Cross-Linking of Epidermal Growth Factor Receptors in Intact Cells: Detection of Initial Stages of Receptor Clustering and Determination of Molecular Weight of High-Affinity Receptors†

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Received March 26, 1986; Revised Manuscript Received July 8, 1986

ABSTRACT: A method was developed to label epidermal growth factor (EGF) receptors with ^{125}I-EGF in whole cells using chemical cross-linking reagents. Polyacrylamide gel electrophoresis resolved an M_r ~180000 EGF–receptor complex and larger M_r ≥360000 aggregates. The formation of the larger complexes was time and temperature dependent and appeared to represent the initial events of EGF receptor clustering. Alteration of the ratio of ^{125}I-EGF-labeled high- (K_d ~ 0.16 nM) and low- (K_d ~ 1.5 nM) affinity complexes by competition with unlabeled EGF or by induction of additional high-affinity sites with dexamethasone suggested that both sites were represented by the M_r ~180000 ^{125}I-EGF–receptor complexes. Digestion of cells before cross-linking detected a small population of trypsin-resistant M_r ~180000 receptors, which could represent previously described cryptic and/or high-affinity receptors. Few of the M_r ~360000 receptors were trypsin resistant. Glucocorticoid induction of high-affinity EGF receptors failed to induce detectable changes in the microclustering of EGF receptors but did result in a 50% increase in EGF-induced receptor phosphorylation in HeLa S_3 cell membranes at 4 °C. Thus, glucocorticoids increase high-affinity EGF binding sites, EGF-induced receptor phosphorylation, and cell growth.

Epidermal growth factor (EGF)[1] is a 6045-Da polypeptide able to stimulate a cascade of cellular events that culminate in mitosis [for review, see Carpenter & Cohen, 1979)]. The actions of EGF and the related type α transforming growth factors are mediated by a transmembrane receptor (M_r ~ 175000) that has homology with the *erb B* gene product (Carpenter & Cohen, 1979; Carpenter, 1984; Ullrich et al., 1984; Roberts & Sporn, 1986). While the exact nature of the mitogenic signal remains elusive, Schreiber et al. (1983) have reported that antibody-induced clustering of the receptors can substitute for EGF in the stimulation of cellular proliferation, implying that clustering is a required step for mitosis to occur. In addition, Cohen and co-workers (1980) have demonstrated an EGF-stimulated tyrosine kinase activity that is intrinsic to the receptor protein. The physiological substrates for the kinase appear to include the EGF receptor itself and an M_r ~35000 cytoplasmic protein (Cohen et al., 1980; Carpenter, 1984; Cohen & Fava, 1985). This kinase activity is modulated under conditions that alter the growth response in vitro (Buss et al., 1982) and in vivo (Rubin et al., 1982), as is a similar kinase activity in insulin receptors (Grunberger et al., 1984; Kadowaki et al., 1984).

Scatchard analysis (Fanger et al., 1984), double-reciprocal plots (King & Cuatrecasas, 1982), and antireceptor monoclonal antibodies (Kawamoto et al., 1983) have demonstrated the existence of two classes of EGF binding sites on a number of different cell lines. Several lines of evidence suggest that the high-affinity sites, although they comprise a very small fraction of the total receptor population, are responsible for the mitogenic response. Kawamoto et al. (1983) demonstrated that the growth of A431 cells was stimulated by very low (3–100 pM) concentrations of EGF, which would preferentially bind to and stimulate the high-affinity receptors, whereas higher concentrations (3 nM), which would also bind to the much larger number of low-affinity receptors, actually inhibited growth. A similar biphasic growth response was reported by Fernandez-Pol (1985) using monoclonal antibodies that also induced an increase in EGF receptor affinity, kinase activity, and clustering. Gregoriou and Rees (1984) found that antibodies that specifically inhibited binding to the low-affinity receptors did not affect EGF-induced kinase or mitogenic activity. Rees et al. (1984) also determined that high-affinity EGF receptors have restricted lateral diffusion and suggested that high- and low-affinity receptors were responsible for mitosis and clustering, respectively.

The number of high-affinity EGF receptors can be decreased by platelet-derived growth factor (Collins et al., 1983; Davis & Czech, 1985), phorbol esters (Shoyab et al., 1979; Davis & Czech, 1984; Fearn & King, 1985), and diacylglycerol (Sinnett-Smith & Rozengurt, 1985) via stimulation of protein kinase C (Nishizuka, 1984; Fearn & King, 1985), which phosphorylates the EGF receptor on threonine residue 654 (Hunter et al., 1984; Davis & Czech, 1985). In contrast, this laboratory (Fanger et al., 1984) and Baker and Cunningham (1978) have shown that glucocorticoids preferentially increase the number of high-affinity EGF receptors in HeLa S_3 and human fibroblast cells, respectively. EGF and glucocorticoids are the critical factors controlling the growth of HeLa cells

† Supported by NIH Grants AM32458, AM32459, AM32460 (J.A.C.), and AM31683 (H.S.E.). A preliminary report of these data was presented at the 68th FASEB Annual Meeting, St. Louis, MO, 1984. Work performed in partial fulfillment of the requirements for a degree of Doctor of Philosophy (to B.O.F.) from the Department of Biochemistry at the University of Vermont College of Medicine.

