Preparation and Characterization of a Bifunctionally Spin-Labeled Mutant of Murine Epidermal Growth Factor for Saturation-Transfer Electron Paramagnetic Resonance Studies of the Growth Factor/Receptor Complex†

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ABSTRACT: In this report we describe the production of a [Lys3,Tyr22] murine epidermal growth factor (mEGF) mutant for spin-labeling with bis(sulfo-*N*-succinimidyl)-[^{15}N,$^{2}H_{16}$]doxyl-2-spiro-4′-pimelate ([^{15}N,$^{2}H_{16}$]BSSDP) in order to study the rotational dynamics of the EGF/EGF receptor complex by saturation-transfer electron paramagnetic resonance (ST-EPR). Previous results [Faulkner-O'Brien et al. (1991) *Biochemistry 30*, 8976–8985] indicated that the reaction of [^{15}N,$^{2}H_{16}$]BSSDP with wild-type mEGF did not yield a product useful for ST-EPR studies of the EGF/EGF receptor complex because the major product, in which [^{15}N,$^{2}H_{16}$]BSSDP was attached only at the amino terminus of mEGF, lacked rigid motional coupling of the spin probe to the protein, and the more tightly coupled bidentate product was unstable. Using oligonucleotide-mediated site-directed mutagenesis of a synthetic gene for mEGF, we replaced Tyr3 with Lys and His22 with Tyr in wild-type mEGF to produce a mutant mEGF suitable for [^{15}N,$^{2}H_{16}$]BSSDP labeling. The [Lys3,Tyr22] mEGF was expressed in *Escherichia coli* HB101 transformed with a pIN-III-ompA3-[Lys3,Tyr22] mEGF plasmid and was purified from the bacterial periplasm using a simple two step purification method. The [^{15}N,$^{2}H_{16}$]BSSDP reacted with [Lys3,Tyr22]mEGF in high yield, and EPR analysis of the major product revealed tight motional coupling between the spin probe and the protein. Biological activity, as assessed by stimulation of EGF receptor autophosphorylation and dimerization, was not affected by either the mutations or the addition of the spin label. The [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was shown to be equipotent with mEGF in EGF receptor competition binding assays using A431 cells; in EPR studies, mEGF also was shown to specifically block [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF binding to the EGF receptor in A431 membrane vesicles. Using the [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF, we now report the first measurement of the rotational dynamics of the EGF/EGF receptor complex in A431 membrane vesicles by ST-EPR.

Epidermal growth factor (EGF)[1] is a small (6040 MW) polypeptide hormone (Cohen, 1962) that can modulate cell growth and differentiation by binding a specific membrane receptor [for reviews, see Carpenter and Cohen (1990), Carpenter and Wahl (1990), and Staros et al. (1989)]. The receptor for epidermal growth factor is a 170-kDa transmembrane glycoprotein consisting of a single polypeptide chain of 1186 amino acids which can be divided into three domains: an extracellular ligand-binding domain, a single hydrophobic transmembrane segment, and an intracellular domain which includes a tyrosyl-residue specific protein kinase (Ushiro & Cohen, 1980; Buhrow et al., 1982, 1983; Ullrich et al., 1984). Binding of EGF to its receptor stimulates the receptor kinase (Carpenter et al., 1979) resulting in receptor autophosphorylation (Cohen et al., 1982; Buhrow et al., 1982) and phosphorylation of several cellular substrates [reviewed in Carpenter and Wahl (1990) and Ullrich and Schlessinger (1990)]. Kinase activity of the receptor is required for EGF to exert its mitogenic effects (Chen et al., 1987; Honegger et al., 1987; Moolenaar et al., 1988). EGF binding also stimulates receptor oligomerization in intact cells, membrane preparations, and solubilized receptor preparations [for reviews, see Ullrich and Schlessinger (1990) and Carpenter and Wahl (1990)]. There is a large body of evidence that suggests that

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[1] Abbreviations: BSA, bovine serum albumin; BS^3, bis(sulfo-*N*-succinimidyl)suberate; BSSDP, bis(sulfo-*N*-succinimidyl)doxyl-2-spiro-4′-pimelate; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; EGF, epidermal growth factor; mEGF, murine epidermal growth factor; EPR, electron paramagnetic resonance; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; IPTG, isopropyl thio-β-D-galactoside; LB, Luria broth; LD-TOF, laser desorption time of flight; OAc, acetate; MSL, *N*-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl)maleimide; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PTH, phenylthiohydantoin; ST-EPR, saturation-transfer electron paramagnetic resonance; TEA, triethylamine; TFA, trifluoroacetic acid; V_1, first-harmonic, in-phase, absorption EPR signal; V_2', second-harmonic out-of-phase absorption EPR signal.

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ligand-induced receptor oligomerization is an essential part of the mechanism through which the EGF receptor kinase is activated (Yarden & Schlessinger, 1987; Carraway et al., 1989; Canals, 1992); however, contrary evidence has also been presented (Biswas et al., 1985; Koland & Cerione, 1988). The exact mechanism linking EGF binding and receptor kinase activation remains unclear.

There have been many applications of structural and morphological techniques to study the interactions of EGF with its receptor. Since modification of the single primary amino group at the amino terminus of murine EGF (mEGF) does not significantly affect biological activity [reviewed in Carpenter and Wahl (1990)], many reporter molecules have been covalently attached to this protein to study the interactions and dynamics of the hormone/receptor complex. A ferritin–mEGF conjugate has been used for electron microscopic studies of mEGF/EGF receptor clustering (Haigler et al., 1979; McKanna et al., 1979). Fluorescent mEGF conjugates have been used to study receptor microaggregation in cells and isolated plasma membranes (Schlessinger et al., 1978; Haigler et al., 1978; Carraway et al., 1989; Azevedo & Johnson, 1990), and phosphorescent mEGF conjugates have been used to study the rotational dynamics of the EGF/EGF receptor complex in isolated membranes and in intact cells (Zidovetsky et al., 1981, 1986, 1991). We previously reported development of a spin-labeled mEGF conjugate that was utilized in an EPR study of the kinetics of mEGF dissociation from solubilized EGF receptor (Faulkner-O'Brien et al., 1991).

In order to use EPR spectroscopy to study the rotational dynamics of proteins, an EPR probe, or spin label, has to be rigidly linked to the protein so that the motion of the spin label accurately reflects the motion of the protein. One method available to help ensure tight motional coupling between a spin label and a protein is to utilize a bifunctional spin label which covalently attaches to the protein in two places (Beth et al., 1986; Anjaneyulu et al., 1988). In the case of the EGF/EGF receptor system, previous spin-labeling studies using wild-type mEGF (Faulkner-O'Brien et al., 1991) showed that the bifunctional spin label bis(sulfo-*N*-succinimidyl)-[^{15}N,^{2}H$_{16}$]doxyl-2-spiro-4′-pimelate, or [^{15}N,^{2}H$_{16}$]BSSDP (Anjaneyulu et al., 1988), primarily forms a single stable covalent linkage at the amino terminus of mEGF. The solution structure of mEGF (Montelione et al., 1987, 1992) revealed two amino acyl residues, His22 and Tyr3, that appear to be within the span of the second reactive end of the reagent and could be potential targets for intramolecular cross-linking. Though Tyr3 appeared to react with the second end of the reagent, the reaction product proved to be unstable (Faulkner-O'Brien et al., 1991). The presence of the histidinyl residue is problematic since imidazole side chains react with the sulfo-*N*-sucinimidyl esters to form unstable covalent adducts (Anjaneyulu & Staros, 1987). Therefore, neither potential target forms a stable second covalent bond with the reagent (Faulkner-O'Brien et al., 1991). While the mEGF conjugate spin-labeled at the amino terminus proved useful for kinetic studies, its lack of a tight motional coupling between the spin label and the protein made this conjugate unsuitable for EPR studies of the rotational dynamics of the EGF/EGF receptor complex.

