Structure–Function Studies of Murine Epidermal Growth Factor: Expression and Site-Directed Mutagenesis of Epidermal Growth Factor Gene†

Prabir Ray,‡,§ Franklin J. Moy,‖ Gaetano T. Montelione,‖,⊥ Jin-Fu Liu,‡ Saran A. Narang,# Harold A. Scheraga,‖ and Ray Wu*,‡

Section of Biochemistry, Molecular and Cell Biology and Baker Laboratory of Chemistry, Cornell University, Ithaca, New York 14853, and Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario K1A 0R6, Canada

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ABSTRACT: Wild-type murine epidermal growth factor (mEGF) and mutants with Leu^{47} replaced by serine and valine, respectively, have been produced by recombinant DNA methodology. A synthetic gene for mEGF was fused to the coding sequence for the signal peptide of the outer membrane protein A (ompA) of *Escherichia coli* in the secretion vector pIN-III-ompA3, and the recombinant plasmid was used to transform *E. coli*. Upon induction of gene expression, mEGF and the mutants were expressed and secreted into the periplasmic space. Purification of the wild-type Leu^{47}-mEGF and the mutants was carried out by reversed-phase and anion-exchange high-performance liquid chromatography (HPLC). Amino acid analysis and Western blot analysis further confirmed the identities of the proteins. Specific activities for wild-type and mutant proteins were measured in both mEGF receptor binding and autophosphorylation assays. The recombinant mEGF has specific activities identical with that of mEGF purified from mouse submaxillary glands, while both mutants have reduced specific activities in both bioassays. The data demonstrate the importance of the highly conserved Leu^{47} residue in mEGF for full biological activity.

Epidermal growth factor (EGF)[1] is a potent stimulator of cell proliferation and differentiation in vitro (Cohen & Elliot, 1963; Cohen, 1965; Savage & Cohen, 1973) and an inhibitor of gastric acid secretion in vivo (Gregory, 1975). First identified in mouse submaxillary glands (Cohen, 1962), EGF has been the subject of extensive biological studies over the last 25 years. It has been proposed that EGF and EGF-like proteins play a central role in oncogenesis (Sporn & Todaro, 1980; Sporn & Roberts, 1985) and in wound healing (Buckley et al., 1985). EGF-like proteins are known to act synergistically with type β transforming growth factor to induce phenotypic cellular transformation (Sporn & Roberts, 1985). For these reasons, we are interested in understanding the molecular basis of EGF function and in developing methods to study the relationship between its three-dimensional structure and biological function.

The biological effects of EGF are mediated by a membrane-bound receptor protein. This receptor has been purified (Downward et al., 1984) and its gene cloned (Ullrich et al., 1984). It is homologous to both the v-*erb* B (Downward et al., 1984) and *neu* (Schechter et al., 1984; Coussens et al., 1985) oncogenes. The EGF receptor is associated with a tyrosine-specific kinase activity (Buhrow et al., 1982). EGF binding to the receptor results in a series of molecular events, including autophosphorylation of the receptor (Carpenter et al., 1979), culminating in mitogenesis.

Amino acid sequence data are available for murine (Savage et al., 1972), human (Gregory, 1975), and rat (Simpson et al., 1985) EGF. Both human and murine EGF contain 53 amino acid residues while rat EGF is smaller, lacking 5 carboxy-terminal residues. Other proteins have amino acid sequences that are homologous to EGF; these include type α transforming growth factor (TGFα) (Derynck et al., 1984; Lee et al., 1985), segments of vaccinia virus growth factor (Blomquist et al., 1984; Brown et al., 1985), Shope fibroma virus growth factor (Chang et al., 1987), Myxoma virus growth factor (Upton et al., 1987), and parts of several proteolytic enzymes involved in the molecular physiology of blood clotting (Banyai et al., 1983). Although crystal structures are not yet available for EGF or for any of its homologues, three-dimensional solution structures for murine (Montelione et al., 1986, 1987) and human (Carver et al., 1986; Cooke et al., 1987) EGF have been determined by NMR spectroscopy.

Site-directed mutagenesis provides a specific and versatile approach for structure–function studies. With this aim, we

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* Author to whom correspondence should be addressed.

‡ Section of Biochemistry, Molecular and Cell Biology, Cornell University.

§ Present address: Department of Molecular Oncology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021.

‖ Baker Laboratory of Chemistry, Cornell University.

⊥ Damon Runyon-Walter Winchell Cancer Foundation Postdoctoral Fellow, 1987. Present address: Biophysics Division, Institute for Science and Technology, University of Michigan, Ann Arbor, MI 48105.

Division of Biological Sciences, National Research Council of Canada.

