High-Yield Covalent Attachment of Epidermal Growth Factor to Its Receptor by Kinetically Controlled, Stepwise Affinity Cross-Linking†

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ABSTRACT: We report here the use of a stepwise affinity cross-linking technique in the specific covalent attachment of epidermal growth factor (EGF) to its receptor. A heterobifunctional cross-linking reagent, sulfo-*N*-succinimidyl 4-(fluorosulfonyl)benzoate (SSFSB), which contains a rapidly reacting sulfo-*N*-succinimidyl active ester and a much more slowly reacting aromatic fluorosulfonyl moiety, was synthesized and characterized. Murine EGF (mEGF) was modified by the cross-linker to yield as the major product a derivative of mEGF having the (fluorosulfonyl)benzoyl moiety attached covalently at the amino terminus. SSFSB-modified, ^{125}I-labeled mEGF was separated from unreacted SSFSB by size-exclusion chromatography and applied to shed membrane vesicles from A431 human carcinoma cells. Binding of derivatized ^{125}I-mEGF to vesicles led to high yields (>60%) of covalent linkage of ^{125}I-mEGF to the EGF receptor, as determined by measurement of the fraction of specifically bound radiolabel which comigrated with the EGF receptor in NaDodSO$_4$–polyacrylamide gels. The specificity of affinity cross-linking was evident in the negligible degree of labeling of species other than the EGF receptor and in the retention of EGF-stimulated receptor kinase activity after cross-linking.

Epidermal growth factor (EGF),[1] a single-chain polypeptide hormone with a molecular weight of approximately 6000, was initially isolated from murine submandibular glands on the basis of its activity in promoting early eyelid opening in newborn mice (Cohen, 1962). Specific membrane receptors [for reviews, see Staros et al. (1989) and Carpenter and Wahl (1990)] which modulate the effects of EGF in cell growth and differentiation have been demonstrated in various tissues (Carpenter & Cohen, 1979; Carpenter, 1981). The binding of EGF to its receptor initiates responses which include the stimulation of a protein kinase activity (Carpenter et al., 1979) specific for tyrosyl residues (Ushiro & Cohen, 1980), which has been shown to be intrinsic to the EGF receptor (Buhrow et al., 1982, 1983; Ullrich et al., 1984) and which leads to receptor autophosphorylation (Cohen et al., 1982; Buhrow et al., 1982) and the phosphorylation of intracellular substrates [reviewed in Carpenter and Wahl (1990)]. This kinase activity is essential for the mitogenic effects of EGF (Chen et al., 1987; Honegger et al., 1987; Moolenaar et al., 1988). Details of the recognition of EGF by its receptor and the mechanism by which ligand–receptor interactions bring about their effects continue to be matters of considerable study [for reviews, see Gill et al. (1987), Staros et al. (1989), Ullrich and Schlessinger (1990), and Carpenter and Wahl (1990)].

In this report, we describe a method for affinity cross-linking EGF to the EGF receptor which combines the advantages of the relatively high yields obtainable using cross-linkers with spontaneously reactive groups and the relative specificity allowed by the stepwise techniques used most often with heterobifunctional photo-cross-linking reagents (Ji, 1983; Bayley & Staros, 1984). In a stepwise affinity cross-linking experiment, we have employed a novel cross-linker, sulfo-*N*-succinimidyl 4-(fluorosulfonyl)benzoate (SSFSB), which contains two spontaneously reactive electrophilic groups. Our technique exploited the kinetic differences between these groups to modify rapidly, covalently, and in high yield ^{125}I-mEGF at its amino terminus, the only primary amino group of this ligand, via reaction with the sulfo-*N*-succinimidyl group of SSFSB. The modified ^{125}I-mEGF proved sufficiently stable for separation from unreacted SSFSB by P2 gel filtration. The binding of derivatized ^{125}I-mEGF to shed membrane vesicles from EGF receptor-rich A431 human carcinoma cells led to reaction with the EGF receptor, promoted by the effectively increased local concentration of the otherwise slowly reacting fluorosulfonyl group (Staros, 1988).

Under optimal conditions, yields of specific covalent linkage of EGF to the EGF receptor with this technique were found to exceed 60%; the specificity of the technique is evident in that the EGF receptor was virtually the only species detectably labeled in the presence of optimal concentrations of ^{125}I-mEGF. Phosphorylation experiments with [γ-^{32}P]ATP demonstrated that the EGF-stimulated autophosphorylation of the EGF receptor was unimpaired in receptors which had been covalently linked to EGF by this technique.