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[1] Abbreviations: dexamethasone, 9-fluoro-16α-methyl-11β,17α,21-trihydroxy-1,4-pregnadiene-3,20-dione; DSP, dithiobis(succinimidyl propionate); DSS, disuccinimidyl suberate; EGF, epidermal growth factor; Me_2SO, dimethyl sulfoxide; $NaDodSO_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinebis(ethanesulfonic acid).

0006-2960/86/0425-6414$01.50/0

in serum-free medium (Barnes & Sato, 1980), and the increase in high-affinity receptors may be one mechanism by which the growth-promoting effects of dexamethasone are transduced. The importance of these two hormones in growth is further emphasized by the findings that the receptors for glucocorticoids and EGF are related to the *erb A* and *erb B* oncogenes, respectively (Ullrich et al., 1984; Weinberger et al., 1985).

If the high-affinity EGF receptors do indeed mediate the mitogenic effects, then they should generate the cellular signal(s) responsible for the stimulation of proliferation, and their modulation could similarly influence the cellular response. Therefore, EGF receptors in control and dexamethasone-treated cells have been compared to identify which properties and actions may pertain to the high-affinity population of EGF receptors. The data presented here suggest that neither the molecular weight of affinity cross-linked EGF receptors nor their ability to cluster is affected by glucocorticoids; however, EGF-induced phosphorylation of the receptor is measurably increased. Furthermore, this report demonstrates that cross-linking can be used to detect the initial stages of EGF receptor clustering, which involves the transient formation of M_r ~360 000 complexes.

Experimental Procedures

Reagents. DSS and DSP were purchased from Pierce Chemical Co. (Rockford, IL). High molecular weight standards were obtained from Sigma Chemical Co. (St. Louis, MO), and laminin was provided by Dr. David Woodley (Department of Dermatology, University of North Carolina at Chapel Hill). Tissue culture grade trypsin (0.25%) was obtained from Armour Pharmaceutical Co. (Tarrytown, NY). The purification and radiolabeling of EGF (to 500 μCi/μg), the growth of HeLa S_3 cells, and the preparation and storage of steroids have been described previously (Earp & O'Keefe, 1981; Fanger et al., 1984).

Chemical Cross-Linking. HeLa S_3 cells were grown to confluency in T-25 culture flasks in the presence or absence of dexamethasone as indicated in the figure legends. The binding and covalent attachment of EGF was performed in whole cells at 4 °C in the presence of 4 mM iodoacetic acid to minimize phosphatase activity as well as receptor metabolism and proteolysis (Carpenter & Cohen, 1976; Schlessinger et al., 1978; Haigler et al., 1979; Zidovetzki et al., 1981; Cassel & Glaser, 1982). Approximately 10 fmol of ^{125}I-EGF (1–2 μCi), with or without a ≥100-fold excess of unlabeled EGF, was added to each flask in 1 mL of PBS and allowed to bind for 4 h. Following this incubation, unbound EGF was removed by washing. Cross-linkers were prepared in 50% Me_2SO–PBS and immediately added to cells; after 15 min the reaction was stopped by the addition of 1/5 volume of 500 mM glycine. After a 5-min incubation, cells were washed twice with PBS, solubilized in 500 μL of complete Fairbanks solution (Fairbanks et al., 1971), and scraped into tubes with a rubber policeman. This solution was homogenized with a 1-s burst of a Tekmar ultra turax (Tekmar Co., Cincinnati, OH) and boiled for 3–5 min.

Samples were either stored at −20 °C or separated immediately by $NaDodSO_4$–polyacrylamide gel electrophoresis on 3–10% linear gradient gels. Gels were fixed in 40% methanol/10% acetic acid, stained with 0.1% Commassie Brilliant Blue R in 45% ethanol/10% acetic acid, destained in 10% acetic acid, and dried overnight in a fume hood between sheets of dialysis membrane backing. Autoradiography was performed at −80 °C using XAR-5 film (Eastman Kodak Co., Rochester, NY); autoradiograms were scanned with a GS300 densitometer (Hoefer Scientific Instruments, San Francisco, CA).