In an effort to produce a spin-labeled mEGF conjugate useful for EPR studies of the EGF/EGF receptor complex, we designed a mutant mEGF protein which would react in high yield with both ends of [^{15}N,^{2}H$_{16}$]BSSDP to form a spin-labeled mEGF conjugate in which the motion of the spin label was tightly coupled to that of the protein. On the basis of studies of the reactivities of sulfo-*N*-succinimidyl esters (Anjaneyulu & Staros, 1987), studies of the reactive groups of [^{15}N,^{2}H$_{16}$]BSSDP, and previous labeling results (Faulkner-O'Brien et al., 1991), we mutated the Tyr3 residue of mEGF to a Lys and the His22 residue to a Tyr. The first mutation places a second primary amino group in a position to react with the second end of [^{15}N,^{2}H$_{16}$]BSSDP; the second mutation removes the His residue, which should only form a transient covalent bond with the second end of the labeling reagent (Anjaneyulu & Staros, 1987). Mutation of Tyr3 to Lys, while not a conservative change, was not expected to alter the binding properties or biological activity of mEGF, since residues near the amino terminus of EGF do not appear to be necessary in EGF/EGF receptor interactions [reviewed in Carpenter and Wahl (1990)]. Also, alignment of known EGF sequences (Staros et al., 1989) showed four different residues at this position in four different species. Mutation of the His22 of mEGF to Tyr is a conservative change, as the residue at position 22 in human EGF (Gregory, 1975) and rat EGF (Simpson et al., 1985) is Tyr.

In this paper we describe the preparation and characterization of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. Using oligonucleotide-mediated site-directed mutagenesis of a synthetic gene for mEGF (Ray et al., 1988), we constructed the DNA encoding [Lys3,Tyr22]mEGF which was then expressed in *Escherichia coli*. The protein product was purified to homogeneity and shown to be equipotent with wild-type mEGF in several assays of biological activity. Reaction of purified [Lys3,Tyr22]mEGF with [^{15}N,^{2}H$_{16}$]BSSDP produced a single dominant product in which the motion of the spin label was tightly coupled to that of the protein. Labeling yields in this reaction were improved greater than 10-fold as compared to previous results using wild-type mEGF. [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was shown to be equipotent with wild-type mEGF in competition binding assays, EGF receptor dimerization assays, and EGF receptor autophosphorylation assays, and this spin-labeled derivative was shown by EPR to be highly specific in binding to the EGF receptor. Using [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]-mEGF specifically bound to EGF receptors in membrane vesicles from A431 cells, we report the first use of ST-EPR to observe the rotational dynamics of the occupied receptor complex in plasma membranes.

MATERIALS AND METHODS

Murine EGF (mEGF) was prepared as previously described (Savage & Cohen, 1972); ^{125}I-mEGF was prepared by the method of Carpenter and Cohen (1976). [^{15}N,^{2}H$_{16}$]BSSDP was synthesized as described in Anjaneyulu et al. (1988). [γ-^{32}P]ATP (6000 Ci/mmol for oligonucleotide labeling, 3000 Ci/mmol for EGF receptor kinase assays) and [α-^{35}S]dATP (500 Ci/mmol) were from NEN. HPLC-grade acetonitrile was from Burdick and Jackson. TFA (HPLC grade) and TEA (Sequanal grade) were from Pierce.

Bacterial Strains and Plasmid. *E. coli* TG1 was used for all phage experiments. *E. coli* JM101 was used for plasmid propagation, and *E. coli* HB101 was used for all expression experiments. Construction of the pIN-III-ompA3-mEGF plasmid was described in Ray et al. (1988).

Oligonucleotide-Mediated Site-Directed Mutagenesis of mEGF. The synthetic oligonucleotides 5′-CAGGCCAACTC-CAAGCCCGGATGTCCATCG-3′ and 5′-GGTGTTTG-TATGTACATCGAATCT-3′, purified by polyacrylamide gel electrophoresis (1.5-mm-thick 15% gel) under denaturing conditions (Sambrook et al., 1989), were used for mutating

Tyr3 to Lys and His22 to Tyr, respectively. Oligonucleotide-directed mutagenesis on the *Xba*I/*Bam*HI fragment of pIN-III-ompA3-mEGF subcloned into M13mp18 was performed simultaneously for both sites using the method of Zoller and Smith (1987). The presence of the double mutation in M13mp18 single-stranded DNA preparations was verified by dideoxy DNA sequencing using [α-^{35}S]dATP and Sequenase (U.S. Biochemical Corp. protocol). The doubly-mutated ompA–mEGF fusion gene was excised by *Xba*I/*Bam*HI digestion of the mutated M13mp18 replicative DNA forms (Sambrook et al. 1989) and was religated into the *Xba*I/*Bam*HI-digested and purified pIN-III-ompA3 expression vector. Purified plasmid was subjected to double-stranded sequencing to determine that the correct mutant sequences were present and had been properly ligated into the vector.

Expression and Purification of [Lys3,Tyr22]mEGF from E. coli HB101. *E. coli* HB101, transformed with pIN-III-ompA3-[Lys3,Tyr22]mEGF, were grown in 6-L shaker flasks in 2-L cultures of LB medium with 100 μg/mL ampicillin at 30 °C. When cells reached an OD_{600} of 0.7, protein expression was induced by the addition of 400 μM IPTG (final concentration); induction continued for another 4–5 h at 30 °C. During induction, the cell density usually reached an OD_{600} of ~1.6. After the induction period, the bacteria were pelleted by centrifugation at 10 000 rpm for 5 min at 4 °C in a GSA rotor (Dupont, Sorvall). The bacteria were then subjected to osmotic shock [according to Ray et al. (1988)] by resuspending the pellets in ice-cold 10 mM Tris, pH 7.4, and 16 mM EDTA buffer ($^1/_{10}$ the volume of the original culture). Following incubation on ice for 15 min, the bacteria were repelleted by centrifugation at 13 000 rpm for 25 min at 4 °C in a GSA rotor, and the supernate was collected. The presence of the [Lys3,Tyr22]mEGF in the supernate initially was monitored by tricine $NaDodSO_4$–PAGE (Schägger & von Jagow, 1987) using 16% separating gels and western blotting using polyclonal antibody directed against mEGF.

A two-step purification was developed, based on the method described in Ray et al. (1988). First, acetonitrile (AcCN) and triethylamine (TEA) were added to the supernate to 5% and 0.1%, respectively, and the pH was adjusted to 6 with glacial acetic acid. The supernate was loaded onto a 5- × 7-cm LiChroprep RP-C8 column equilibrated with 95:5 water/AcCN buffered with 0.1% TEA/acetate, pH 6.0, and the column was washed with 5 column volumes of the equilibration buffer. The fraction containing the [Lys3,Tyr22]mEGF was then eluted with 3 column volumes of 50:50 water/AcCN buffered with 0.1% TEA/acetate, pH 6.0. This fraction was lyophilized and redissolved in 50 mM HEPES (pH 7.4), and 100 mM NaCl buffer (5 mL of buffer for every 200 mL of supernate at the start of purification). Insoluble matter was removed by centrifugation at 38 000 rpm for 30 min at 4 °C in a Ti-75 rotor (Beckman). This crude fraction was then subjected to a second purification step of semipreparative reversed-phase HPLC (RP-HPLC) using a Dynamax-300A 1- × 25-cm C8 column (5 μm) and 0.1% TFA in water (buffer A), and 0.1% TFA in 80:20 AcCN/water (buffer B) as mobile phases. The solvent delivery system consisted of two Waters 510 pumps, and absorbance was monitored at 220 and 280 nm by a Waters 490e programmable multiwavelength detector. The system was controlled from a Maxima 820 workstation. For each HPLC run, the column was preequilibrated at 95:5 buffer A/buffer B and loaded with 5 mL of the Lichroprep-purified fraction. The column was developed with a 70-min linear gradient of 5%–50% B at a flow rate of 4 mL/min. In the initial purification, the peak which corresponded to [Lys3,Tyr22]mEGF was identified by dot-blotting all peak fractions directly onto a nitrocellulose filter and assaying for mEGF reactivity by immunostaining with an anti-mEGF polyclonal antiserum. In subsequent purifications, the peak containing the [Lys3,Tyr22]mEGF was hand-collected and lyophilized. Final purity of the [Lys3,Tyr22]mEGF was determined by analytical RP-HPLC. Samples were injected onto a 4.6- × 220-mm Brownlee Aquapore RP-300 C-8 cartridge equilibrated with 95:5 buffer A/buffer B and were eluted with a linear gradient of 5%–50% buffer B over 70 min at a flow rate of 1 mL/min. Purified [Lys3,Tyr22]mEGF was stored in 20 mM HEPES, pH 7.4, at –20 °C.