[1] Abbreviations: mEGF, murine epidermal growth factor; Leu^{47}-mEGF, wild-type mEGF; hTGFα, human type α transforming growth factor; ompA, outer membrane protein A; YT, yeast extract–tryptone; RF, replicative form; gd, gapped duplex; ss, single stranded; DTT, dithiothreitol; NAD, nicotinamide adenine dinucleotide; dNTP, deoxynucleoside triphosphate; SSC, sodium saline citrate (0.15 M NaCl/0.015 M sodium citrate); SDS, sodium dodecyl sulfate; tRNA, transfer RNA; RPC, reverse-phase chromatography; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl thio-β-D-galactoside; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; BRL, Bethesda Research Laboratories.

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have expressed a synthetic gene for mEGF (Sung et al., 1986) in *Escherichia coli* using the pIN-III-ompA3 secretion vector (Ghrayeb et al., 1984). In this paper, we describe the construction of an mEGF secretion vector and the expression and purification of wild-type and mutant mEGF proteins. The mutant mEGF molecules are immunologically active and are recognized by antibodies against wild-type mEGF on Western blots (data not shown).

Simpson et al. (1985) have suggested that Leu^{47}, which is strongly conserved among the EGF-like growth factors, may play an essential role in determining the biologically active structure of mEGF. The homologous leucine (Leu^{48}) in hTGFα has been shown to be important for biological activity (Lazar et al., 1988). Our data demonstrate that the leucine residue at position 47 of mEGF is important for full mEGF activity. Changing this residue to either valine or serine appreciably reduces the affinities of receptors on the A-431 cell membrane or intact cells for the mutant proteins. However, substantial amounts of receptor binding activity remain.

Materials and Methods

Bacterial Strains and Plasmids. *E. coli* JA221 (Nakamura & Inouye, 1979) was used as the host strain in all expression studies with the recombinant plasmids. *E. coli* JM101 was used as the host strain for all experiments with phage M13 other than mutagenesis. The *E. coli* K-12 strain HB2154 (*ara*, Δ[lac-pro], *thi*-/F′ $proA^+B^+$, $lacI^q$, lacZΔM15, mutL::Tn10) obtained from Anglian Biotechnology (London) was used as the host strain for generating deletion and site-specific mutants. JA221 cells were grown in YT medium containing 8 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter. For growing cells containing plasmid, the medium was supplemented with 50 μg of ampicillin/mL. Plasmid pIN-III-ompA3 (Ghrayeb et al., 1984) was used as the starting material for all secretion vectors.

Synthesis of Oligonucleotides. The synthesis of the gene for mEGF has been described by Sung et al. (1986). Oligonucleotides used for site-specific mutations were synthesized by the phosphoramidite method (Beaucage & Caruthers, 1981) on an Applied Biosystems 38LB automated oligonucleotide synthesizer.

Oligonucleotide-Directed Deletion and Site-Specific Mutagenesis. A synthetic oligonucleotide, 5′-GTAGCGCAGGCCAACTCCTACCCC-3′, was used in deletion mutagenesis to remove the extraneous sequence between the ompA signal sequence and the mEGF gene. M13mp18 phage containing the mEGF gene and the signal peptide sequence cloned at the *Xba*I-*Bam*HI sites (M13mp18 mEGF1) was used as the starting material for deletion mutagenesis following essentially the gapped duplex (gd) method of Kramer and Fritz (1987). Duplex DNA, containing the mutagenic primer, was used to transfect competent HB2154 cells. The resulting plaques were blotted onto nitrocellulose filters and probed with 5′ ^{32}P-labeled primers (Maniatis et al., 1982) to screen for the desired mutants in which the extraneous DNA sequence between the ompA signal peptide and the mEGF gene had been deleted. Hybridization was carried out in a solution containing 6 × SSC, 1× Denhardt's solution, 20 μg/mL tRNA, and 0.05% sodium pyrophosphate at 65 °C. After hydridization, the filters were washed in 2× SSC containing 0.1% SDS twice for 20 min each at 75 °C.

For generation of mutations at amino acid position 47 (Leu) of mEGF, a 21-mer, 5′-ACCAGAGACGTGAGGTGGTGG-3′, was used for mutating Leu to Val and another 21-mer, 5′-ACCAGAGACTCGAGGTGGTGG-3′, for converting Leu to Ser. Transfection with gd DNA was carried out as for deletion mutagenesis. Mutants were screened by sequencing random plaques.

DNA Sequencing. Single-stranded DNA sequencing, used to verify the site-directed mutations, was carried out by following the dideoxynucleotide chain termination procedure of Sanger et al. (1977). For preliminary screening of mutants, only two of the usual four lanes were used, viz., T and G sequencing reactions for the Leu-to-Val mutation.