Preparation and Phosphorylation of Membrane Ghosts. HeLa S_3 cells (~10^8), grown in T-150 culture flasks in the presence of 10^{-7} M steroid or vehicle as indicated in the figure legends, were detached at 37 °C with 0.2% EDTA in PBS. Membrane ghosts were prepared by the method of Atkinson (1973) using 4 mM iodioacetic acid to inhibit receptor proteolysis. Membranes were suspended in ~200 μL of 20 mM Pipes, and ~20-μL aliquots were used immediately for determinations of protein (Bradford, 1976) or EGF-dependent phosphorylation (Rubin et al., 1982). Membranes (25 μg of protein) were incubated for 10 min at 4 °C in the presence or absence of 1 μg/mL EGF. The reaction, in the presence of 30 mM $MgCl_2$ and 20 mM Pipes (pH 7.0), was initiated by adding 1 μM ATP (5 μCi of [γ-^{32}P]ATP per assay) and stopped by boiling in electrophoresis sample buffer. Samples were separated by $NaDodSO_4$–polyacrylamide gel electrophoresis on 8% gels and autoradiographed. Radioactivity was quantitated by densitometric scanning of the autoradiogram or by removing the bands from the gel, digesting, and counting in a scintillation counter. Phosphatase activity was minimal under the conditions of this assay. Dephosphorylation of ^{32}P-labeled EGF receptors from purified liver membranes, assayed under these conditions, was <10% after 1 min at 4 °C. In HeLa S_3 cells, phosphatase activity at alkaline pH was minimal even at 37 °C.

Results

Molecular Weight of High- and Low-Affinity EGF Receptors. Cross-linking, chromatographic, and sequencing data have determined that the molecular weight of the EGF receptor is 170 000–175 000 (Das et al., 1977; Baker et al., 1979; Hock et al., 1979; Linsley et al., 1979; Miller et al., 1983; Ullrich et al., 1984), with smaller species of EGF receptors produced by proteolytic degradation (Cassel & Glaser, 1982; Cohen et al., 1982). Because of the presence of both high- and low-affinity EGF receptors (King & Cuatrecasas, 1982; Kawamoto et al., 1983; Fanger et al., 1984), and the different molecular weights of the high- and low-affinity nerve growth factor receptors (Hosang & Shooter, 1985), high-affinity EGF receptors were investigated to determine whether they had a different molecular weight from the low-affinity receptors. EGF receptors on intact cells were affinity cross-linked with ^{125}I-EGF using DSS, a bifunctional cross-linking reagent that attaches amino groups within 1.1 nm of each other (Pilch & Czech, 1979). Whole cells were employed to maintain the normal relation of the receptor, lipid bilayer, and submembranous structures, as well as to reduce proteolysis that can occur during the preparation of membranes (Cassel & Glaser, 1982; Cohen et al., 1982). Initially, cells were kept at 4 °C to minimize EGF receptor clustering and internalization (Carpenter & Cohen, 1976; Schlessinger et al., 1978; Haigler et al., 1979; Zidovetzki et al., 1981), and 4 mM iodoacetic acid was present to inhibit EGF receptor proteolysis (Cassel & Glaser, 1982). A 15-min exposure to 1 mM DSS was found to be optimal, and it was also determined that 50% Me_2SO allowed solubilization of hydrophobic cross-linkers at 4 °C while providing better attachment than other solvents tested (data not shown). Cross-linkers were prepared just prior to their use to minimize hydrolysis.

Figure 1 (lane 0) shows that several bands of radioactivity are resolved by $NaDodSO_4$–polyacrylamide gel electrophoresis after affinity cross-linking. The M_r ~60 000 band is detected in the presence of 300 ng/mL unlabeled EGF (Figure 1, lane 300) and thus represents a nonsaturable interaction. The band

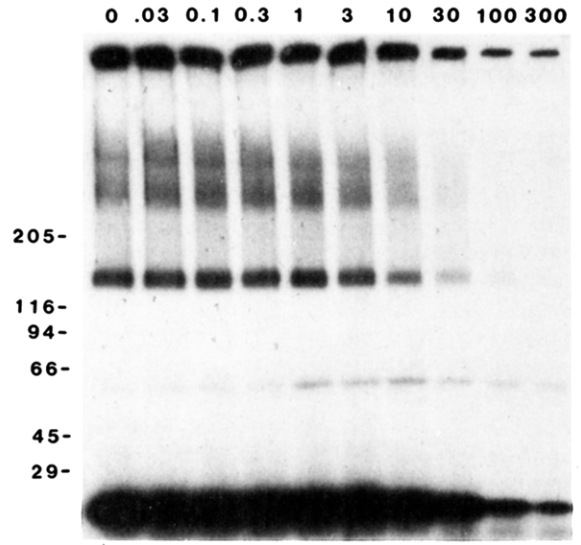

FIGURE 1: Competition of EGF with ^{125}I-EGF–receptor complexes. HeLa S_3 cells were treated for 24 h with 10^{-7} M dexamethasone; then, unlabeled EGF (0–300 ng/mL) was incubated with HeLa S_3 cells for 4 h at 4 °C in PBS. ^{125}I-EGF was added for an additional 2 h, and EGF–receptor complexes were cross-linked, solubilized, separated, and exposed to film. The position of the molecular weight standards ($\times 10^{-3}$) and the concentration of unlabeled EGF (ng/mL) are indicated; this is representative of five different experiments.