The purified [Lys3,Tyr22]mEGF was quantitated spectrophotometrically by absorbance at 280 nm and analyzed by mass spectrometry. Matrix-assisted laser desorption time-of-flight (LD-TOF) mass spectra (Karas & Hillenkamp, 1988) were acquired with a Vestec VT2000 LD-TOF mass spectrometer (Vestec Corp., Houston, TX), operating at 30 kV accelerating voltage and equipped with a N_2 UV laser (LSI Inc., Cambridge, MA). For each sample, the LD-TOF mass spectrum was obtained using a matrix of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (Beavis & Chait, 1989). Sample was ionized by 337-nm irradiation, with 8-ns laser pulses at a repetition rate of 5 Hz. Each spectrum represents the sum of 40 laser shots (Juhasz et al., 1992). The presence of the mutated amino acyl residues was determined by protein sequencing by the general method of Hewick et al. (1981) following reduction and carboxamidomethylation of the protein (Stone et al., 1989).

Preparation and Purification of [^{15}N,$^2H_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF. [Lys3,Tyr22]mEGF was lyophilized and redissolved in 100 mM HEPES, pH 7.6, for a final concentration of 1 μg/μL. This solution was then added directly to dry [^{15}N,$^2H_{16}$]BSSDP to give a 15-fold molar excess of spin label, and the reaction was allowed to proceed for 4 h at room temperature. Products were separated by RP-HPLC using a 4.6- × 220-mm Brownlee Aquapore RP-300 C-8 cartridge with 20 mM TEA/acetate, pH 5.5 (buffer C) and 100% AcCN (buffer D) as mobile phases. The reaction mix was loaded onto the column which had been equilibrated at 90:10 buffer C/buffer D. Products were eluted with a 10%–30% linear gradient of increasing buffer D over 90 min at a 1 mL/min flow rate; product peaks were detected by monitoring 220- and 280-nm absorbance. Product peaks were collected, lyophilized, and reinjected using the same method. The purified products were then assayed for spin-label incorporation by EPR. The desired product was quantitated by 280-nm absorbance using the molar extinction coefficient of 18 700 cm^{-1} M^{-1} for wild-type mEGF (Taylor et al., 1972), lyophilized, and rehydrated in 20 mM HEPES, pH 7.4, to a final concentration of 1 μg/μL. This product was then aliquoted and stored under argon at –70 °C until use. A sample of this product was also analyzed by mass spectrometry.

Biological Characterization of [Lys3,Tyr22]mEGF and [^{15}N,$^2H_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF. The ability of [^{15}N,$^2H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF to compete with ^{125}I-mEGF for EGF receptor binding relative to unlabeled mEGF was determined in competition binding assays based on established binding assay methods (Carpenter, 1985). A431 cells, grown to confluence in 12-well plates (Costar), were washed twice with Hanks' balanced salt solution (Sigma). After the second wash, 0.5 mL of binding medium (Dulbecco's modified Eagle's medium supplemented with 0.2% BSA) was then added to each well, and the plates were cooled on ice for 25 min. Each data point determination used four

wells; three wells to determine the degree of competition and one well for nonspecific binding correction. Nonspecific binding was determined by preblocking binding sites with 200-fold excess unlabeled mEGF. At the start of the assay, 0, 12.5, 25, 50, 75, or 100 ng of mEGF was mixed with 50 ng of ^{125}I-mEGF in four wells. In a parallel set of wells, 0, 12.5, 25, 50, 75, or 100 ng of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was mixed with 50 ng of ^{125}I-mEGF. Plates were incubated 2 h at 4 °C, and then the wells were washed three times with 1 mL of ice-cold Hanks' balanced salt solution supplemented with 0.2% BSA. After the final wash, cells were solubilized by the addition of 1 mL of 1 N NaOH and incubation at 37 °C for 1 h. Contents of each well were then loaded into counting tubes and counted on a Gamma 4000 (Beckman).

The ability of [Lys3,Tyr22]mEGF and [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF to stimulate receptor autophosphorylation and dimerization was determined using solubilized EGF receptor from A431 cells prepared as described in Fanger et al. (1989), with the substitution of 1% $C_{12}E_8$ for 1% Triton X-100 as the detergent. For autophosphorylation assays, 1 μL of [Lys3,Tyr22]mEGF or [^{15}N,$^{2}H_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF (1 μg/μL stocks) was added to 99 μL of solubilized A431 cells. Samples were then incubated at room temperature for 10 min and cooled on ice for 5 min. Autophosphorylation was started by adding 54 μL of sample to 6 μL of 10× phosphorylation buffer to give final phosphorylation buffer concentrations of 20 mM HEPES, pH 7.4, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 0.1 mM sodium orthovanadate, and 20 μM ATP with 0.9 μCi of [γ-^{32}P]ATP per assay. Autophosphorylation proceeded for 2 min at 4 °C and was quenched by the addition of 20 μL of 4× electrophoresis sample buffer (4× sample buffer: 0.25 M Tris, pH 6.8, 8% $NaDodSO_4$, 40% glycerol, 20% β-mercaptoethanol, and 0.008% bromophenol blue) and heating to 100 °C for 10 min. Samples were separated by $NaDodSO_4$–PAGE, and autophosphorylation was assessed by autoradiography. The ability of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF to stimulate EGF receptor autophosphorylation was also performed using A431 membrane vesicles (Cohen et al., 1982) as the receptor source. In these assays, each receptor sample consisted of 2 μL of A431 vesicles diluted to 100 μL with 20 mM HEPES, pH 7.4, to which 0.1 μg of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF (0.1 μg/μL stock) was added. The kinase assay was subsequently performed as described above. Dimerization assays using both [Lys3,Tyr22]mEGF and [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF were performed and analyzed as described in Fanger et al. (1989).

Electron Paramagnetic Resonance Spectroscopy. X-band EPR and ST-EPR spectra were recorded with a Bruker ESP-300 spectrometer equipped with an ER-4103 TM_{110} cavity and an ER-4111VT variable-temperature controller. Microwave and field modulation at the sample were calibrated using peroxylaminedisulfonate (Beth et al., 1983). Sample temperature was maintained at 2 °C by blowing precooled nitrogen through the front optical port of the cavity, and samples were measured in a WG-813 (Wilmad Glass Co.) flat cell designed for aqueous samples. For samples in capillary tubes, the cavity was fitted with a capillary tube holder. V_1 EPR spectra were recorded using 100-kHz field modulation of 0.5-G or 1.0-G amplitude (peak-to-peak) with a microwave power of 10 mW which produced a 0.06-G field in the rotating frame. V_2' ST-EPR spectra were recorded using 50-kHz field modulation (100-kHz detection) of 5.0-G amplitude (peak-to-peak) with a microwave power of 100 mW which produced a 0.2-G field in the rotating frame. The detection phase for the 90° out-of-phase signal was determined using the self-null method with a microwave power of 1 mW (Thomas et al., 1976). Spectra were signal-averaged 100-G scans consisting of 1024 data points.

Computer-simulated nitroxide EPR spectra (Balasubramanian & Dalton, 1979) and ST-EPR spectra (Beth et al., 1983) were fit to the acquired spectra using an isotropic rotational model. The incorporation of these simulations into a nonlinear least-squares analysis program is described in Hustedt et al. (1993). The **A** and **g** tensors were determined from the best-fit simulation of the V_1 EPR spectrum of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF bound to the EGF receptor in A431 membrane vesicles, where the motion of the nitroxide is at the linear EPR no-motion limit.[2] For **A** and **g** tensor determination, the parameter $\bar{g}$ [described in Hustedt et al. (1993)] was fixed to 2.005500, and the value of A_{zz} was fixed to 47.00 G. The correlation times (τ_r) for [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF in solution and bound to the EGF receptor in membranes were determined from the best-fit simulation of the EPR and ST-EPR spectra.