Isolation and Purification of mEGF from Periplasmic Fraction. *E. coli* JA221 cells, transformed with the recombinant expression vector pompA-mEGF2, were grown in YT medium containing 50 μg of ampicillin/mL. Cells were grown at 30 °C with shaking up to an OD_{550} of 1.6 (approximately 6×10^8 cells/mL). For induction of expression of mEGF, IPTG was added to a final concentration of 2 mM. The cells were incubated with shaking for another 2 h. Cells were collected by centrifugation at 6000*g* for 15 min at 4 °C and resuspended in $^1/_{10}$ volume of chilled 10 mM Tris-HCl, pH 7.5. Next, EDTA was added to a final concentration of 16 mM, and the suspension was incubated at 0 °C for 15 min; it was then centrifuged at 6000*g* for 30 min. The supernatant (100 mL from 1 L of cell suspension) was collected for purification of mEGF. A three-step purification procedure was developed on the basis of methods described by Burgess et al. (1982, 1983) for isolating mEGF from murine submaxillary glands. In the first step, concentration and crude purification of mEGF was obtained by applying the supernatant fraction (approximately 100 mL) directly to a 1.1 cm × 30 cm preparative LiChroprep (Bodman Chemicals) C8 reversed-phase chromatographic (RPC) column previously equilibrated with H_2O. A separate LiChroprep C8 column was used for the wild-type mEGF and for each of the mutants to avoid cross contamination. The column bed was then washed stepwise with 20-mL volumes of 0.1% TFA in H_2O/CH_3CN solvent mixtures containing 0%, 10%, 20%, 50%, and 100% CH_3CN, respectively. SDS–PAGE analysis indicated that most of the wild-type monomeric mEGF is eluted from this column in 20–50% CH_3CN.

The second purification step involved HPLC anion-exchange chromatography. The crude reversed-phase fractionated protein was lyophilized and redissolved in 5 mL of 20 mM Bis-Tris buffer at pH 6.5. A small amount of insoluble protein was removed by centrifugation at 1600*g*, and then the sample (with its pH adjusted to 6.5) was injected onto a 1 cm × 10 cm Mono Q (Pharmacia) anion-exchange HPLC column previously equilibrated with the same pH 6.5 Bis-Tris buffer. The mEGF protein was then eluted with a linear gradient of 0–100 mM NaCl at 2 mL/min over 60 min.

The final purification step involved desalting and purification with reversed-phase HPLC. The Mono Q purified pooled protein (with its pH adjusted to 2 with HCl) was applied directly to a 0.5 cm × 10 cm ProRPC (Pharmacia) C8 reversed-phase column equilibrated with 5% CH_3CN in 0.1% TFA. The mEGF protein was then eluted with a linear gradient of 5–30% CH_3CN at 1 mL/min over 40 min. The protein peak eluting with the same retention time as natural mEGF was collected, lyophilized, and characterized by amino acid analysis and bioassay.

Estimation of Protein Concentration and Amino Acid Analysis. The concentrations of solutions of wild-type Leu^{47}-mEGF were determined spectrophotometrically by measuring the absorbance at 278 nm in aqueous 0.1% TFA (ϵ_{278} = 19000 cm^{-1} M^{-1}; G. T. Montelione and H. A. Scheraga, unpublished result). Samples for amino acid

analysis were hydrolyzed in constant-boiling HCl and analyzed on a Waters Pico Tag amino acid analyzer.

SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Blot Analysis. SDS–polyacrylamide gels were run according to the method of Laemmli (1970), either in the presence (i.e., reducing) or in the absence (i.e., nonreducing) of 2-mercaptoethanol. For reducing gels, the disulfides of the samples were reduced with 5% 2-mercaptoethanol and boiled for 3 min. Silver staining of gels was carried out according to the manufacturer's (ICN) procedures. Western blot analysis was carried out by using the Bio-Rad Immuno-blot (GAR-HRP) assay kit containing goat anti-rabbit IgG horseradish peroxidase–conjugate, according to the manufacturer's procedures. Rabbit antiserum to mEGF and authentic (wild-type) mEGF were purchased from CalBiochem.

Biological Assays. EGF binding to membrane fractions was monitored as described by Carpenter et al. (1979). Membranes from A-431 cells were isolated, as described by Thom et al. (1977), and solubilization of the membranes was carried out as described by Cohen et al. (1980). The membrane fraction was incubated with varying amounts of mEGF prior to the addition f a constant amount (approximately 2 ng) of ^{125}I-mEGF. ^{125}I-mEGF (specific activity 150–200 μCi/μg) was purchased from New England Nuclear Corp. The reaction mixture contained 20 mM Hepes buffer, pH 7.4, and 0.1% BSA in a volume of 200 μL. Incubation was carried out at 25 °C for 30 min, after which labeled mEGF was added. After incubation for an additional 30 min, the reaction mixture was filtered rapidly on Millipore GSWP filters and washed extensively with the same buffer, and the radioactivity retained on the filters was determined. The procedure described by Schreiber et al. (1981) was used to study binding of mEGF and its mutants to intact A-431 cells. EGF-stimulated phosphorylation of proteins was examined in the Triton-solubilized membrane fraction essentially as described by Carpenter et al. (1979).