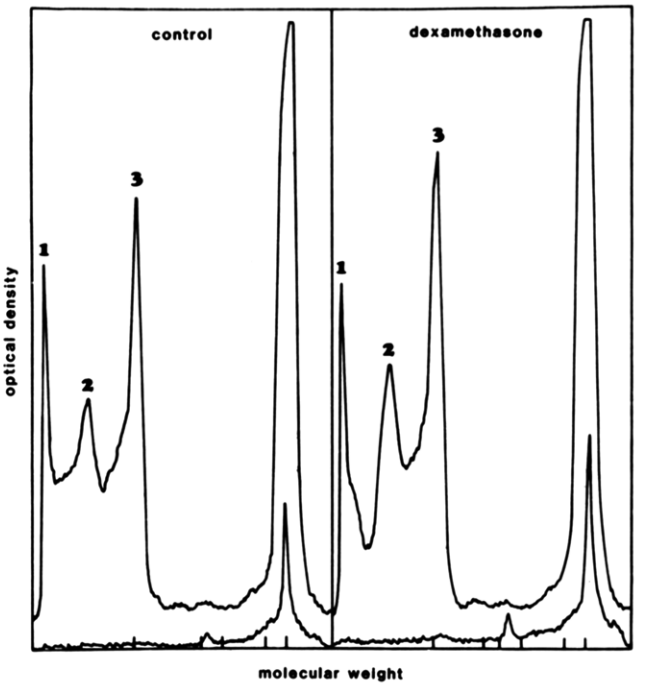

FIGURE 2: Dexamethasone induction of EGF–receptor complexes. HeLa S_3 cells were treated for 24 h with 10^{-6} M dexamethasone or vehicle and cross-linked. A densitometric scan of the exposed film is shown with nonsaturable binding indicated by the lower line. The numbers indicate the M_r ~180 000 (peak 3) and M_r ~360 000 (peak 2) complexes and complexes at the top of the spacer gel (peak 1); the position of the molecular weight standards (as in Figure 1) are indicated on the x axis. This is representative of more than 10 experiments in which dexamethasone-treated and control cells were compared.

at the bottom of the gel comigrates with ^{125}I-EGF and represents ligand that was bound but not covalently attached; its apparent saturability is due to the removal of unbound EGF before cross-linking. The M_r ~180 000 band is not detected in the presence of 300 ng/mL EGF and undoubtedly represents affinity cross-linked EGF receptors. The larger, more diffuse M_r ~360 000 band (in this gel, two bands were resolved), which migrates just below the M_r ~400 000 subunit of laminin, and the very large complexes unable to enter the 3% spacer gel probably represent microclusters of EGF receptors cross-linked together by DSS (see below).

To determine whether any of the saturable bands represented high-affinity EGF receptors, the relative labeling of high- and low-affinity ^{125}I-EGF–receptor complexes was altered by occupation with 0–300 ng/mL unlabeled EGF for 4 h followed by the addition of trace amounts of ^{125}I-EGF for 2 h. The labeling of high-affinity receptors should be reduced as compared to low-affinity receptors due to their preferential occupation during the first 4 h at the lower EGF concentrations and by the reduced dissociation of unlabeled EGF and subsequent occupation by ^{125}I-EGF during the last 2 h. In addition, the number of high-affinity receptors was increased by pretreating cells with 10^{-7} M dexamethasone for 24 h (Fanger et al., 1984). However, there is no visible change in the amount of radioactivity in the bands at concentrations of EGF (0.3–1 ng/mL) that should displace ^{125}I-EGF from high-affinity receptors (Figure 1). This impression was confirmed by scanning the autoradiogram with a densitometer and by removing the areas of the gel that contained these bands and counting them in a γ counter (data not shown). There were also no faint bands detectable on the autoradiogram or its densitometric scan that might represent a minor population of high-affinity receptors, despite its relatively intense exposure. These data suggest that high-affinity receptors do not constitute a unique molecular weight species and are most likely present as minor components in the M_r ~180 000 and/or larger complexes.

This conclusion was tested further by comparing ^{125}I-EGF–receptor complexes obtained from control cells and cells treated for 24 h with dexamethasone, which more than doubles the number of high-affinity receptors, while low-affinity receptors increase only slightly (Fanger et al., 1984). Figure 2 shows that there is only a minor increase in the amount of radioactivity in the saturable bands. These data were confirmed by removing areas of the gel corresponding to radioactive bands and counting them in a γ counter (data not shown). The lack of any new bands in the dexamethasone-treated cells and the small increase in the amount of radioactivity in each band supports the concept that high-affinity EGF receptors are the same size as low-affinity receptors.