Spectroscopic Characterization of [^{15}N,$^{2}H_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF. The labeling stoichiometry of the spin-labeled [Lys3,Tyr22]mEGF was determined by plotting the integrated value of the V_1 EPR signal from a known concentration of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,-Tyr22]mEGF with a standard curve of integrated values of V_1 EPR signal vs concentration for [^{15}N,^{2}H]-*N*-(1-oxyl-2,2,6,6,-tetramethyl-4-piperidinyl)maleimide ([^{15}N,^{2}H]MSL; Beth et al., 1981). To observe any independent motion of the spin label relative to the protein, [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was precipitated with ammonium sulfate, and the motion of the spin label was determined by linear and saturation-transfer EPR. For this experiment 20 μg of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was mixed with 200 μg of unmodified wild-type mEGF in 220 μL of 20 mM Hepes, pH 7.4, and then lyophilized to dryness overnight. This sample was rehydrated in 20 μL of 20 mM Hepes, pH 7.4, and the proteins were precipitated by the addition of a saturated ammonium sulfate solution to a final ammonium sulfate concentration of 60%. The resulting precipitate was loaded into a 50-μL glass capillary tube (Corning), and the tube was sealed with Critoseal. The precipitate was packed in the bottom of the tube by centrifugation at 500 rpm for 2 min in a GLC-1 centrifuge (Dupont/Sorvall). Spin-label motion was determined by recording the V_1 EPR and the V_2' ST-EPR spectra.

EPR and Saturation-Transfer EPR Measurements of [^{15}N,$^{2}H_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF Bound to EGF Receptor in A431 Vesicles. Membrane vesicles were prepared from A431 cells by the method of Cohen et al. (1982). For each EPR experiment, a 0.3-mL aliquot of the vesicle preparation was incubated with 3.3 μM [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF (final concentration) for 10 min at 4 °C. Binding specificity was assessed in a parallel sample by adding a 15-fold excess of unlabeled wild-type mEGF to the vesicles before addition of [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. Following the binding incubation, the vesicles were pelleted by centrifugation at 28 000 rpm for 10 min at 4 °C in a TLA-100.3 rotor (Beckman), and the supernate was drawn off. For EPR experiments to determine the binding specificity of the labeled mutant, the vesicles were immediately resuspended to 0.3 mL final volume with ice-

[2] D. L. Rousseau, Jr., Ph.D. Dissertation, Vanderbilt University.

cold phosphate-buffered saline (Gibco) and a V_1 EPR spectrum was collected. To remove most of the unbound [^{15}N,$^{2}H_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF in samples used for ST-EPR measurements, the vesicles were resuspended once in 0.5 mL of ice-cold PBS and repelleted. The supernate was drawn off and the vesicles were resuspended to 0.3 mL final volume with ice-cold PBS and were then used for ST-EPR measurements. For ST-EPR, the low signal strength of these samples prevented direct determination of the detection phase for the 90° out-of-phase signal using the self-null method. To determine the detection phase, a sample of [^{15}N,^{2}H]MSL-BSA (~0.1 mM) was used in the flat cell as a sample for the self-null method. The phase determined for this sample was then used for data collection on the [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF samples. The [^{15}N,^{2}H]MSL-modified BSA sample was prepared by allowing equimolar amounts of [^{15}N,^{2}H]MSL and BSA, each 1 mM final concentration in 100 mM phosphate buffer, pH 8.0, to react for a period of 2 h on ice after which the reaction mixture was applied to a 1- × 5-cm P-2 gel-filtration column to separate the reacted and unreacted label. The excluded volume from this column was diluted 5-fold and used to determine the detection phase.

NaDodSO$_4$–PAGE and Western Blot Analysis. NaDodSO$_4$–PAGE was performed according to the method of Laemmli (1970). Tricine NaDodSO$_4$–PAGE was performed according to the method of Schägger & von Jagow (1987). Electrophoretic transfer of proteins to nitrocellulose (Schleicher & Schuell) was carried out with a Sartoblot II (Sartorius) semidry transfer apparatus according to the manufacturer's instructions, using a current of 4 mA/cm^2 for 25 min. Immunostaining of the transferred proteins was carried out using the Bio-Rad Immuno-Blot goat anti-rabbit alkaline phosphatase assay kit (Bio-Rad) following the manufacturer's instructions, except for the preparation of the primary antibody. The primary antibody was anti-mEGF antiserum from rabbit (gift of Professor Stanley Cohen), which was presorbed against an HB101 extract prepared as described below.

HB101 extract was prepared from a 1-L culture of untransformed *E. coli* HB101 grown overnight in LB medium at 37 °C on an orbital shaker (250 rpm). Bacteria from this culture were pelleted by centrifugation at 10 000 rpm for 5 min at 4 °C in a GSA rotor (Sorvall). The pellet was resuspended in 10 mL of double-distilled water and boiled for 10 min. This 10-mL solution was then used as a 100X stock of HB101 extract. The anti-mEGF antiserum was presorbed with this HB101 extract by diluting the antiserum 1:100 into buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 3% BSA (w/v), and 1X HB101 extract (final concentration). The antiserum in this buffer was incubated overnight at 4 °C on a rocker platform. After incubation, precipitates were cleared by centrifugation at 100000*g* for 30 min at 4 °C in a Ti-50 rotor (Beckman). The supernate was decanted and used as the primary antibody for immunostaining.

RESULTS

Expression and Purification of [Lys3,Tyr22]mEGF. The [Lys3,Tyr22]mEGF was expressed in freshly transformed HB101 and released from the bacterial periplasm by osmotic shock as described in Materials and Methods. Initially, expression of the mutant was detected by Tricine NaDodSO$_4$–PAGE and western blotting; however, quantitation of expression by this method was unreliable. Therefore, once the [Lys3,Tyr22]mEGF had been identified and purified by HPLC, a simple assay was developed which allowed easy quantitation of [Lys3,Tyr22]mEGF expression before large-scale purification proceeded. In this assay, 5 mL of osmotic shock supernate was loaded onto a C_{18} Sep-Pak cartridge (Waters) which had been equilibrated with 95:5 water/AcCN buffered with 0.1% TEA/acetate, pH 6.0. The Sep-Pak was then washed with 5 mL of equilibration buffer and eluted with 3 mL of 50:50 water/AcCN buffered with 0.1% TEA acetate, pH 6.0. This eluted fraction was lyophilized and subjected to analytical RP-HPLC as described in the mutant purification section of Materials and Methods. The peak corresponding to the [Lys3,Tyr22]mEGF was then integrated, and the mEGF concentration was determined from a standard integration curve of known mEGF concentrations. Using this method, we found that our expression levels reached 0.5 mg/L of bacterial culture.

The [Lys3,Tyr22]mEGF was purified from the osmotic shock supernate using a two-step procedure. In the first step, the supernate was loaded onto a Lichroprep RP-C8 column, and the fraction containing [Lys3,Tyr22]mEGF was eluted with 50:50 water/AcCN + 0.1% TEA/acetate, pH 6.0. [Lys3,Tyr22]mEGF was further purified from this fraction by semipreparative RP-HPLC. The peak corresponding to [Lys3,Tyr22]mEGF was hand collected and assayed for purity by analytical RP-HPLC (Figure 1A). Final purification yielded 100–120 μg of purified [Lys3,Tyr22]mEGF from a 1-L expression culture. [Lys3,Tyr22]mEGF was subjected to 23 cycles of gas-phase Edman sequencing, which confirmed the presence of Lys as the third residue and Tyr as the twenty-second residue. Mass spectrometric analysis (see below) determined a mass of 6032.4 for this purified mutant, which is in excellent agreement with its calculated mass of 6031.8. EGF receptor autophosphorylation assays and dimerization assays demonstrated that the ability of the mutant to activate the EGF receptor kinase or to stimulate EGF receptor dimerization was indistinguishable from that of wild-type mEGF.[2]

Purification and Physical Characterization of [^{15}N,$^{2}H_{16}$]-BSSDP-Modified [Lys3,Tyr22]mEGF. Figure 1B is the HPLC chromatogram of the reaction mixture of [^{15}N,$^{2}H_{16}$]-BSSDP with [Lys3,Tyr22]mEGF after a 4-h incubation at room temperature. A single dominant product, which eluted at 67.8 min, was formed in this reaction. Under these HPLC conditions, unlabeled [Lys3,Tyr22]mEGF would elute at 64.1 min. Figure 1C is an HPLC chromatogram of the purified product which was used in the subsequent studies. The V_1 EPR spectrum of this product and the best-fit EPR simulation are shown in Figure 2A. The EPR spectrum was fit to a single isotropic component spectrum with a $\tau_r = 2.9$ ns, which was consistent with an [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,-Tyr22]mEGF molecule in which the spin label was rigidly coupled to a protein the size of mEGF. The identity of this product was confirmed by mass spectrometry (Figure 3). The experimentally determined mass of 6273.1 closely matched the calculated mass of 6273.0 for [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF, confirming that the [^{15}N,$^{2}H_{16}$]BSSDP was covalently linked to the [Lys3,Tyr22]mEGF at two positions. The spin density for this product was 1.1 mol of spin/mol of protein,[2] determined as described in Materials and Methods. The overall yield of purified [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was 54%, based on the starting concentration of [Lys3,Tyr22]mEGF in the labeling reaction. Other products of the reaction did have EPR signal, but the low yields of these products prevented further characterization.