Results

Construction of ompA3-mEGF Secretion Plasmid. The plasmid vector pIN-III-ompA3 developed by Ghrayeb et al. (1984) was used as the starting material for the construction of an mEGF secretion vector. In this plasmid, any foreign DNA fragment can be cloned at the unique *Eco*RI, *Hin*dIII, or *Bam*HI sites immediately after the ompA signal peptide coding sequence. This places the cloned foreign gene under the control of both the lpp promoter and the lac promoter-operator. The synthetic mEGF gene (Sung et al., 1986), containing the two restriction sites *Eco*RI and *Bam*HI at its two ends, was ligated with *Eco*RI- and *Bam*HI-digested pIN-III-ompA3, to generate the recombinant plasmid pompA-mEGF1 (Figure 1). In order to remove the extraneous sequence between the ompA signal peptide and the first codon of the mEGF gene, the following steps were carried out, as described in the legend of Figure 1. Briefly, an *Xba*I–*Bam*HI fragment of pompA-mEGF1, containing the mEGF gene, was cloned into the *Xba*I and *Bam*HI sites of M13mp18 RF DNA to give M13mp18 mEGF1. To remove the extraneous sequence, the gapped-duplex method for deletion mutagenesis (Kramer & Fritz, 1987) was used, as described under Materials and Methods. After confirmation of the desired deletion by DNA sequencing, the restructured *Xba*I–*Bam*HI fragment of M13mp18 mEGF2 RF DNA, containing the ompA signal peptide sequence and the mEGF gene in the correct reading frame without any extraneous junction sequence, was reintroduced into the plasmid pIN-III-ompA3. This new plasmid was designated pompA-mEGF2. Plasmid

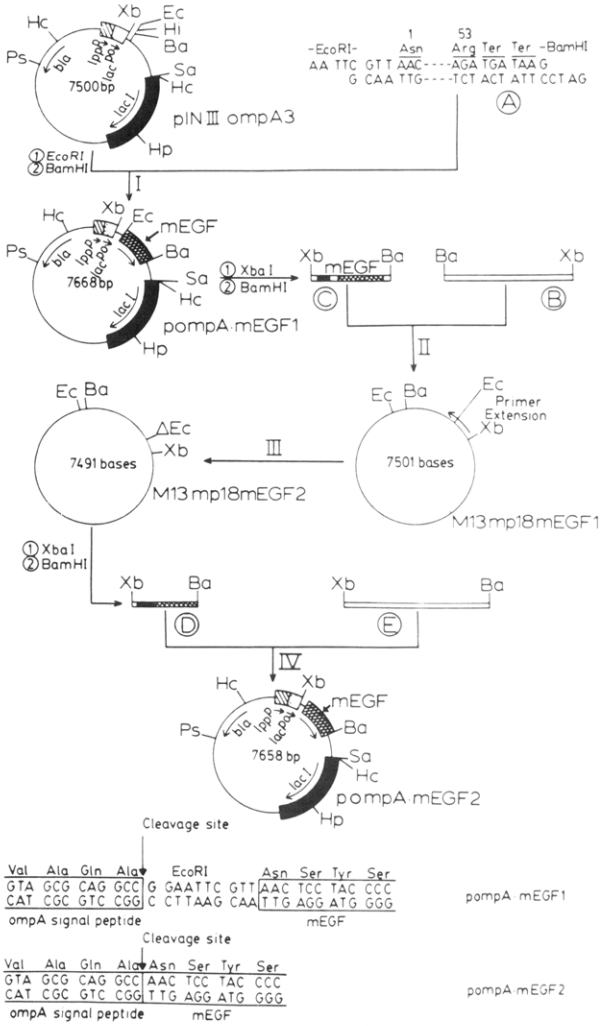

FIGURE 1: Construction of pIN-III-ompA3-based expression-secretion plasmid pompA-mEGF1 and pompA-mEGF2. The starting plasmid pIN-III-ompA3 was constructed by Ghrayeb et al. (1984); lpp^{P} and lac^{PO} denote the lpp promoter and lac promoter-operator, respectively. bla indicates the β-lactamase gene and lacI the lac repressor gene. Other abbreviations: Ba, *Bam*HI; Ec, *Eco*RI; Hc, *Hin*cII; Hi, *Hin*dIII; Hp, *Hpa*I; Ps, *Pst*I; Sa, *Sal*I; Xb, *Sba*I. In step I, *Eco*RI- and *Bam*HI-digested pIN-III-ompA3 was ligated with a synthetic mEGF gene (A) to give pompA-mEGF1. The cross-hatched region between the *Eco*RI and *Bam*HI sites contains the mEGF gene. In step II, M13mp18 RF DNA fragment, flanked by *Xba*I and *Bam*HI sites (B), was ligated with an *Xba*I–*Bam*HI fragment of pompA-mEGF1 (C) containing the mEGF gene (cross-hatched region) and the ompA signal peptide sequence (filled-in region). After transformation of *E. coli* JM101 cells with this ligation mix, recombinant ss phage DNA (M13mp18 mEGF1) was isolated. Deletion of the extraneous sequence in M13mp18 mEGF1 in step III generated M13mp18 mEGF2. The *Xba*I–*Bam*HI fragment of M13mp18 mEGF2 RF DNA, containing the mEGF gene and the ompA signal peptide coding sequence (D), was digested with *Xba*I and *Bam*HI and ligated with pIN-III-ompA3 (E) in the last step (IV) to give the final plasmid pompA-mEGF2.

pompA-mEGF2 was used to transform *E. coli* JA221 cells for expression studies.