Buxser et al. (1983) have reported that high-affinity nerve growth factor receptors are resistant to trypsin digestion, while King and Cuatrecasas (1982) reported that trypsin digestion did not degrade high-affinity EGF receptors, suggesting that the high-affinity receptors are either resistant to trypsin or located in a protected environment (internal compartment). Therefore, HeLa S_3 cells were enriched in high-affinity receptors by a 48-h treatment with 10^{-7} M dexamethasone, digested for 0–50 min with 0.1% trypsin, and cross-linked as before. Figure 3 shows that trypsin produces a progressive reduction in the amount of affinity cross-linked complexes; this was confirmed by removing areas of the gel corresponding to radioactive bands and counting them in a γ counter. These receptors do not represent remnants of incomplete trypsin digestion, since equivalent radioactivity is detected after 50 min of digestion (data not shown). The M_r ~180 000 trypsin-resistant complexes (peak 3) may represent the previously described cryptic or high-affinity EGF receptors (King & Cuatrecasas, 1982). There are very few trypsin-resistant complexes in the M_r ~360 000 band (peak 2).

Cross-Linking of Clustered EGF Receptors. One explanation for the size heterogeneity of ^{125}I-EGF–receptor com-

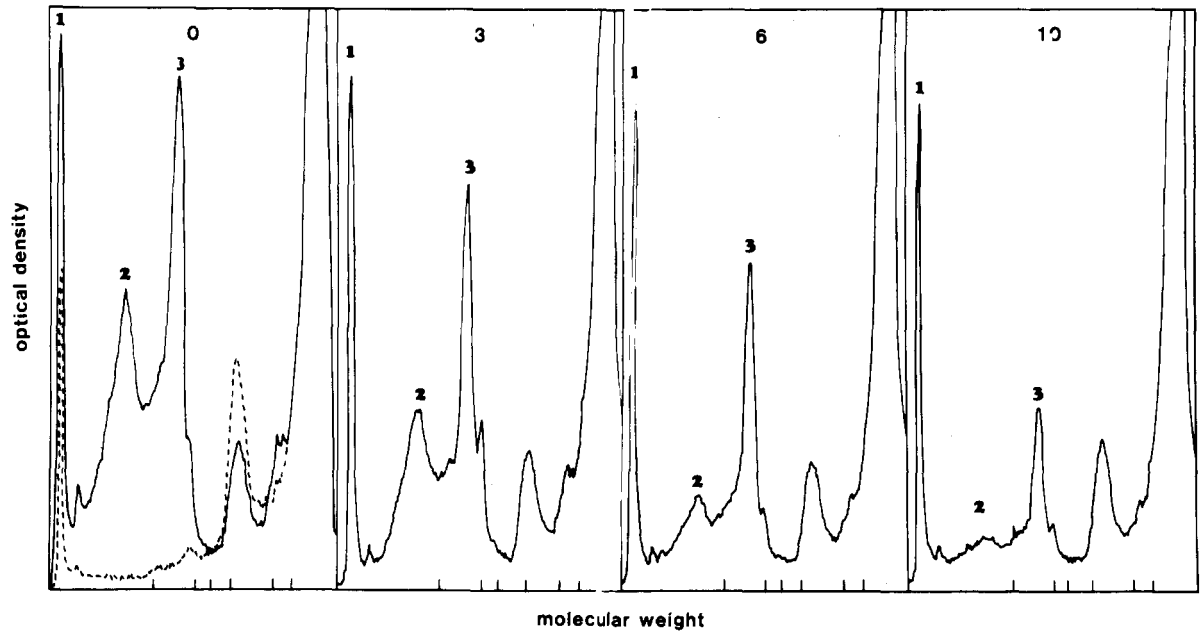

FIGURE 3: Time course of the trypsin digestion of EGF receptors. HeLa S_3 cells were treated for 2 days with 10^{-7} M dexamethasone and digested for 0–50 min at 37 °C with 0.1% trypsin. Cells were washed in 10 volumes ice-cold PBS with 4 mM iodoacetic acid; then, ^{125}I-EGF–receptor complexes were cross-linked, solubilized, separated, and exposed to film. Densitometric scans of the lanes of 0–10-min digestions are shown, with nonsaturable binding indicated by the dashed line. The positions of the complexes and the molecular weight standards are indicated as in Figure 2. This is representative of two different experiments.

plexes was that the larger ones represent clusters of EGF receptors, an idea also suggested by O'Connor-McCourt and Hollenberg (1983). EGF receptor clustering has been reported to be inhibited at 4 °C (Carpenter & Cohen, 1976; Schlessinger et al., 1978; Haigler et al., 1979; Zidovetzki et al., 1981); however, the detection of clusters of EGF receptors could reflect those formed by EGF in culture at 37 °C, before addition of exogenous EGF at 4 °C, or could be formed during the cross-linking procedure, possibly as a result of the heat generated by the addition of Me_2SO to aqueous solvent. To test the latter possibility, the DSS solution was incubated for 15 min at 4 or 25 °C before its addition to cells. The large complexes in peaks 1 and 2 were not detected when DSS precooled to 4 °C was used but are present after cross-linking at 25 °C with both control and dexamethasone-treated cells (data not shown).