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[1] Abbreviations: DCC, dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; EGF, epidermal growth factor; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; FAB-MS, fast atom bombardment mass spectrometry; FSBAcid, 4-(fluorosulfonyl)benzoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HOSu(SO$_3$)Na, sodium salt of *N*-hydroxysulfosuccinimide; HPLC, high-performance liquid chromatography; LD-TOF, laser desorption time of flight; mEGF, murine EGF; NaDodSO$_4$, sodium salt of dodecyl sulfate; NMR, nuclear magnetic resonance; SSFSB, sulfo-*N*-succinimidyl 4-(fluorosulfonyl)benzoate; TBAF, tetrabutylammonium formate; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

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EXPERIMENTAL PROCEDURES

Materials and Routine Procedures. Murine EGF was prepared as previously described (Savage & Cohen, 1972); ^{125}I-mEGF was prepared as described (Carpenter & Cohen, 1976), with 1 mg/mL nonradiolabeled mEGF used as a carrier for radiolabeled ligand. Shed membrane vesicles were prepared (Cohen et al., 1982) from A431 human carcinoma cells. [γ-^{32}P]ATP (specific activity 3000 Ci/mmol) was purchased from New England Nuclear. HPLC-grade methanol and acetonitrile were from Burdick and Jackson. TFA (HPLC grade) was obtained from Pierce and tetrabutylammonium hydroxide (HPLC grade) from Fisher. Formic acid (99%) was obtained from Sigma.

Derivatives of mEGF were analyzed by reverse-phase HPLC using a 2.1 × 220 mm, 300-Å pore size Brownlee C-8 column (Applied Biosystems) and mobile phases of 0.1% TFA in water (solvent A) and 0.1% TFA in 80:20 acetonitrile–water (solvent B). Separations were performed with increasing solvent B delivered by Waters 510 pumps. Absorbance at 220 and 280 nm was monitored with a Waters 490E programmable multiwavelength detector. The system was controlled by a Maxima 820 workstation. The stability of 4-(fluorosulfonyl)-benzoic acid (FSBAcid) was measured using the isocratic system previously described (Staros, 1982) in conjunction with a HP3390A integrator, using a reverse-phase 4.6 × 150 mm Econosphere C-18 column (Alltech) and a mobile phase of 50:50 tetrabutylammonium formate (TBAF), pH 3.5–methanol.

All reagents and solvents used were ACS-certified grade or better. FSBAcid (97%) and DCC were purchased from Aldrich. FSBAcid was recrystallized from a mixture of benzene and acetonitrile before use. The sodium salt of *N*-hydroxysulfosuccinimide [HOSu(SO_3)Na] was prepared from *N*-hydroxymaleimide as reported previously (Staros, 1982). Proton NMR spectra of SSFSB in dry deuterated Me_2SO were recorded on an IBM/Bruker NR-300 spectrometer. Negative-ion fast atom bombardment mass spectrometry (FAB-MS) was performed on a VG-70/250 gas chromatography–mass spectroscopy instrument. 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). Elemental analysis was carried out by Galbraith Laboratories (Knoxville, TN).

Synthesis of SSFSB. SSFSB was prepared by DCC-mediated coupling of FSBAcid to HOSu(SO_3)Na by the general method described (Anderson et al., 1964). To a solution of DCC (0.3 g, 1.4 mmol) in 4 mL of dry DMF were added HOSu(SO_3)Na (0.22 g, 1.0 mmol) and FSBAcid (0.3 g, 1.4 mmol) under inert atmosphere. The reaction mixture was stirred at 3 °C for 2 h and then at room temperature overnight. The precipitated dicyclohexylurea was removed by filtration and washed with a small quantity of dry DMF. Addition of a mixture of ethyl acetate and ethyl ether (3:2) to the filtrate resulted in precipitation of the product. Recrystallization from a mixture of benzene and acetonitrile (1:1) gave 0.19 g (47%) of white crystalline SSFSB: NMR (Me_2SO-d_6) δ 8.44 (d, J = 8.5 Hz, 2, ArH, ortho to FSO_2 group), 8.38 (d, J = 8.5 Hz, 2, ArH), 4.05 (d, J = 8.0 Hz, 1, CH), 2.9 (m, 2, CH_2); FAB-MS m/z [relative intensity 380 [89, M – Na], 203 [71, M – Su(SO_3)Na], 159 [61, M – Su(SO_3)Na – CO_2]. Anal. Calcd for $C_{11}H_7FNO_9S_2Na$: C, 32.75; H, 1.73. Found: C, 32.58; H, 1.98.