Expression of mEGF. E. coli JA221 cells were transformed with either pompA-mEGF1 (used as a negative control) or pompA-mEGF2. To confirm the identity of the new M_r 6000 protein produced in cells harboring pompA-mEGF2, 5 mL of periplasmic extract was fractionated on a C_{18} Sep-Pak cartridge. The fractions were lyophilized and dissolved in 100 μL of water, and 10 μL from the 30% and 50% fractions was analyzed by SDS–PAGE (Figure 2A). The single band observed in the 30% CH_3CN fraction was analyzed further by Western blotting along with total periplasmic extract

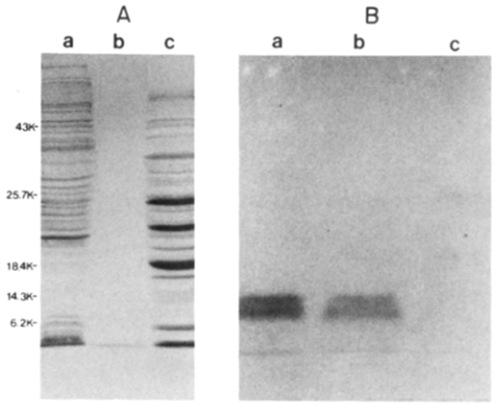

FIGURE 2: (A) SDS–PAGE analysis of total periplasmic extract of cells carrying pompA-mEGF2 (lane a) and 30% and 50% CH_3CN fractions of the same extract fractionated on a C_{18} Sep-Pak cartridge (lanes b and c, respectively). (B) Western blot analysis of 2 μg of authentic (receptor-grade) mEGF (lane a), 30% CH_3CN-fraction of periplasmic extract from 20 mL of cells harboring pompA-mEGF2 (lane b), and total periplasmic extract of cells carrying pompA-mEGF1 (lane c). In this case, the sample buffer did not contain 2-mercaptoethanol.

prepared from cells carrying pompA-mEGF1 (Figure 2B, lanes b and c). The result of the Western blot analysis showed the presence of an immunoreactive band in the 30% CH_3CN fraction (Figure 2B, lane b) of size identical with authentic (wild-type) mEGF run as a standard (Figure 2B, lane a). No such immunoreactive band was detected in lane c of Figure 2B, which contained the extract of the control pompA-mEGF1 expression vector in which the mEGF gene is not in a proper reading frame.

Site-Directed Mutagenesis of Leu^{47} *of mEGF to Ser*^{47} *and Val*^{47}. The amino acid leucine at position 47 of mEGF is highly conserved within the family of EGF-like polypeptides (Simpson et al., 1985; Upton et al., 1987) and may be essential for biological activity (Burgess et al., 1988; Lazar et al., 1988). To define the functional importance of this residue more precisely, we altered the codon TTG (for Leu) to TCG (for serine) and to GTG (for valine), respectively, by oligonucleotide-directed mutagenesis. The mutations were carried out as described under Materials and Methods. Mutations generated at the specific site were confirmed by DNA sequencing. *Xab*I–*Bam*HI fragments from recombinant M13mp18 containing sequences corresponding to ompA-mEGF2-Ser^{47} and ompA-mEGF2-Val^{47} were reinserted at the *Xba*I and *Bam*HI sites of the pIN-III-ompA3 vector. Expression of the mutant proteins was checked as described previously for wild-type mEGF by SDS–PAGE and Western blot analysis of periplasmic extracts.

Purification and Amino Acid Analysis of Secreted mEGF Molecules. Wild-type Leu^{47}-mEGF and its mutants (Ser^{47} and Val^{47}) were purified from periplasmic extracts according to a three-step procedure, as described under Materials and Methods. The mEGF peak, collected on the ProRPC-C8 column, was rerun on ProRPC-C8 to demonstrate its purity (Figure 3). A total of 100–150 μg of pure mEGF could be obtained from 1 L of *E. coli* cell culture containing 6×10^{11} cells. Amino acid analysis of the three purified mEGF molecules further confirmed the identities of the proteins (Table I).