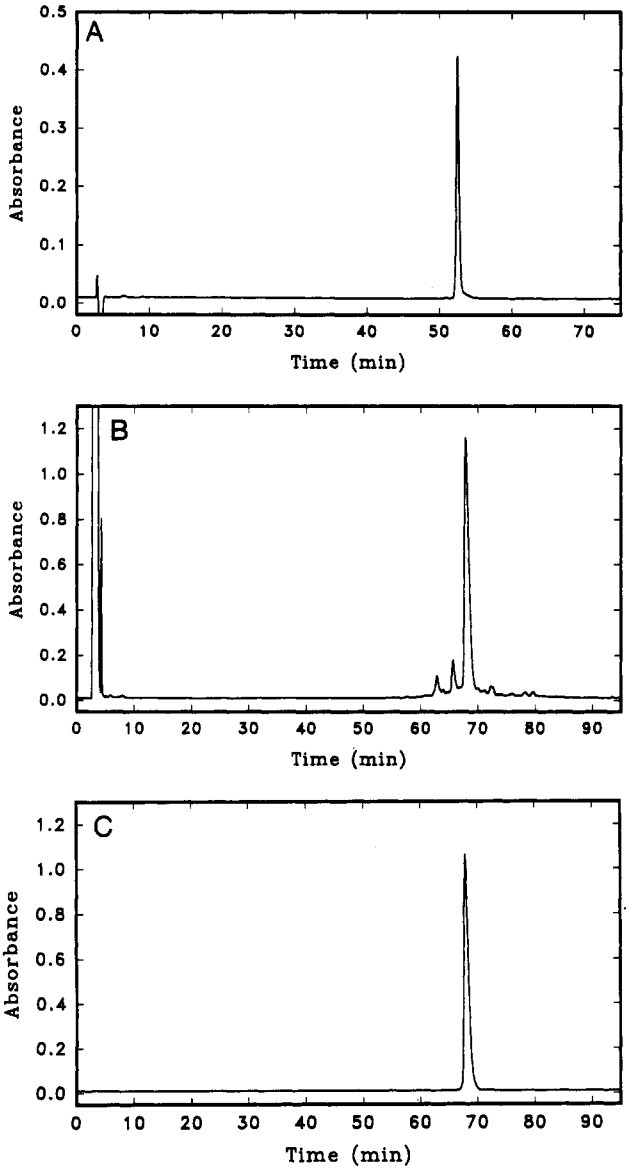

FIGURE 1: HPLC purification of [Lys3,Tyr22]mEGF and [^{15}N,^{2}H$_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF. Panel A is the A_{220} chromatogram of an analytical RP-HPLC injection of the purified [Lys3,Tyr22]mEGF. The identity of this product was confirmed by amino acid sequencing and LD-TOF mass spectrometric analysis. Panel B is the A_{280} chromatogram of the reaction mix of [^{15}N,^{2}H$_{16}$]BSSDP- and [Lys3,Tyr22]mEGF after 4 h at room temperature. The predominant peak at 67.8 min was hand-collected, lyophilized, redissolved in 20 mM HEPES, pH 7.4, and reinjected using the same RP-HPLC method. Panel C is the A_{280} chromatogram of the purified sample. The 67.8 min peak in panel C was identified as [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF by LD-TOF mass spectrometry and EPR spectroscopy.

In order to assess the motional coupling of the spin label to the protein, [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was immobilized by precipitation with ammonium sulfate. Precipitation was carried out in the presence of a 100-fold excess of unlabeled mEGF to decrease the spin–spin interactions between spin labels on the modified [Lys3,Tyr22]-mEGF. The V_1 EPR spectrum of the precipitated [^{15}N,^{2}H$_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF is shown in Figure 4A. This spectrum shows a dominant motional component which is immobilized on the linear EPR time scale. There is a small degree of line broadening probably due to spin–spin interactions between closely spaced [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF molecules in the precipitate. The V_2' ST-EPR spectrum of this sample is shown in Figure 4B.

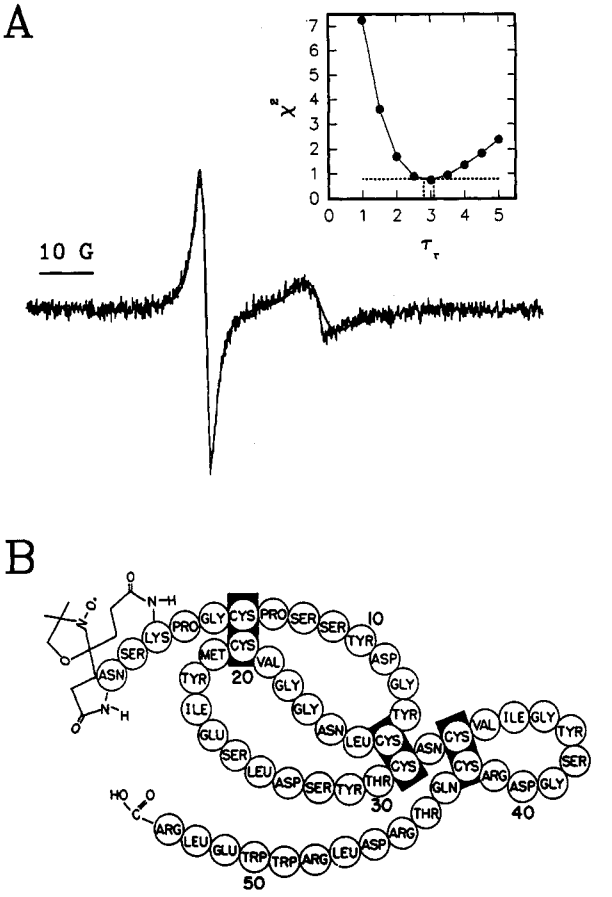

FIGURE 2: V_1 EPR spectrum and fit of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF at 2 °C. Panel A shows the V_1 EPR spectrum and fit of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF at 2 °C. The inset is a plot of χ^2 value as a function of τ_r. The dashed line marks the 66.6% confidence interval for the τ_r determination. The EPR spectrum was recorded on a Bruker ESP-300 spectrometer using a 0.5-G modulation amplitude, a microwave power of 10 mW, and a 100-G sweep width. The solid line spectrum is the best-fit computer-simulated EPR spectrum, assuming isotropic rotation, calculated using the following parameters: g_{xx} = 2.008132; g_{yy} = 2.005923; g_{zz} = 2.002446; A_{xx} = 10.359 G; A_{yy} = 10.191 G; A_{zz} = 47.000 G; T_{1e} = 27 μs; T_{2e} = 145 ns; H_m = 0.5 G (peak to peak); H_1 = 0.06 G (rotating frame). From this fit, a value of τ_r = 2.9 ns was determined for [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. Panel B is the proposed covalent structure of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,-Tyr22]mEGF. The position of the spin-label on the mutant mEGF is proposed on the basis of labeling studies using wild-type mEGF (Faulkner O'Brien et al., 1991) and on the reaction kinetics of the reactive ends (Anjaneyulu & Staros, 1987); however, the position has not been directly determined.

The degree of immobilization of the spin label in this spectrum again suggests a very tight motional coupling between the spin label and the peptide backbone of the protein, and there is little evidence for any large-amplitude independent motion of the spin label relative to the precipitated protein.