Measurement of the Stability of FSBAcid in Aqueous Solution. FSBAcid was dissolved to a final concentration of 0.5 mM in 100 mM HEPES, pH 8.0, which also contained 0.4 or 0.8 mM *N*-carbobenzoxy-L-threonine as an internal absorbance standard (Limbird et al., 1983). Solutions were incubated at 4 or 23 °C with periodic removal of 10-μL samples for analysis using the isocratic TBAF–methanol HPLC system described above. The pH of FSBAcid solutions was found to remain at 8.0 throughout the experiment.

Preparation and Characterization of SSFSB-Modified mEGF. A freshly dissolved solution of 40 mM SSFSB was added to 0.13 mg of mEGF in HEPES buffer to give final concentrations of 10 mM SSFSB, 0.09 mM mEGF, and 20 mM HEPES, pH 7.4, in a volume of 0.24 mL. The mixture was incubated at 23 °C, and the pH was observed not to fall below 6.5, as measured with pH indicator paper, over the course of the reaction. SSFSB-modified mEGF was separated from small molecules by gel filtration chromatography of an aliquot of the reaction mixture using a column containing 2 mL of water-swelled P2 gel filtration resin (Bio-Rad) in a bed length of 9.5 cm and maintained at 4 °C. The column was washed with ice-cold water, the excluded fraction was collected in a volume of 0.4 mL and diluted to 0.6 mL with water, and 0.5 mL was immediately injected on the Waters HPLC system, which had been preequilibrated in 2% solvent B. Elution of mEGF species was accomplished at a flow rate of 0.4 mL/min using a gradient which increased solvent B in linear segments to 28% at 10 min, 35% at 90 min, and 98% at 105 min after sample injection.

Alternately, SSFSB-modified mEGF prepared as described above but not purified by gel filtration was diluted to 0.6 mL with water and subjected to HPLC as before. Absorbances at 220 and 280 nm were monitored simultaneously in real time, and fractions of maximally 1.5 mL per fraction were collected by hand when significant absorbances appeared. For each lyophilized fraction, a LD-TOF mass spectrum was obtained using a matrix of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (Beavis & Chait, 1989). Sample ionization was achieved by 337-nm irradiation, with 8-ns laser pulses at a repetition rate of 5 Hz; each spectrum is the sum of 40 laser shots (Juhasz et al., 1992).

Stepwise Affinity Cross-Linking of ^{125}I-mEGF to the EGF Receptor. A freshly dissolved solution of 40 mM SSFSB was added to 19 μg of buffered ^{125}I-mEGF (specific activity 260 000 cpm/μg) to give a final concentration of 10 mM SSFSB, 0.08 mM ^{125}I-mEGF, and 20 mM HEPES, pH 7.4, in a volume of 40 μL. As a control, an equal amount of ^{125}I-mEGF of the same specific activity was incubated in an equal volume of buffer, but in the absence of SSFSB. After 20 min at 23 °C, 30 μL was removed from each reaction and applied to columns containing 2 mL of water-swelled P2 gel filtration resin (Bio-Rad) in a bed length of 9.5 cm and maintained at 4 °C. The reaction mixture was washed through the column with ice-cold water, and the excluded fraction containing modified ^{125}I-mEGF species was collected in a volume of 0.4 mL. Various amounts of the excluded fraction were applied to 3 μL of membrane vesicles from A431 cells suspended in 50 mM HEPES, pH 8.0, to give a total volume of 0.3 mL. To determine the specificity of cross-linking, an excess of nonradiolabeled, unmodified mEGF was present in parallel assays under all conditions. All assays were performed in duplicate.

After reaction had proceeded overnight at 4 °C, vesicles were pelleted in a microfuge (Eppendorf, Model 5414) for 6 min at 4 °C, and supernatants were discarded. Pellets were washed three times by suspension in 0.5 mL of ice-cold 50

mM HEPES, pH 8.0, followed by centrifugation and supernatant removal as before. Washed vesicles were solubilized with the addition of 60 μL of 1 × electrophoresis sample buffer containing 62.5 mM Tris, pH 6.8, 2% $NaDodSO_4$, 10% glycerol, 50 mM dithiothreitol, and 0.001% bromphenol blue and boiled for 10 min. The degree of covalent cross-linking was assayed by polyacrylamide gel electrophoresis and autoradiography. The radioactivity present in bands excised from dried polyacrylamide gels was determined with a Gamma 4000 counter (Beckman Instruments, Inc.).