These data suggest that the larger complexes represent clusters of EGF receptors that form at temperatures >4 °C. However, the cross-linking reaction itself could be affected by temperature or be affecting the EGF receptors, particularly considering that Me_2SO has been shown to stimulate EGF receptor phosphorylation (Rubin & Earp, 1983). To demonstrate that large complexes could be induced under similar cross-linking conditions, cells were warmed for 0–2 min at 37 °C immediately prior to cross-linking at 4 °C with an ice-cold DSS solution. Figure 4 illustrates the progressive formation of M_r ~360 000 complexes (peak 2), and then the larger receptor complexes (peak 1), at the expense of the smaller complexes (peak 3). The reduction of the M_r ~180 000 complexes, the transient increase in the M_r ~360 000 complexes, and the final increase in the largest complex is typical of a precursor–intermediate–product relationship and suggests that these complexes represent the initial steps in the clustering and internalization of EGF receptors. There was no significant difference in the clustering of EGF receptors in control and dexamethasone-treated cells (data not shown).

To confirm that the larger complexes are indeed composed of the smaller complexes, EGF receptors were first cross-linked

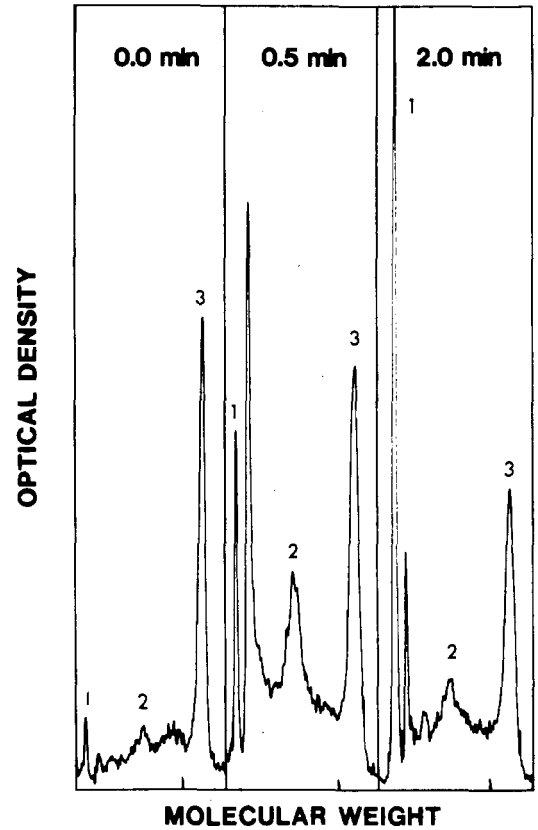

FIGURE 4: Effect of clustering on the size of EGF–receptor complexes. HeLa S_3 cells were treated for 24 h with 10^{-7} M dexamethasone, incubated for 4 h with ^{125}I-EGF in PBS, then incubated for 0–2 min at 37 °C as indicated, cooled, and cross-linked with ice-cold DSS. A densitometric scan of the top half of the exposed film is shown—nonsaturable binding was determined to be insignificant. The positions of the complexes are indicated as in Figure 2; the migration of myosin (M_r ~205 000) is indicated on the x axis. This is representative of five experiments performed on both dexamethasone-treated and control cells.

with precooled DSS at 4 °C to form monomer complexes. The DSS was then washed from the cells at 4 °C, and the larger

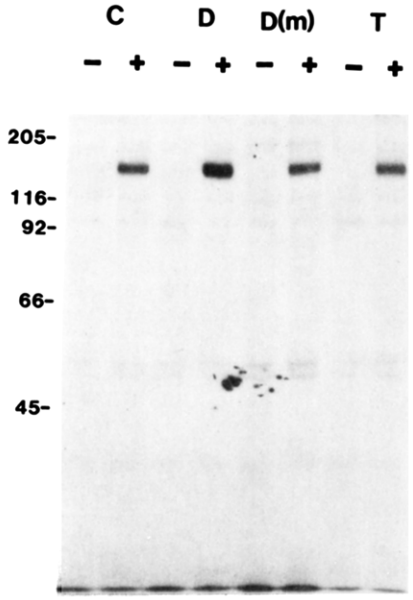

FIGURE 5: Specificity of the EGF-dependent phosphorylation induced by dexamethasone. HeLa S_3 cells were inoculated into T-150 flasks at one third confluency in 50 mL of medium and grown for 24 h followed by an additional 24 h in the presence of 10^{-7} M steroid or vehicle. The cells ($\sim 10^8$) from five flasks were combined and used to prepare membrane ghosts (Atkinson, 1973), which were immediately assayed for protein (Bradford, 1976) and phosphorylated in the absence (−) or presence (+) of 1 μg/mL EGF (Rubin et al., 1982). The glucocorticoid-induced increase detected in this experiment is consistent with that detected by five other determinations. Key: C, control, no steroid added; D, dexamethasone; D(m), 10^{-7} M dexamethasone added to membranes after their preparation from control cells; T, 5α-dihydrotestosterone.

complexes were formed by the addition of prewarmed (37 °C) dithiobis(succinimidyl propionate) (DSP), a compound whose structure is identical with DSS save for the presence of a disulfide bond (Lomant & Fairbanks, 1976). The very large complexes in peak 1 were dissociated into the smaller complexes in peaks 2 and 3 upon reduction (data not shown), demonstrating that the larger complexes are indeed made of monomer ($M_r \sim 180\,000$) EGF–receptor complexes.