Biological Characterization of [^{15}N,^{2}H$_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF. The relative abilities of wild-type mEGF and [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF to compete with ^{125}I-mEGF for receptor binding were compared in competitive binding assays based on general binding assay methods described in Carpenter (1985). This binding assay used the A431 cell line, an epidermoid carcinoma cell line which overexpresses the EGF receptor (Haigler et al., 1978). Figure 5A shows that wild-type mEGF and [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF were indistinguishable in their competitive binding properties. The

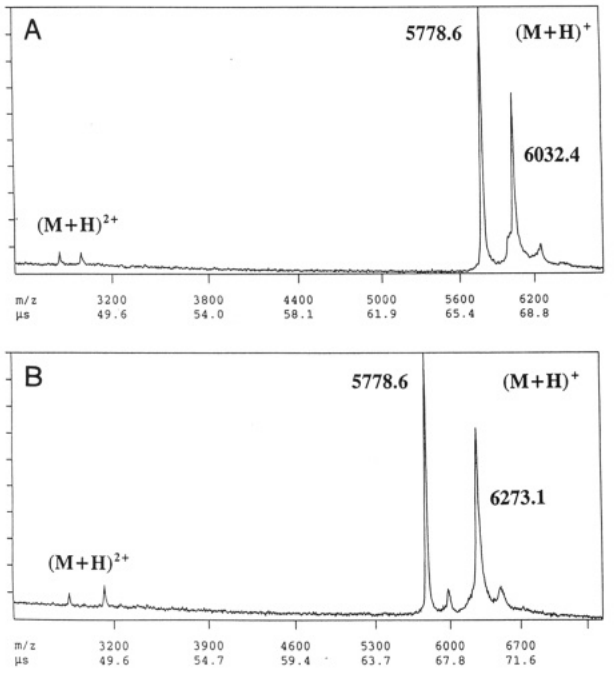

FIGURE 3: LD-TOF mass spectra of [Lys3,Tyr22]mEGF and [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. The spectrum in panel A is of purified [Lys3,Tyr22]mEGF. The m/z = 6032.4 determined for the protonated [Lys3,Tyr22]mEGF closely matched the calculated value of 6031.8. Panel B is the spectrum for purified [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. The determined mass of m/z = 6273.1 for the protonated form of this product was in excellent agreement with its calculated mass of 6273.0. In both spectra, the m/z = 5778.6 peak represents porcine insulin, which was added to each sample as an internal calibration standard.

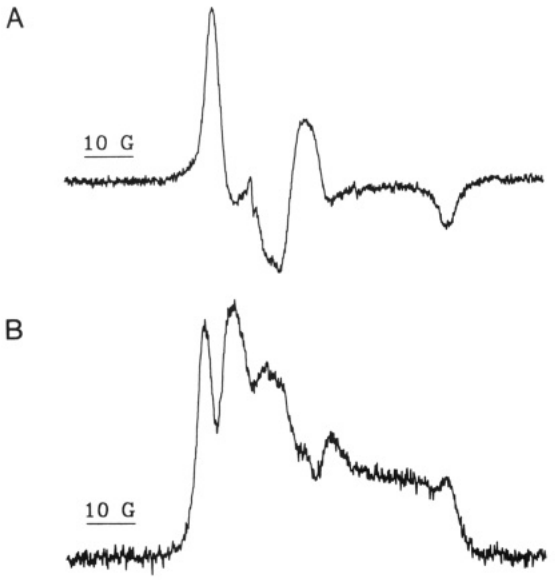

FIGURE 4: Determination of the spin-label motion on immobilized [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was immobilized by ammonium sulfate precipitation in the presence of a 100-fold excess of unlabeled wild-type mEGF. The unlabeled mEGF was added to decrease the spin–spin interactions between the labeled molecules. Panel A is a V_1 linear EPR spectrum of the precipitate recorded using a 0.5-G modulation amplitude and a microwave power of 10 mW. Panel B is a V_2' ST-EPR spectrum of the same sample recorded using a 5.0-G modulation amplitude and a microwave power of 100 mW. Both spectra are 100 G in width. The degree of immobilization of the spin-label in both of these spectra indicates that it has little independent motion relative to the protein.

ability of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF and wild-type mEGF to stimulate EGF receptor autophosphorylation was compared in phosphorylation assays using both soluble (Figure 5B) and membrane-bound receptors (Figure 5C). Finally, [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,-Tyr22]mEGF and wild-type mEGF were comparable in their ability to induce dimerization of solubilized EGF receptor in

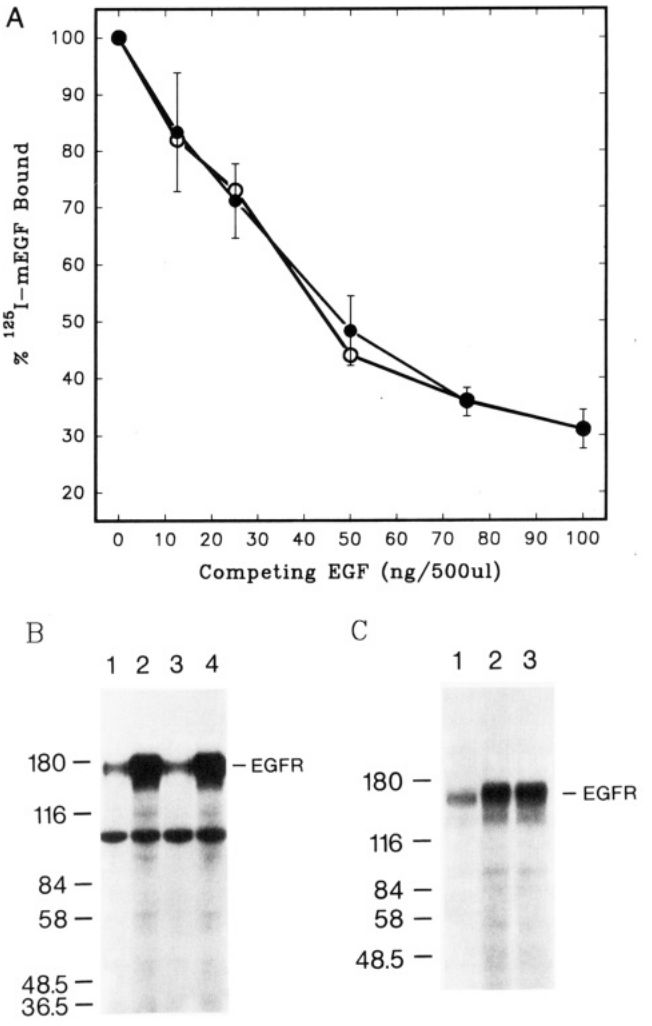

FIGURE 5: Biological characterization of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. Comparison of the ability of wild-type mEGF (●) and [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF (○) to compete with ^{125}I-mEGF for EGF receptor binding on A431 cells is shown in panel A. In the assay, 0, 12.5, 25, 50, 75, or 100 ng of competing wild-type mEGF was added with 50 ng of ^{125}I-mEGF (31 500 cpm/ng) to A431 cells in 12-well plates. Following incubation for 2 h at 4 °C, the cells were washed extensively, solubilized with 1 N NaOH, and counted. Nonspecific binding at each concentration was determined by addition of a 100-fold excess of unlabeled wild-type mEGF and was always less than 10%. Each data point was determined in triplicate in two separate experiments. The resulting six values were corrected for nonspecific binding and then averaged. The percent ^{125}I-mEGF bound was calculated by dividing the specific bound counts of ^{125}I-mEGF at each concentration of competing EGF by the specific bound counts of ^{125}I-mEGF in the absence of competing EGF. The error bars represent the standard deviation of the mean for the averaged data points on the wild-type mEGF (●) plot. Panel B is an autoradiogram of solubilized EGF receptor autophosphorylation under basal conditions (lanes 1 and 3) and with stimulation by 1.6 μM wild-type mEGF (lane 2) or 1.6 μM [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF (lane 4). Panel C is an autoradiogram of EGF receptor autophosphorylation in A431 membrane vesicles under basal stimulation (lane 1) and stimulation by 0.16 μM wild-type mEGF (lane 2) or 0.16 μM [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF (lane 3). Autophosphorylation assays for both receptor preparations were performed as described in Materials and Methods. Samples were separated by NaDodSO$_4$–PAGE (Laemmli, 1970) using 7.5% separating gels. The autoradiograms were produced by overnight exposure of the dried gels on Kodak XAR-5 film.