Effects of Pretreatment of ^{125}I-mEGF with Methyl Acetimidate on Cross-Linking to the Receptor. A dried preparation of 40 μg of ^{125}I-mEGF (specific activity 210 000 cpm/μg) was dissolved in 20 μL of 2 M HEPES, pH 8.2; to this was added 20 μL of a freshly prepared, neutralized solution of 2 M methyl acetimidate hydrochloride (Aldrich). After incubation for 2 h at 50 °C, ^{125}I-mEGF species were separated from small molecules in the reaction mixture using P2 gel filtration at 23 °C as described above. The excluded P2 fraction (0.4 mL) was dried in a Speed Vac concentrator (Savant Instruments, Inc.), in parallel with an equal amount of unreacted ^{125}I-mEGF of the same specific activity. Both samples were subsequently treated with SSFSB, and ^{125}I-mEGF species were separated from the reaction mixture by gel filtration as described above. Various concentrations of modified ^{125}I-mEGF were applied to 5 μL of A431 membrane vesicles in a total volume of 0.2 mL of final concentration 50 mM HEPES, pH 8.0, and cross-linking was allowed to proceed for 3 h at 4 °C in the presence or absence of an excess of nonradiolabeled, unmodified mEGF. Vesicles were pelleted but not washed prior to solubilization, boiling for 5 min, electrophoresis, autoradiography, and measurement of radioactivity as described above. All assays were performed in duplicate.

Assays of EGF Receptor Autophosphorylation in the Presence of Cross-Linked mEGF. A431 vesicles were labeled essentially as described in the Stepwise Affinity Cross-Linking section, with the following modifications. Nonradiolabeled, SSFSB-modified mEGF (final concentration of 0.6 μM) was applied to 5 μL of A431 membrane vesicles suspended in 0.5 mL of a solution of 20 mM HEPES, pH 7.4, 0.1 mM sodium orthovanadate, and 1 mM EGTA. Equal volumes of vesicles containing no mEGF, or containing 0.6 μM nonradiolabeled, unmodified mEGF, were incubated in parallel in the same buffer. Incubation was carried out overnight at 4 °C. Subsequently, EGF receptor autophosphorylation was initiated in the entire reaction volume of unwashed vesicles on ice with the addition of 100 μL of phosphorylation buffer, to give final concentrations of 20 mM HEPES, pH 7.4, 1 mM $MnCl_2$, 5 mM $MgCl_2$, 0.1 mM sodium orthovanadate, and 40 μM ATP with 1.25 μCi of [γ-^{32}P]ATP per assay. EGF receptor autophosphorylation was quenched after 2 min with the addition of 0.2 mL of 4 × electrophoresis sample buffer (described above), followed by boiling for 15 min. All assays were performed in duplicate. Samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis, and autophosphorylation was assessed by autoradiography and by Cerenkov counting, using a Beckman LS 7500 counter, of EGF receptor bands excised from dried gels.

RESULTS AND DISCUSSION

Preparation of SSFSB. We prepared the cross-linking reagent SSFSB (Figure 1) and characterized this novel compound by negative-ion FAB-MS, NMR, and elemental analysis. Sulfo-*N*-succinimidyl esters in aqueous solutions react rapidly and in high yield with protein amino groups to form stable products (Anjaneyulu & Staros, 1987). The fluorosulfonyl group which comprises the second moiety of this cross-linker, however, is relatively less reactive in aqueous solutions (Limbird et al., 1983) but will react with lysyl (Roy & Colman, 1979; Zoller & Taylor, 1979; Likos et al., 1980; Weng et al., 1980; Russo et al., 1985), cysteinyl (Annamalai & Colman, 1981; Tomich et al., 1981; Togashi & Riesler, 1982), tyrosyl (Esch & Allison, 1978; Likos et al., 1980; Annamalai & Colman, 1981), and, by analogy to the chlorosulfonyl group, histidinyl and activated seryl (Paulos & Price, 1974) residues when bound at a site containing such a nucleophilic group.

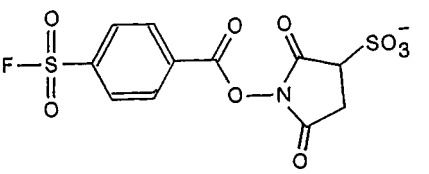

FIGURE 1: Structure of SSFSB.

