Methods using 80 μg of protein per lane Digestion with papain (a) and (b) or bromelain (c) a) Coomassie blue-stained gel, b) and c) autoradiograms Concentration of enzyme, μg per ml Papain, A) 0, B) 0 01, C) 0 05, D) 0 1, E) 0 2, F) 1, G) 5 Bromelain, H) 0 2, I) 2, J) 20, K) 100, L) 500

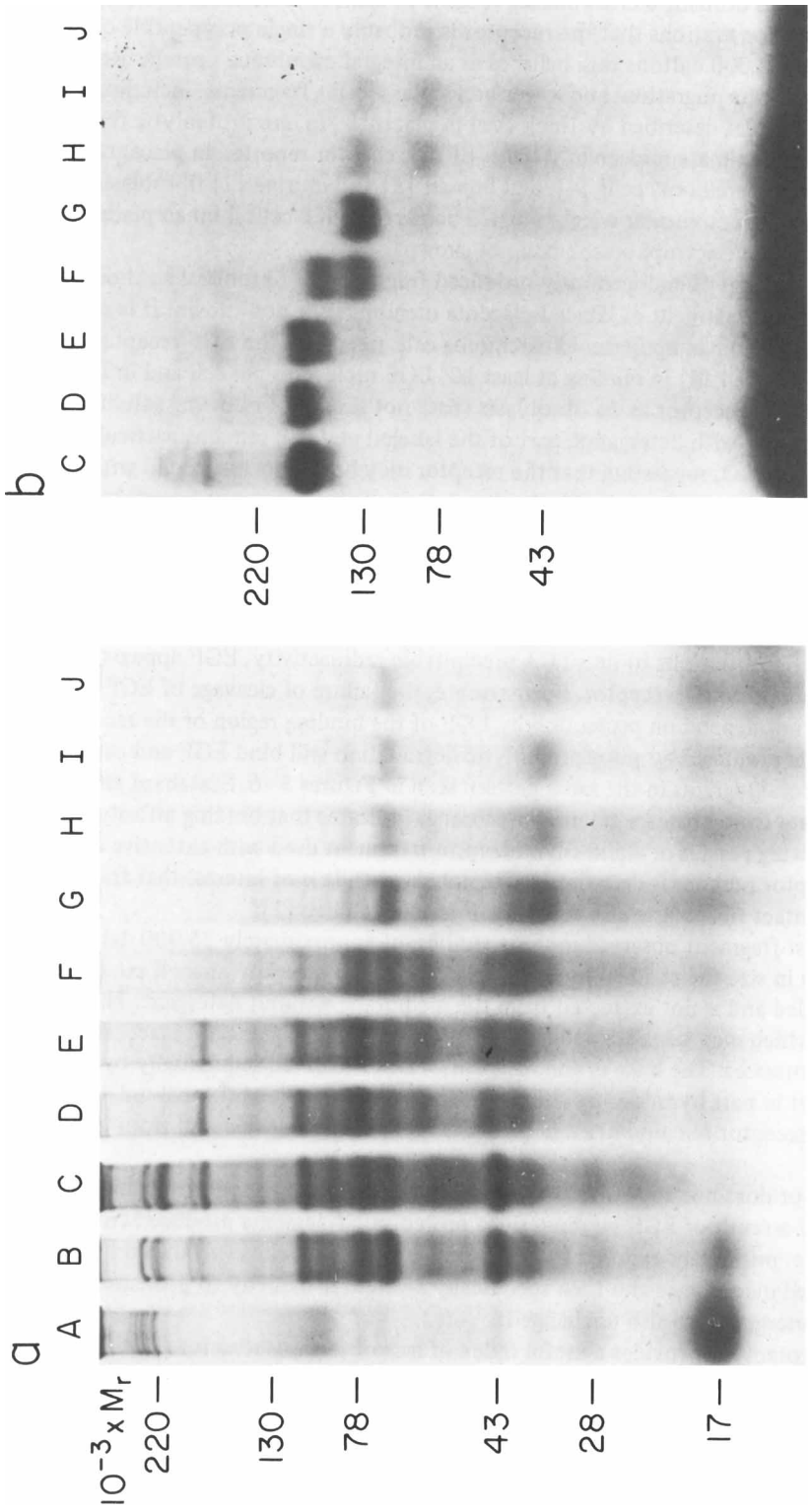

Fig 6 Digestion of solubilized labeled EGF receptor by alpha chymotrypsin Labeled placenta membranes resuspended in 25 mM Tris buffer, pH 7 4, were solubilized in 5 volumes of 2% Ammonyx LO for 20 minutes at 4° and centrifuged at 180,000g_{av} for 60 minutes Samples of supernatant were digested with enzyme for 20 minutes at 24° added to electrophoresis buffer, and heated and electrophoresed as described in Methods a) Coomassie blue stained gel, b) autoradiogram A) erythrocyte standard, B) unlabeled membrane standard, C) control labeled membranes, D–J) supernatant from solubilized labeled membranes Alpha chymotrypsin concentration, μg per ml D) 0, E) 0 1 F) 0 5, G) 2, H) 10, I) 50, J) 100

These studies utilizing a cross-linking reagent to affinity-label the EGF receptor confirm previous demonstrations that the receptor is probably a single polypeptide chain of approximately 180,000 daltons that behaves as an integral membrane protein. Reducing agents do not alter its migration, and lower molecular weight fragments, including the component of the doublet described by Hock et al in placenta [1], are proteolytic fragments. Although the approximate molecular weights of the receptor reported in placenta [1], A-431 epidermoid carcinoma cells [4], and human [3] and murine [2] fibroblasts differ, we find that the high molecular weight labeled bands from KB cells, human placenta, and human fibroblasts coelectrophorese (data not shown).