EGF–Receptor Phosphorylation. Dexamethasone had little effect on the process of clustering in spite of its ability to induce high-affinity EGF receptors and, presumably, increase the mitogenic signal that they stimulate. Therefore, we examined the effect of dexamethasone on another process believed to mediate the effects of EGF: receptor phosphorylation. These studies used membrane ghosts prepared by the method of Atkinson (1973), which was used because it was developed specifically for HeLa cells, it is relatively fast and thus provides fresh membranes, and it minimizes alterations in the membrane environment as compared to other methods. Membranes were prepared from cells grown in the presence of 10^{-7} M steroid or vehicle and assayed for EGF-induced phosphorylation by incubating with [γ-^{32}P]ATP at 4 °C. The autoradiogram in Figure 5 shows little receptor phosphorylation in the absence of EGF, whereas the presence of EGF dramatically stimulates phosphorylation of the $M_r \sim 175\,000$ EGF receptor. The amount of EGF-induced receptor phosphorylation is enhanced by 50% in membranes prepared from cells treated for 24 h with 10^{-7} M dexamethasone, but not in cells similarly treated with 5α-dihydrotestosterone or in membranes from control cells to which 10^{-7} M dexamethasone was added after their preparation. Neither progesterone nor 17β-estradiol influenced the extent of EGF receptor phosphorylation. In addition, similar results were seen when membranes were solubilized in 0.2% Triton X-100 prior to the initiation of phosphorylation (data not shown). These data indicate that glucocorticoids, which specifically increase the number of high-affinity EGF receptors, also specifically increase the kinase activity of EGF receptors or in some other way influence the extent of phosphorylation of these receptors. It is unlikely that this increase is due to a change in phosphatase activity, since there is little phosphatase activity present under the conditions of the kinase assay (data not shown).

Discussion

This report describes a method to affinity cross-link EGF receptors in intact cells. The advantage of this method is that it allowed analysis of receptor populations under conditions in which the normal submembranous interactions of the receptor's cytoplasmic domain remain intact. This method was used to demonstrate the rapid formation of high molecular weight EGF–receptor complexes that appear to represent the initial stages of receptor clustering. In addition, these studies suggest that high-affinity receptors have the same molecular weight as low-affinity receptors and that glucocorticoids, which preferentially induce high-affinity receptors, increase EGF-dependent receptor phosphorylation.

Previous reports from this laboratory (Fanger et al., 1982) indicated that high-affinity EGF receptors comprise 12% of the total receptor population in dexamethasone-treated HeLa S_3 cells; yet, no unique population of ^{125}I-EGF–receptor complexes was found in cells that corresponded to this population of receptors, as determined by glucocorticoid induction (Figure 2) or displacement by low concentrations of unlabeled EGF (Figure 1). These data suggest that any difference between the size of high- and low-affinity receptors lies within the range of separation by $NaDodSO_4$–polyacrylamide gel electrophoresis, which we estimate to be ≤10% ($M_r \sim 220\,000$ and 240 000 subunits of fibronectin can be resolved).

Although no differences in high- and low-affinity sites were obvious, the interrelationship of the different sizes of EGF–receptor complexes is made clear by the demonstration that the size of the complex is altered under conditions in which receptors are known to cluster (Figure 4; O'Connor-McCourt & Hollenberg, 1983). At least a portion of the monomeric complexes certainly represents EGF receptors in their "native" state. This is supported by the demonstration that only the monomer complexes are formed when the temperature is kept strictly at 4 °C (Figure 4) and that the larger complexes can be dissociated into the smaller ones (data not shown). Further evidence comes from the detection of trypsin-resistant monomer complexes (Figure 3) that may represent newly exposed receptors (King & Cuatrecasas, 1982).

The data in Figure 4 show the progressive formation of large aggregates, indicating a relocation of EGF receptors from small to intermediate to large complexes. It is unlikely that the $M_r \sim 360\,000$ complexes result only from incomplete attachment of larger receptor aggregates. First, relatively extensive cross-linking is performed (15 min at 1 mM), due to the presence of only a single primary amino group (the N-terminus) on murine EGF (Carpenter & Cohen, 1979). For comparison, transforming growth factor type β, which has 16 lysine residues (Derynck et al., 1985), will become covalently attached to the binding subunit of its receptor after only 2 min under similar conditions, and after 15 min significant attachment of receptor subunits occurs (Fanger et al., 1986). The EGF receptor also contains multiple lysine residues (Ullrich et al., 1984) and should be readily attached to an associated protein. Second, the formation of the $M_r \sim 360\,000$ complexes is rapid and transient (Figure 4). It is unlikely that

a complex resulting from incomplete attachment would follow such a pattern, unless receptors moved from an initial state of "loose" clustering to a subsequent "tight" association. In this case, the transient formation of M_r ~360 000 complexes would still reflect a unique stage of EGF receptor aggregation.