Stability of the Fluorosulfonyl Group of FSBAcid in Aqueous Solution. Since SSFSB was designed to test a kinetically driven, stepwise affinity cross-linking technique, further characterization of the rate of hydrolysis of the fluorosulfonyl group was undertaken. At both 23 and 4 °C, the hydrolysis of FSBAcid was observed to follow first-order kinetics. At 23 °C, the rate constant was 2.6×10^{-3} min^{-1} (average of two experiments, with average $r = -0.998$), corresponding to a half-life of 4.5 h. A rate constant of 1.7×10^{-4} min^{-1} ($r = -0.91$) was obtained at 4 °C; the extrapolated half-life would be 2.8 days. These data suggested that the inherent stability of the fluorosulfonyl group of SSFSB-modified mEGF, particularly at low temperatures, was such that purification of the ligand modified with this group should be feasible. Since reaction of sulfo-*N*-succinimidyl active esters with amines is enhanced relative to hydrolysis by elevated temperatures (Anjaneyulu & Staros, 1987), a rational strategy for stepwise affinity cross-linking using SSFSB appeared to be rapid modification of ^{125}I-mEGF at its amino terminus by reaction with the sulfo-*N*-succinimidyl ester in a short incubation at room temperature, followed by removal of unreacted SSFSB by size-exclusion chromatography, performed at 4 °C to stabilize the fluorosulfonyl group incorporated into ^{125}I-mEGF. The strategy of the second linkage step called for applying SSFSB-modified ^{125}I-mEGF to the EGF receptor, with the low inherent reactivity of the fluorosulfonyl group offset by the high local concentration of nucleophiles present at the binding interface to give rise to high yields of specific cross-linking.

Preparation and Characterization of SSFSB-Modified mEGF. LD-TOF mass spectrometry data for the fractions corresponding to the two most prominent peaks from the separation of the reaction of SSFSB with mEGF (Figure 2) are presented in Figure 3. The major species in Figure 3a ($m/z = 6040.7$) was identified as unmodified mEGF; hence, it is clear that reaction of mEGF with SSFSB did not go to completion under these conditions. The peak in Figure 3b ($m/z = 6227.3$) is within an estimated error of 0.01% of the expected m/z value of 6226.9 for mEGF which has undergone reaction with the sulfo-*N*-succinimidyl moiety of SSFSB to yield mEGF bearing a single (fluorosulfonyl)benzoyl moiety, the desired reaction product. No products corresponding to mEGF which had reacted with the fluorosulfonyl group of SSFSB to yield a mEGF species bearing an intact sulfo-*N*-succinimidyl active ester were detected in HPLC fractions.

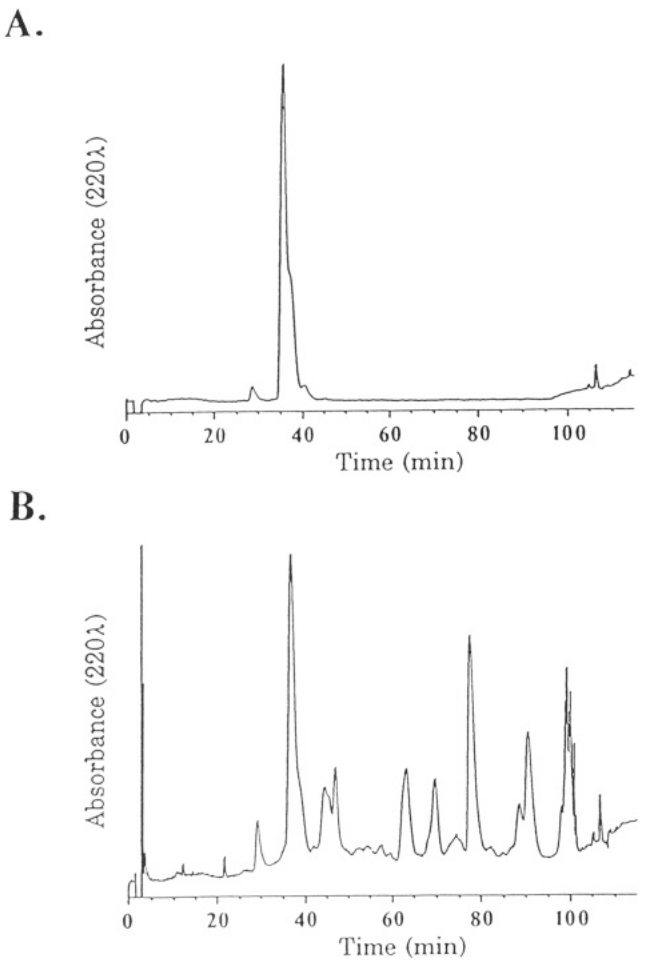

FIGURE 2: HPLC chromatograms of the products of the reaction of SSFSB with mEGF. The 220-nm absorbances of species present in mEGF preparations are depicted in panel A; these are intact mEGF (mEGFα), eluting at 37 min; des-Asn^1-mEGF (mEGFβ), eluting as a shoulder to the right of mEGFα; des-Asn^1,Ser^2-mEGF (mEGFγ), eluting as a secondary shoulder to the right of mEGFβ; and methionine sulfoxide forms of all mEGF species, eluting at 28–31 min. Panel B depicts the A_{220} profile of the products of the reaction of SSFSB and preparations of mEGF from which small molecules were removed by P2 gel filtration; the most prominent product (at 78 min) which does not comigrate with mEGF represents 13% of the A_{220} of all mEGF species. In a separate experiment in which the P2 gel filtration step was omitted, fractions corresponding to the peaks at 37 and 78 min were collected, lyophilized, and analyzed by LD-TOF mass spectrometry.