The relationship of endogenously produced fragments in fibroblasts to those seen following protease treatment of labeled placenta membranes is not known. It is of interest that the KB cell, a human epidermoid carcinoma cell, resembles the EGF receptor "hyperproducing" A-431 cell [4] in binding at least 10^6 EGF molecules per cell and in failing to degrade the labeled receptor as do fibroblasts (data not shown). Following solubilization of the labeled receptor with detergents, part of the labeled material remains particulate even in 10% Ammonyx-LO, suggesting that the receptor may be in part associated with structural or other proteins not released with the dissolution of the membrane by mild detergents. Similar findings are also obtained using either Triton X-100 or Ammonyx-LO with KB cells, in which physiologic significance is more likely than in membrane preparations.

Proteases cleave the receptor, producing several unlabeled fragments, without removing bound ^{125}I-labeled EGF. Since EGF alone is readily degraded by papain and other proteases at low concentrations to non-TCA precipitable radioactivity, EGF appears to be protected when bound to the receptor. Furthermore, the failure of cleavage of EGF from the receptor does not depend on protection by EGF of the binding region of the receptor, since fragments produced by prior proteolytic degradation still bind EGF and can be visualized on autoradiograms in the same pattern seen in Figures 3–6. Scatchard analysis of the binding data from protease-treated membranes indicates that binding affinity is unchanged following papain or alpha-chymotrypsin treatment even with extensive degradation, but receptor number is decreased (data not shown). It is of interest that fragments generated in intact fibroblasts also retain EGF binding ability [2].

The smallest fragment observed in these studies, of approximately 25,000 daltons, may approach in size the residual hydrophobic core of the receptor, since it could not be further degraded and is not extracted from the membrane without detergent. The EGF binding site, which may be preserved on this fragment, may be sterically inaccessible to these larger proteases. The bulk of the receptor, which is cleaved sequentially by proteases, may be at least in part hydrophilic, although the possibility that additional unlabeled segments of the receptor remain buried in phospholipid cannot be evaluated from these studies.

The receptor does not appear to undergo any dramatic conformational change in this preparation as a result of EGF binding, since proteolytic fragments produced from labeled membranes (ie, previously exposed to EGF) are identical with those produced by proteolysis of unlabeled membranes and then specifically labeled. Sensitivity to proteases and extraction by detergents are also unchanged.

Limited proteolysis provides a useful index of loss of tertiary structure of the EGF receptor [13] and may be helpful in defining conditions during solubilization and purification, which are not denaturing. Binding of labeled EGF can also be used for this purpose, but binding affinity is substantially reduced in detergent [14] and requires more rigorous conditions for measurement, such as decreased detergent and salt concentrations, than does limited proteolysis.

ACKNOWLEDGMENTS

These studies were supported by U.S. Public Health Service grant AM24871 to E. J. O'Keefe and by grants from the Dermatology Foundation, the Burroughs Wellcome Co., and the Medical Faculty Grants Committee and University Research Council of the University of North Carolina.

REFERENCES

1. Hock RA, Nexø E, Hollenberg MD: Nature 277:403, 1979.
2. Das M, Fox CF: Proc Natl Acad Sci USA 75:2644, 1978.
3. Baker J, Simmer RL, Glenn KC, Cunningham DC: Nature 278:743, 1979.
4. Wrann MM, Fox CF: J Biol Chem 254:8083, 1979.
5. Linsley PS, Blifeld C, Wrann M, Fox CF: Nature 278:745, 1979.
6. Savage CR Jr, Cohen S: J Biol Chem 247:7609, 1972.
7. Hunter WM, Greenwood FC: Nature 194:495, 1962.
8. Fairbanks G, Steck TL, Wallach DFH: Biochemistry 10:2606, 1971.
9. Davies GE, Stark GR: Proc Natl Acad Sci USA 66:651, 1970.
10. O'Keefe E, Hollenberg MD, Cuatrecasas P: Arch Biochem Biophys 164:518, 1974.
11. Savage CR Jr, Ingami T, Cohen S: J Biol Chem 247:7612, 1972.
12. Gregory H, Preston BM: Int J Peptide Protein Res 9:107, 1977.
13. Tanford C, Reynolds JA: Biochim Biophys Acta 457:133, 1976.
14. Carpenter G: Life Sci 24:1691, 1979.
15. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.