Biological Activity of Recombinant mEGF and Its Mutants. The biological properties of recombinant mEGF and the Ser^{47} and Val^{47} derivatives were compared with those of authentic (wild-type) mEGF in (a) receptor binding to isolated A-431 cell membranes, (b) receptor binding to intact cells, and (c)

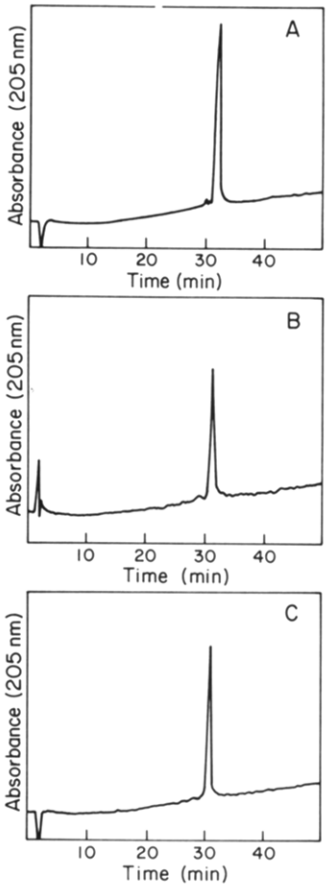

FIGURE 3: Panels A, B, and C are analytical reversed-phase HPLC chromatograms of wild-type Leu^{47}-mEGF, Val^{47}-mEGF, and Ser^{47}-mEGF, respectively, on a 0.5 cm × 10 cm Pharmacia ProRPC-C8 column eluted with a linear gradient of 5–40% acetonitrile at 1 mL/min over 45 min. Elution times for Leu^{47}-mEGF, Val^{47}-mEGF, and Ser^{47}-mEGF were 32.1, 31.3, and 30.6 min, respectively. Since the mutant proteins are resolved on the ProRPC-C8 column, these analytical reversed-phase chromatograms provide documentation for the absence of cross-contamination among the recombinant mEGF samples.

Table I: Amino Acid Analyses[a] of Wild-Type and Mutant Recombinant mEGF

	wild-type Leu^{47}-mEGF	Val^{47}-mEGF	Ser^{47}-mEGF
Asx	7.1 (7)	6.7 (7)	6.9 (7)
Glx	2.1 (3)	3.5 (3)	3.5 (3)
Ser	6.1 (6)	6.1 (6)	7.3 (7)
Gly	6.5 (6)	6.6 (6)	6.5 (6)
His	1.0 (1)	0.85 (1)	0.98 (1)
Arg	3.9 (4)	3.7 (4)	3.8 (4)
Thr	2.1 (2)	2.0 (2)	2.0 (2)
Ala	trace (0)	trace (0)	trace (0)
Pro	2.2 (2)	2.3 (2)	2.2 (2)
Tyr	4.5 (5)	4.7 (5)	4.5 (5)
Val	2.2 (2)	3.2 (3)	2.1 (2)
Met	1.0 (1)	1.1 (1)	0.84 (1)
Ile	2.1 (2)	2.1 (2)	1.9 (2)
Leu	3.9 (4)	3.2 (3)	3.2 (3)
Phe	0.1 (0)	0.01 (0)	0.05 (0)
Lys	0.1 (0)	0.04 (0)	0.04 (0)

[a] Cysteine and tryptophan were not determined.

membrane phosphorylation assays.

(*a*) *Competitive Binding to A-431 Cell Membrane Receptors*. Competitive binding assays were first carried out to determine the concentrations of the different mEGF's that inhibited 50% of the binding of ^{125}I-mEGF to receptor in isolated human A-431 cell membranes. The specific activities of authentic and recombinant (wild-type) mEGF's were found

Table II: Receptor Binding of mEGF and Its Mutants to Membranes Isolated from Human A-431 Cells and to Intact Cells

EGF	binding affinity (nM)	
	membranes[a]	intact cells[b]
authentic mEGF	1.2	1.0
Leu^{47}-mEGF	1.2	1.1
Val^{47}-mEGF	3.6	5.2
Ser^{47}-mEGF	8.3	18.0

[a] The binding affinity (IC 50) is taken as the EGF concentration causing 50% displacement of ^{125}I-mEGF (10 ng/mL) from membranes of human A-431 cells. The binding assay was carried out as described under Materials and Methods. Binding of ^{125}I-mEGF to membranes gave approximately 20 000 cpm in the absence of competing mEGF. [b] The binding assay with A-431 cells was the same as with isolated membranes, except that the concentration of the competing ^{125}I-mEGF was 5 ng/mL. Binding of ^{125}I-mEGF to cells gave approximately 10 000 cpm in the absence of competing mEGF. Data from three experiments varied by ±20% from the average results reported here.

to be identical. Approximately 1.2 nM recombinant Leu^{47}-mEGF was found to cause 50% displacement of labeled mEGF from the receptors (Table II), similar to authentic mEGF. However, the concentrations of Val^{47}-mEGF and Ser^{47}-mEGF required to cause 50% inhibition of binding of labeled mEGF were found to be 3-fold and 7-fold higher, respectively (Table II). In other words, Val^{47}-mEGF was three times less effective than Leu^{47}-mEGF in competing with ^{125}I-mEGF for binding to membrane receptors.