Stepwise Affinity Cross-Linking of ^{125}I-mEGF to the EGF Receptor. The formation of covalent cross-links between SSFSB-modified ^{125}I-mEGF and the EGF receptor is evident in Figure 4 from the comigration of radiolabel with the EGF receptor in denaturing gels. The specificity of the linkage is indicated by the competition for sites of cross-linking exerted by nonradiolabeled, unmodified mEGF. Spontaneous cross-linking of radiolabeled EGF to the EGF receptor has been reported to occur at low yields under some conditions (Baker et al., 1979; Linsley et al., 1979; Linsley & Fox, 1980; Comens et al., 1982; Marti et al., 1989). However, the cross-linking obtained here is mediated essentially entirely by SSFSB, since no significant cross-linking is observed when ^{125}I-mEGF is not modified with SSFSB before application to EGF receptor-containing vesicles (Figure 4, lanes 9 and 10).

The presence of a signal from ^{125}I-mEGF in the low molecular weight region of the autoradiograph of Figure 4 indicates that some ^{125}I-mEGF species bound to A431 membrane vesicles without subsequent covalent cross-linking to the EGF receptor, and the specificity of such binding is reflected in the ability of nonradiolabeled, unmodified mEGF to compete away this signal as well.

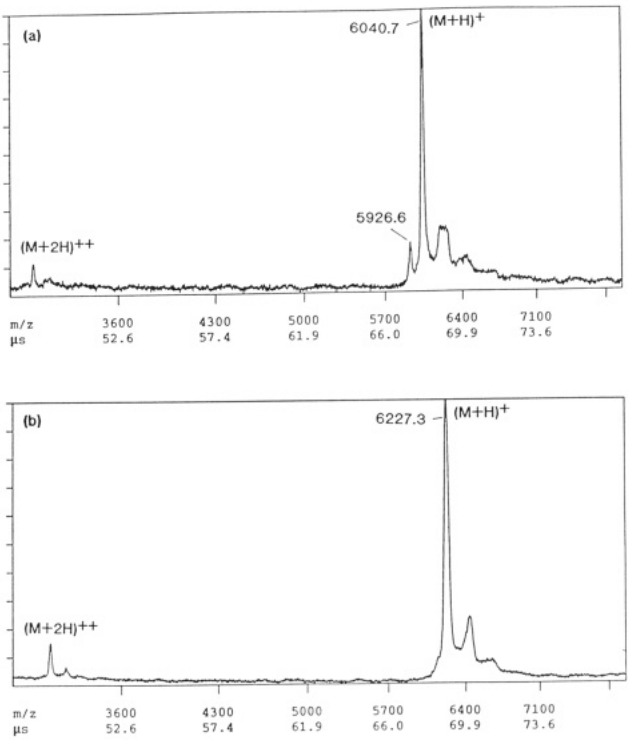

FIGURE 3: LD-TOF mass spectra of the two major HPLC fractions collected after reaction of SSFSB with mEGF. The peak at 206 units above the $(M + H)^+$ ion is due to the sinapinic acid matrix adduct. The HPLC fraction from which the spectrum of panel a is derived coeluted with unmodified mEGF standards, and the m/z = 6040.7 is that calculated for protonated mEGF. The m/z = 5926.6 value of the minor peak corresponds to that expected for protonated des-Asn-mEGF, a common contaminant of mEGF prepared as described. Panel b depicts the spectrum of the major product not coeluting with mEGF or the hydrolysis products of SSFSB in HPLC chromatograms.