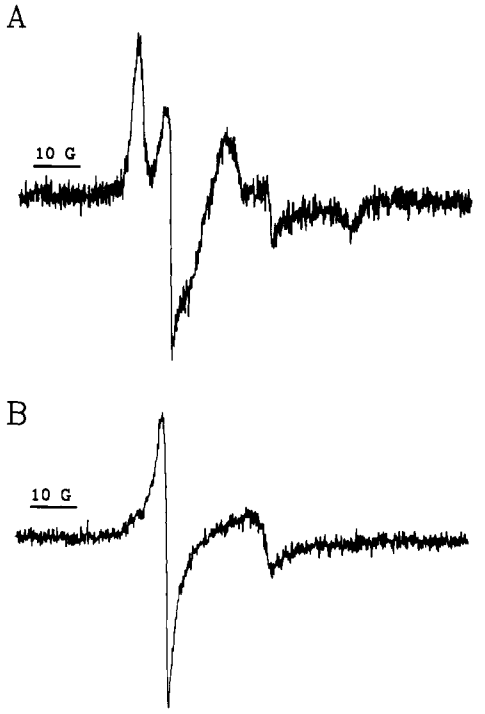

FIGURE 6: Binding of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]-mEGF to EGF receptor in A431 membrane vesicles. Panel A is the V_1 EPR spectrum of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF in the presence of EGF receptor in A431 membrane vesicles. Panel B is the V_1 EPR spectrum of a parallel sample in which a 15-fold excess of unlabeled wild-type mEGF was added before incubation with [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. The immobilized component of the upper spectrum can be attributed to the receptor-bound spin-labeled mEGF as this component is virtually absent in the parallel sample where receptor sites were preblocked with unlabeled wild-type mEGF. The low level of immobilized signal in the lower spectrum is indicative of the high specificity of the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF for the EGF receptor. Both spectra were recorded at 2 °C using a modulation amplitude of 0.5 G, a microwave power of 10 mW, and a 100-G sweep width.

cross-linking studies using the method of Fanger et al. (1989) (data not shown). These studies each suggest that [^{15}N,^{2}H$_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF is indistinguishable from wild-type mEGF in its ability to compete for receptor binding, stimulate receptor autophosphorylation, and induce receptor dimerization.

EPR and Saturation-Transfer EPR of [^{15}N,^{2}H$_{16}$]BSSDP-Modified [Lys3,Tyr22]mEGF Bound to EGF Receptor in A431 Membranes. V_1 linear EPR spectra of [^{15}N,^{2}H$_{16}$]-BSSDP-modified [Lys3,Tyr22]mEGF with A431 membrane vesicles in the absence or presence of competing wild-type mEGF are shown in Figure 6. The spectrum in the upper panel (Figure 6A) was recorded in the absence of unlabeled wild-type mEGF and consists of two well-resolved motional components which represent receptor-bound and unbound [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. The slow motion component exhibits a characteristic line shape with 47-G splitting of the exterior features, consistent with that of an immobilized probe on the linear EPR time scale (Beth & Robinson, 1989). For the lower spectrum (Figure 6B), the sample included a 15-fold excess of unlabeled wild-type mEGF. This spectrum is dominated by a motional component representing unbound [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,-Tyr22]mEGF. The almost complete absence in Figure 6B of the slow motional component seen in Figure 6A demonstrates the specificity of interaction of the labeled mutant with the EGF receptor in A431 membranes.

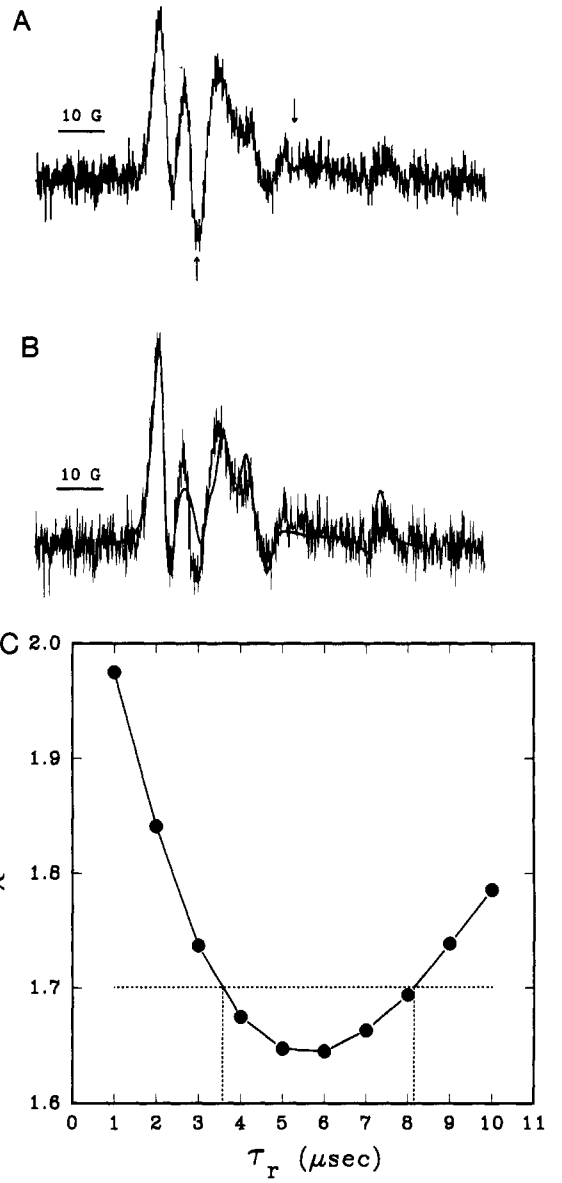

FIGURE 7: ST-EPR spectrum of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF bound to EGF receptor in A431 membrane vesicles. Panel A is a V_2' ST-EPR spectrum of spin-labeled hormone bound to its receptor in membrane vesicles. The 100-G spectrum was recorded at 2 °C using a modulation amplitude of 5.0 G and a microwave power of 100 mW. Arrows indicate signal from a small amount of unbound, spin-labeled mEGF present in the samples. For fitting purposes, this spectrum was corrected by digitally subtracting the unbound signal. Panel B shows the corrected spectrum and the best-fit simulation using an isotropic rotational model. Fitting parameters were as follows: g_{xx} = 2.008132; g_{yy} = 2.005923; g_{zz} = 2.002446; A_{xx} = 10.359 G; A_{yy} = 10.191 G; A_{zz} = 47.000 G; T_{1e} = 27 μs; T_{2e} = 24 ns; H_m = 5.0 G (peak to peak); H_1 = 0.2 G (rotating frame). Panel C is a plot of χ^2 value as a function of τ_r. The dashed lines indicate the 66.6% confidence interval for the τ_r determination. From the best-fit simulation a value of τ_r = 6 μs was determined for the spin-labeled hormone/receptor complex in membranes.

Figure 7A shows V_2' ST-EPR spectra of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF bound to the EGF receptor in A431 membranes. The upper spectrum is the uncorrected spectrum recorded from the sample. From the line shape it is apparent that a small amount of unbound signal is still present. For fitting purposes, this unbound signal was digitally subtracted using a V_2' ST-EPR spectrum of unbound [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF. The corrected spectrum and the best-fit simulation are shown in Figure 7B. The fit simulation was calculated assuming an isotropic

rotational model for the EGF/EGF receptor complex. The statistics for this fit are seen in Figure 7C. The best fit of the data gives a rotational correlation time of 6 μs for the hormone/receptor complex in membranes at 2 °C.

DISCUSSION

Previous work in this laboratory demonstrated that labeling wild-type mEGF with [^{15}N,^{2}H$_{16}$]BSSDP gave a predominant product in which the label was attached to mEGF only at the amino terminus. Moreover, the bifunctionally modified product, one in which the label was attached covalently at two sites, was formed in low yield, and the second covalent attachment, presumably at Tyr3, was unstable (Faulkner-O'Brien et al., 1991). While this [^{15}N,^{2}H$_{16}$]BSSDP-labeled wild-type mEGF derivative proved useful for the study of the dissociation kinetics of the EGF/EGF receptor complex, its low yield and lack of rigid motional coupling of the spin label to the protein made this derivative unsuitable for ST-EPR studies of the EGF/EGF receptor complex. One goal of this study was to produce a mEGF molecule which would react with both ends of [^{15}N,^{2}H$_{16}$]BSSDP in high yield and give a spin-labeled mEGF in which the motion of the label was tightly coupled to the motion of the hormone. On the basis of the reactivity of sulfo-*N*-succinimidyl esters (Staros & Anjaneyulu, 1987), two changes in wild-type mEGF were predicted to make it more amenable to labeling with [^{15}N,^{2}H$_{16}$]BSSDP. First, placing a second primary amine close to the amino terminus would provide a stable labeling site for the second end of [^{15}N,^{2}H$_{16}$]BSSDP. Second, changing His22 would remove the imidazole side chain which could catalyze hydrolysis of sulfo-*N*-succinimidyl esters. To accomplish these changes we used oligonucleotide-mediated site-directed mutagenesis to substitute Lys for Tyr3 and Tyr for His22 in mEGF.