(*b*) *Competitive Binding to Intact A-431 Cells.* The concentrations of Val^{47}-mEGF and Ser^{47}-mEGF required to cause 50% inhibition of binding of labeled mEGF were found to be approximately 5-fold and 18-fold higher, respectively. Thus, the results of competitive binding of mutant mEGF's to receptors are within a 2-fold range when isolated cell membranes or intact cells are compared, and the Val^{47}-mEGF was about 3 times as effective as the Ser^{47}-mEGF in these competition assays.

(*c*) *Membrane Phosphorylation Assays.* We next examined the capacity of the different EGF's to stimulate phosphorylation of A-431 cell membrane proteins since this has been found to be an event triggered early by the interaction of EGF with its receptor (Ushiro & Cohen, 1980). We compared the biological activities of the mutant mEGF's, Val^{47}-mEGF and Ser^{47}-mEGF, with that of wild-type, i.e., Leu^{47}-mEGF. Densitometric scanning of the autoradiogram (Figure 4A) showed that the stimulation of phosphrylation effected by both the mutant proteins (lanes b and c) was only ca. 50% and 20%, respectively, of that brought about by the same amount of Leu^{47}-mEGF (lane d). The level of phosphorylation induced by 120 nM Leu^{47}-mEGF (Figure 4B, lane d) could be achieved by using about 200 nM Val^{47}-mEGF and 500 nM Ser^{47}-mEGF (Figure 4B, lanes b and c, respectively). Our result with Leu^{47}-mEGF is consistent with that of Cohen et al. (1980), who showed that 120 nM mEGF was required to elicit maximum stimulation of phosphorylation.

Discussion

mEGF Expression. Although there are many examples of expression of foregin proteins, including mEGF (Oka et al., 1985) and hTGFα (Derynck et al., 1984; Winkler et al., 1986), in the *E. coli* cytoplasm, there are many advantages to obtaining secreted proteins. These include (1) absence of any extra amino acid at the N-terminus, (2) less exposure of the protein to proteases in the periplasmic space, and (3) easier purification procedures. However, a combined expression–secretion system may not lead to the secretion of a desired protein even though the protein may be expressed successfully.

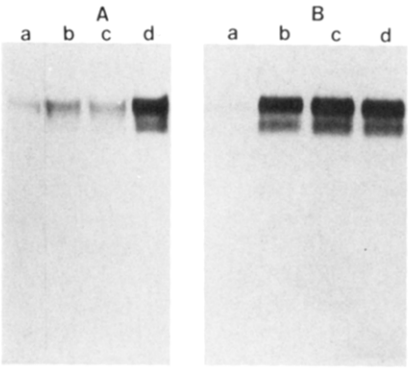

FIGURE 4: SDS–PAGE and autoradiography of Triton X-100 solubilized A-431 membranes following incubation with [γ-^{32}P]ATP and Val^{47}-mEGF, Ser^{47}-mEGF, and Leu^{47}-mEGF, respectively. (A) Triton X-100 extracts of A-431 cell membranes (25 μg of protein) were incubated with [γ-^{32}P]ATP (20 μM, 3 μCi) and 18 nM Val^{47}-mEGF, 40 nM Ser^{47}-mEGF, and 40 nM Leu^{47}-mEGF (lanes b, c, and d, respectively) in a total volume of 70 μL. Lane a is a control sample containing no mEGF. After incubation at 0 °C for 3 min, the reactions were stopped by the addition of an equal volume of 2× SDS sample buffer. The mixtures were boiled for 3 min, and 60 μL from each was subjected to SDS gel electrophoresis and autoradiography. (B) Results of a similar experiment carried out with 200 nM Val^{47}-mEGF (lane b), 500 nM Ser^{47}-mEGF (lane c), and 120 nM Leu^{47}-mEGF (lane d). Lane a is the control sample similarly incubated without mEGF.

In view of the success in high-level expression and proper secretion of *E. coli* β-lactamase (Ghrayeb et al., 1984), staphylococcal nuclease A (Takahara et al., 1985), and human growth hormone (Hsiung et al., 1986), with the pIN-III-ompA secretion vector developed by Inouye and his co-workers (Ghrayeb et al., 1984), we chose this vector as the starting material for constructing an mEGF secretion plasmid. Our results demonstrate synthesis and secretion of both wild-type mEGF and the mutants with this expression vector. In most cases, secreted proteins in *E. coli* can be recovered by the osmotic lysis method of Neu and Heppel (1965). We found that approximately 150 μg of the proteins could be recovered from approximately 6×10^{11} cells simply by suspending the cells in 10 mM Tris-HCl, pH 7.5, containing 16 mM EDTA at ca. 0 °C for 15 min. Since this amount of recovery was sufficient for our experimental purposes, we did not attempt to analyze other cellular fractions (e.g., membrane and cytoplasmic) in greater detail for a higher yield recovery of the proteins. Preliminary experiments which we carried out using SDS–PAGE, however, have shown that more protein can indeed by released by washing the cells with high salt (1 M Tris-HCl, pH 7.5) (data not shown) as was done in the case of staphylococcal nuclease A (Takahara et al., 1985).