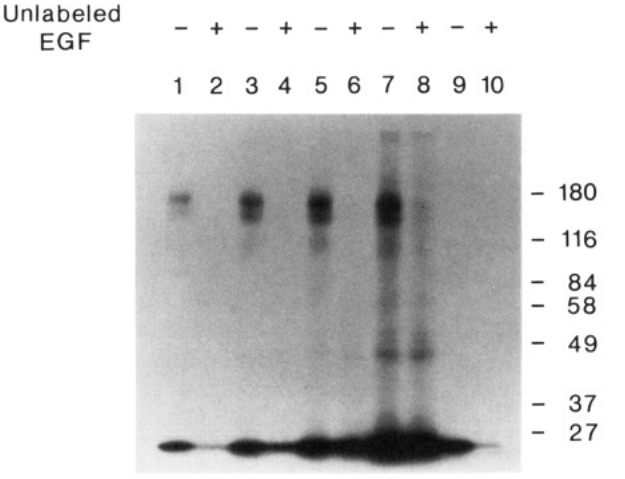

FIGURE 4: Stepwise affinity cross-linking of ^{125}I-mEGF to its receptor. Various concentrations of SSFSB-modified ^{125}I-mEGF were incubated with A431 vesicles in the absence or presence of excess (5.5 μM) nonradiolabeled, unmodified mEGF as indicated. The concentration of SSFSB-modified ^{125}I-mEGF was 0.03 μM for lanes 1 and 2, 0.1 μM for lanes 3 and 4, 0.3 μM for lanes 5 and 6, and 1 μM for lanes 7 and 8. As a control, 1 μM ^{125}I-mEGF of the same specific activity which had not been modified by SSFSB was incubated with A431 vesicles in lanes 9 and 10. Samples were separated by electrophoresis in $NaDodSO_4$-containing 7.5% polyacrylamide gels (Laemmli, 1970). The figure is an autoradiograph of the gel, which was dried without fixing. Molecular weight standards are indicated on the right.

A quantitative treatment of these results was accomplished by excising the region of dried polyacrylamide gels corresponding to the EGF receptor and ^{125}I-mEGF bands and counting the radioactivity associated with each band. The percentage of specifically bound ^{125}I-mEGF which is covalently cross-linked to the EGF receptor can be expressed as

$$[(\text{specific counts in receptor band}) \times 100]/(\text{specific counts in receptor band} + \text{specific counts in } ^{125}\text{I-mEGF band})$$

Specific counts in all cases are obtained by subtracting the signal in a given band in which cross-linking was performed with an excess of nonradiolabeled, unmodified mEGF from the signal in the corresponding band in which cross-linking was done without competing nonradiolabeled, unmodified mEGF. This calculation gives rise to values of 44%, 50%, 69%, and 66% in the presence of 0.03, 0.1, 0.3, and 1 μM SSFSB-modified ^{125}I-mEGF, respectively. In the absence of SSFSB, the value obtained for the specific spontaneous linkage of ^{125}I-mEGF to the EGF receptor was found to be less than 1%.

In all cross-linking experiments reported here, ^{125}I-mEGF and its derivatives are present at sufficiently high concentration to saturate the estimated number of receptors present in assays. Because preparations of SSFSB-modified mEGF contain a significant proportion of unmodified mEGF, it is likely that generally increasing yields of specific cross-linking with increasing concentrations of the modified ligand preparation are due to the higher rates of EGF receptor binding and/or higher turnover rates of ligands at higher concentrations of ^{125}I-mEGF species.

Effects of Pretreatment of EGF with Methyl Acetimidate on Cross-Linking to the EGF Receptor. ^{125}I-mEGF was pretreated with an imido ester, an amino-specific class of reagents, before derivatization with SSFSB and incubation with receptor preparations. If covalent linkage of ^{125}I-mEGF to the EGF receptor proceeds via SSFSB modification of the amino terminus, the only free amino group present in mEGF (Savage et al., 1972), such pretreatment could be expected to reduce cross-linking yields. To avoid denaturation of ^{125}I-mEGF under the conditions of temperature and pH necessary for quantitative modification by imido esters, relatively mild reaction conditions were employed, under which only partial modification of the α-amino group of ^{125}I-mEGF would be expected (Means & Feeney, 1971). A separate control experiment (data not shown) demonstrated that ^{125}I-mEGF heated to 50 °C in 1 M HEPES, pH 8.2, for 2 h could be cross-linked to the EGF receptor in a yield and at a specificity indistinguishable from that of ^{125}I-mEGF maintained at 0 °C in water. However, pretreatment of ^{125}I-mEGF with methyl acetimidate at 50 °C, pH 8.2, reduced the yield of subsequent SSFSB-mediated specific cross-linking of ^{125}I-mEGF to the EGF receptor to 37%, 41%, and 51% of that observed with unblocked controls in the presence of 0.1, 0.25, and 0.5 μM ^{125}I-mEGF species, respectively (Figure 5). As mentioned above, it is likely that exchange of ^{125}I-mEGF species in the binding site of the EGF receptor leads to yields of specific receptor labeling which are greater than the reactive fraction of ^{125}I-mEGF species in the reaction mixture. Hence, the large majority of sites in ^{125}I-mEGF which participate in the cross-link to the EGF receptor appears to have been blocked by methyl acetimidate under the conditions used in the blocking reaction, as a small proportion of ^{125}I-mEGF modified with SSFSB at the amino terminus would result in a higher yield of cross-linking than would be predicted by its proportion in the reaction mixture. Reagent specificity argues, then, that most cross-linking proceeds through the amino terminus of ^{125}I-mEGF. It should also be noted that residual cross-linking observed after prior reaction of ^{125}I-mEGF with methyl acetimidate is not necessarily due to modification by SSFSB of non-amino-terminal sites in ^{125}I-mEGF. The anticipated incomplete modification of ^{125}I-mEGF by methyl acetimidate would also account for residual cross-linking.