The [Lys3,Tyr22]mEGF was expressed in *E. coli* HB101 and directed to the bacterial periplasm using the pIN-III-ompA3-mEGF expression plasmid (Ray et al., 1988). Purification of the protein from periplasm was accomplished in a two-step chromatographic procedure which allowed recovery of 20% of the expressed mutant in purified form. The overall low yield in this purification can be traced to the first step of purification on the LiChroprep column. Since there is no detectable [Lys3,Tyr22]mEGF in the flow through and washes of the column (data not shown), we suspect that the majority of the [Lys3,Tyr22]mEGF losses occurred due to irreversible binding of the mutant mEGF to the column. Current efforts are being directed to optimize yields in this purification scheme. The presence of the mutated amino acid residues in purified [Lys3,Tyr22]mEGF was verified by sequencing the first 23 amino acids of the protein and by mass spectrometric analysis (Figure 3A). The presence of the mutated amino acids did not appear to alter the biological activity of the [Lys3,Tyr22]-mEGF since it was indistinguishable from wild-type mEGF in receptor autophosphorylation and receptor dimerization assays.[2]

Modification of [Lys3,Tyr22]mEGF with [^{15}N,^{2}H$_{16}$]BSSDP was done under conditions similar to those reported previously (Faulkner-O'Brien et al., 1991). However, the purification of the products was modified from previous methods in an attempt to optimize the yield of the labeled product. First, due to the known acid instability of the doxyl ring of the spin label (Gaffney, 1976), the pH of the HPLC buffer was increased to 5.5. Second, the eluant mobile phase was changed from MeOH to AcCN, which appeared to yield a higher recovery of mEGF from the HPLC column. From Figure 1B it is evident that a single dominant product is formed in this reaction and that little unreacted [Lys3,Tyr22]mEGF is left after 4 h of reaction at room temperature. This major product was purified (Figure 1C) and identified by EPR spectroscopy (Figure 2A) and by LD-TOF mass spectrometry (Figure 3B) as the bifunctionally modified [^{15}N,^{2}H$_{16}$]BSSDP-labeled [Lys3,Tyr22]mEGF. Biological activity of this product was determined to be indistinguishable from that of wild-type mEGF (Figure 5). The overall yield of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF was greater than 50%. Our proposed covalent structure of [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF is shown in Figure 2B. We propose that the [^{15}N,^{2}H$_{16}$]BSSDP label is covalently linked to the amino terminus and the Lys3 side chain on the basis of analysis of the reaction products of [^{15}N,^{2}H$_{16}$]BSSDP with wild-type mEGF (Faulkner-O'Brien et al., 1991) and of the known reactivities of sulfo-*N*-succinimidyl esters (Anjaneyulu & Staros, 1987); however, the exact position of the label has not been directly determined.

The spectroscopic properties of the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF were used to determine the motional characteristics of both the probe and the mutant mEGF molecule to which it was covalently linked. The motion of the spin label was shown to be rigidly coupled to that of the mEGF peptide backbone by analysis of the EPR and ST-EPR spectra of ammonium sulfate precipitated protein (Figure 4). Both the EPR and ST-EPR spectra of ammonium sulfate precipitated [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF indicate immobilization of the spin label in the time window of each EPR method. Fitting of the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF EPR spectrum (Figure 2A) to an isotropic rotational model yielded a τ_r of 2.9 ns at 2 °C, which gives the protein a calculated r_h of 11.6 Å. Our τ_r value is in reasonable agreement with the τ_r values of 4 ns (20 °C) (Ghiron et al., 1992) and 2 ns (room temperature) (Greenfield et al., 1989) reported for human EGF using time-resolved fluorescence measurements. Both the τ_r and the r_h values calculated for the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]-mEGF are smaller than expected for a spherical protein of mEGF size, suggesting an anisotropic rotational motion for the protein or the presence of some segmental flexibility of the protein at the [^{15}N,^{2}H$_{16}$]BSSDP labeling sites. The NMR solution structure of mEGF (Montelione et al., 1987, 1992; Kohda & Inagaki, 1992) indicates that the protein is not spherical, so an anisotropic rotational motion for the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF is not unexpected. We are currently working to model the anisotropic motions of the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]-mEGF from the EPR data. This work, in conjunction with time-resolved fluorescence experiments with fluorescein-labeled mEGF currently in progress, should allow a rigorous determination of the anisotropic motion of mEGF.

The specificity of binding of the [^{15}N,^{2}H$_{16}$]BSSDP-modified [Lys3,Tyr22]mEGF to the EGF receptor in A431 membrane vesicles was demonstrated in Figure 6. The preincubation of a 15-fold excess of unlabeled mEGF with the membrane vesicles almost completely eliminated the immobilized component of the EPR spectrum, which indicates that this immobilized signal is specifically bound and is, therefore, accurately reflecting the motion of the EGF/EGF receptor complex. Supporting previous results with solubilized EGF receptor (Faulkner-O'Brien et al., 1991), there is no evidence of dipolar interaction between spin labels in the EPR spectrum of the EGF/EGF receptor complex in membranes, indicating a separation of greater than 16 Å between the spin labels on

adjacent EGF/EGF receptor complexes. The ST-EPR spectrum of the [^{15}N,$^{2}H_{16}$]BSSDP-modified [Lys3,Tyr22]-mEGF/EGF receptor complex in membranes (Figure 7) was fit to an isotropic rotational model, and the best fit simulated spectrum gave an effective τ_r of 6 μs. The value of 6 μs from an isotropic model represents an upper bound for the rotational correlation time for the EGF/EGF receptor complex. The relationship between the effective rotational correlation time determined from isotropic models and the true rotational correlation times for an anisotropically diffusing system has been discussed for the general case of isotropic versus anisotropic motional models in previous work (Beth & Robinson, 1989). Clearly, the rotational diffusion of EGF when complexed with its receptor in membrane vesicles would not be predicted to be isotropic. This statement is strengthened by previous studies employing phosphorescence anisotropy decay of erythrosin-labeled mEGF bound to its receptor in membranes (Zidovetzki et al., 1986), where a measurable residual anisotropy was observed, suggesting orientationally constrained rotational diffusion. It is also supported by the relatively poor fit of the experimental ST-EPR data in Figure 7B. Further work will be required to discriminate between various constrained rotational diffusion models for the EGF/EGF receptor complex. A reasonable starting point would be to consider the rotational diffusion as axial about the membrane normal axis or as a wobble in a cone about an appropriate director axis [these models have been discussed by Thomas (1985, 1986)].

Even at this early stage, it is instructive to compare the current results with previous studies. The τ_r of the EGF/EGF receptor complex in A431 membranes has previously been measured by phosphorescence anisotropy decay of erythrosin-labeled mEGF bound to the EGF receptor (Zidovetsky et al., 1986). Their reported τ_r of 16–20 μs is significantly longer than our value. We considered the possibility that the Thom et al. (1977) method of membrane preparation used by Zidovetzki et al. (1986) could produce membranes with different components and therfore different physical properties than those made using the method of Cohen et al. (1982). In addition, A431 membranes prepared using the Thom et al. (1977) method contain EGF receptor in a 150-kDa form, resulting from proteolysis of the 170-kDa receptor (Cassel & Glaser, 1982; Gates & King, 1982; Cohen et al., 1982), whereas the 170-kDa receptor is the predominant form in membranes prepared by the method of Cohen et al. (1982). However, our preliminary data suggest that the τ_r of the EGF/EGF receptor complex is not affected by the method of membrane preparation or by the presence of a majority of the 150 kD form of the EGF receptor.[2] Currently we cannot explain the differing correlation times measured by the two different methods. However, recent progress in methods to simultaneously analyze phosphorescence anisotropy data and ST-EPR data (Hustedt et al., 1993) should allow us to resolve this apparent discrepancy between the two techniques and will allow a more complete understanding of the rotational properties of the EGF/EGF receptor complex in membranes.

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