It should be noted that DSS can diffuse through the plasma membrane; thus, the formation of high molecular weight complexes could be due to associations with the intracellular as well as the extracellular proteins involved in receptor clustering, or intracellular substrates of the receptor kinase. The M_r ~360 000 complexes could, therefore, represent a unique population of receptor dimers with an important function such as phosphorylation and/or signal transduction. These complexes could also be a short-lived "seed" for the formation of larger receptor clusters or could be EGF receptors associated with a distinct protein. It is worth noting that examination of EGF receptor phosphorylation in membranes also revealed the existence of high molecular weight species. Under nonreducing conditions, a portion of the EGF-induced tyrosine-specific phosphorylation migrated as an M_r ~340 000 band or higher. When this form was eluted from the gel, reduced, and separated by electrophoresis again, the monomeric M_r ~175 000 form of the receptor was present. The proportion of oligomers of the receptor varies depending on the cell type or tissue, ranging from 20 to 50%.[2] This suggests that the M_r ~360 000 complexes may play a role in receptor phosphorylation. Interestingly, Biswas et al. (1985) also detected two sizes of solubilized EGF receptors using sedimentation analysis: a 7.7S (M_r ~170 000) form with EGF-independent kinase activity and a 12S (M_r ~340 000) form without kinase activity that can be converted to the 7.7S form by EGF.

A number of techniques have been used to detect large clusters of EGF receptors, including electron microscopy of ferritin-conjugated EGF (Haigler et al., 1979), light microscopy of fluorescently labeled EGF (Schlessinger et al., 1978), and formation of cross-linked aggregates (O'Connor-McCourt & Hollenberg, 1983). Zidovetzki et al. (1981) suggested that clustering was initiated by the formation of microclusters of 10–50 EGF receptors. The data presented here demonstrate that cross-linking can be used to detect even earlier stages of EGF clustering, which involves the rapid and transient formation of receptor dimers or an association with a similarly sized protein.

If high-affinity EGF receptors are responsible for the generation of the mitogenic signal, then the dexamethasone-induced increase in high-affinity EGF receptors should be paralleled by a similar increase in the mitogenic signal. Yet, no such increase is detected in the number of M_r ~360 000 complexes or the rate of clustering. One explanation is that the mitogenic signal is not generated by the clustering of high-affinity EGF receptors. Rees et al. (1984) have also reported evidence that high-affinity EGF receptors generate their signal without clustering. Other possibilities include that the effect was not detected by this technique, possibly because it involves the larger aggregates or it is masked by the larger number of low-affinity EGF receptors.

In contrast to the inability of dexamethasone to influence receptor clustering, an increase in EGF-induced phosphorylation of its receptor was observed in glucocorticoid-treated cells. This increase in phosphorylation is consistent with the fact that high-affinity receptors are more active kinases (or better substrates). It is also possible that the EGF receptor kinase activity is better preserved or in an activated state in membranes from dexamethasone-treated cells or that the EGF receptor in glucocorticoid-treated membranes is recovered in a state of reduced phosphorylation as compared to those in control membranes and therefore incorporate more ^{32}P during the assay. The latter possibility is unlikely, because membranes were prepared and assayed under conditions that inhibit phosphatase activity.

The concurrent dexamethasone-induced increase in high-affinity receptors and phosphorylation shown here is consistent with studies by Gregoriou and Rees (1984) and Rees et al. (1984), who produced antibodies that acted only upon the low-affinity population of EGF receptors. They demonstrated antibody-induced clustering of EGF receptors without affecting mitogenic or kinase activity and suggested that the high- and low-affinity receptors have separate functions of growth and down-regulation, respectively. Furthermore, Das et al. (1984) and Fernandez-Pol (1985) have also reported that antibodies were able to simultaneously increase receptor affinity and kinase activity. Taken together with the studies of Kawamoto et al. (1983), which correlate high-affinity receptors and the mitogenic response, these data suggest a connection between high-affinity EGF receptors, EGF receptor kinase activity, and the stimulation of the mitogenic response. In addition, the data presented here indicate that glucocorticoids regulate these aspects of EGF action.

[2] R. A. Rubin and H. S. Earp, unpublished observations.

ACKNOWLEDGMENTS

We thank Drs. Lalage M. Wakefield and Maureen O'-Connor-McCourt for critical reading of the manuscript.

Registry No. EGF, 62229-50-9; dexamethasone, 50-02-2.

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