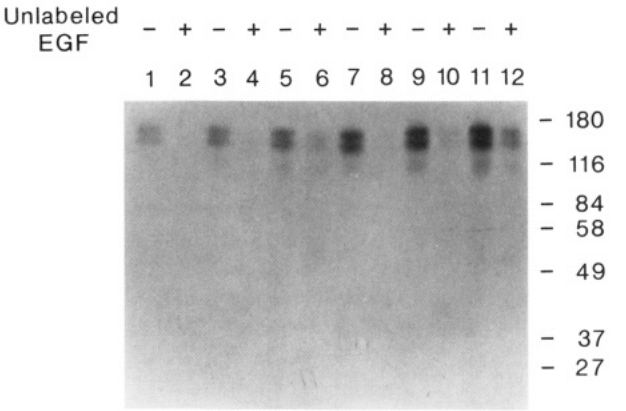

FIGURE 5: Inhibition of cross-linking of ^{125}I-mEGF to the EGF receptor by amino-terminal blockage of ^{125}I-mEGF. ^{125}I-mEGF was modified by SSFSB with (lanes 1–6) or without (lanes 7–12) prederivatization with methyl acetimidate. Various concentrations of product ^{125}I-mEGF (0.1 μM for lanes 1, 2, 7, and 8; 0.25 μM for lanes 3, 4, 9, and 10; and 0.5 μM for lanes 5, 6, 11, and 12) were applied to A431 vesicles in the absence or presence of excess (0.8 μM) nonradiolabeled, unmodified mEGF as indicated. Electrophoresis and autoradiography were performed as described in Figure 3. Molecular weight standards are indicated on the right.

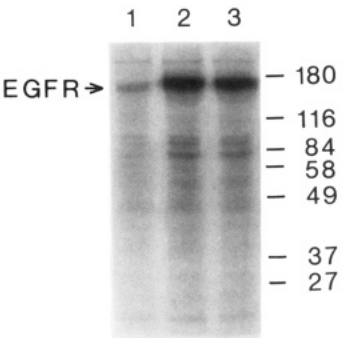

FIGURE 6: Autoradiograph of EGF receptor autophosphorylation in the presence of covalently linked mEGF. A431 vesicles were incubated without mEGF (lane 1), with 0.6 μM SSFSB-modified mEGF (lane 2), or with 0.6 μM unmodified mEGF (lane 3) and then assayed for autophosphorylation activity using [γ-^{32}P]ATP. Electrophoresis of 150 μL of quenched reactions and autoradiography of the dried polyacrylamide gel were performed as described in Figure 3. Molecular weight standards are indicated on the right, and the position of the EGF receptor (EGFR) is indicated on the left.

Assay of EGF Receptor Autophosphorylation in the Presence of Cross-Linked mEGF. A final concern in cross-linking experiments is the degree to which the functionality of cross-linked species remains intact relative to bound but unlinked species. This is related to the question of specificity of labeling addressed above, in that covalent modifications which do not contribute to the formation of specifically cross-linked complexes might impair protein function. The EGF-stimulated autophosphorylation of the EGF receptor was used as a measure of both a receptor function (kinase activity) and an EGF function (the stimulatory effect). Figure 6 demonstrates that nonradiolabeled mEGF which has been covalently linked to the EGF receptor with SSFSB stimulates receptor autophosphorylation to at least the same degree as an

equivalent concentration of unmodified mEGF. Parallel experiments (data not shown) using ^{125}I-mEGF established that the concentration of mEGF present in phosphorylation assays was 0.6 μM and revealed that 55% of specifically bound mEGF was covalently linked to the EGF receptor in the experiment involving SSFSB.

It is hoped that the kinetically driven, stepwise affinity cross-linking techniques developed here will aid in studies presently carried out with traditional cross-linking techniques, for example, in the identification of receptors and binding proteins. Additionally, the high specificity and yield of linkage afforded by the techniques described here may be of use in identification of sites or surfaces of interaction of proteins with high affinity for each other (Woltjer et al., 1992). The short span and relative inflexibility of the adduct of SSFSB with the ligand may aid in such experiments by limiting the functional groups accessible to the reactive group of the bound, modified ligand. Finally, the preservation of the function of cross-linked species may provide a way to examine effects of ligand binding on, for example, the biological activities and metabolic fates of "irreversibly" bound species.

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