Structure–Function Studies of mEGF. In an initial attempt to understand the relationships between the three-dimensional structure and function of EGF, we have compared the amino acid sequences of the several small proteins which are homologous to mEGF. As has been pointed out elsewhere (Simpson et al., 1985), aside from the six cysteine residues, most of the conserved residues common to all members of this EGF family, including Gly^{36}, Tyr^{37}, Gly^{39}, Arg^{41}, and Leu^{47}, are located in the polypeptide segment Cys^{31}–Leu^{47}. This sequence adopts a small "double-hairpin" β-sheet in the solution structure of mEGF (Montelione et al., 1986, 1987). Interestingly, the folding of the polypeptide chain brings the side chains of Tyr^{37} and Leu^{47} close to each other on the surface of the protein, suggesting that these two side chains might form part of a solvent-accessible active site.

Several other studies indicate that Leu^{47} may be essential for mEGF function. While the proteolytic fragments mEGF

(1–51), mEGF (1–48), and mEGF (1–47) retain native biological activity (Savage et al., 1972), the shorter proteolytic fragments mEGF (1–45) and mEGF (1–46) are inactive (Burgess et al., 1988). However, there is only a 3–7-fold diminution in the binding and mitogenic activities of mEGF (1–47). For the homologous human TGFα, site-directed mutagenesis also indicates that the corresponding Leu^{48} residue is important both for receptor binding and mitogenic activities (Lazar et al., 1988). Replacement of Leu^{48} by Ala results in complete loss of hTGFα activity, while both Ile^{48}-hTGFα and Met^{48}-hTGFα have very low activities (Lazar et al., 1988). These data indicate that Leu^{47} is important for mEGF activity, either directly in the interactions between mEGF and its receptor or indirectly in determining the active mEGF conformation. Structural studies of recombinant wild-type and mutant mEGF proteins are in progress.

In the present study, we have used the site-directed mutagenesis approach (with *E. coli*) to produce mEGF analogues in which Leu^{47} is replaced by either Val or Ser. The specific activities of HPLC-purified Val^{47}- and Ser^{47}-mEGF mutants are 20–33% and 6–14%, respectively, that of wild-type mEGF in both of the receptor-binding assays. Hence, replacement of Leu^{47} with a somewhat similar hydrophobic residue, Val, leads to greater biological activity than with a very different hydrophilic residue, Ser. The residual activity of both mutants, however, indicates that, although Leu^{47} may be involved in the mEGF/receptor interaction, other residues may substitute for leucine at position 47 of mEGF and provide substantial levels of biological activity.

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Conformation of Gramicidin in Relation to Its Ability To Form Bilayers with Lysophosphatidylcholine†

J. Antoinette Killian‡ and Dan W. Urry*

Laboratory of Molecular Biophysics, School of Medicine, University of Alabama in Birmingham, P.O. Box 311, Birmingham, Alabama 35294

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ABSTRACT: The ability of gramicidin to induce bilayer formation in lysophosphatidylcholine (LPC) systems was investigated as a function of the conformation of the peptide. The conformation was varied by using different solvents to cosolubilize gramicidin and lipid. Using circular dichroism (CD), it was found that when codissolved in trifluoroethanol (TFE), after drying and subsequent hydration, gramicidin is mainly present in the single-stranded $\beta^{6.3}$-helical configuration, whereas when using chloroform/methanol or ethanol as the solvent, it is proposed that the dominant conformation of gramicidin in the membrane is that of the double-stranded antiparallel dimer. Employing ^{31}P NMR, the stoichiometry for bilayer formation was found to be 6 to 7 lipid molecules per gramicidin monomer, when samples were prepared from TFE, whereas a stoichiometry of 4 was found when chloroform/methanol or ethanol was the solvent. Upon heating the latter samples, a conversion was observed in the CD pattern toward that indicative of the $\beta^{6.3}$-helical configuration. This change was accompanied by an increase in the extent of bilayer formation. Next, it was investigated whether the conformation of gramicidin and its ability to induce bilayer formation were dependent on the lipid acyl chain length. CD measurements of samples prepared from TFE indicated that gramicidin, independent of acyl chain length, was present in the $\beta^{6.3}$-helical configuration but the intensity of the ellipticities at 218 nm increased with the length of the acyl chain. The extent of bilayer formation in these samples was found to be largely chain length independent. In contrast, when ethanol was used as the solvent to codissolve peptide and lipid, the extent of bilayer formation increased with the chain length. CD measurements of these latter samples showed that the gramicidin molecules were now present in a dominantly "non-channel" configuration, most likely as an antiparallel dimer, and that again a chain length dependence was observed in the magnitude of the ellipticities at 218 nm. The results are discussed in terms of the possible mechanism of bilayer formation in dispersions of gramicidin and LPC in relation to the conformational behavior of the peptide.

Gramicidin is a linear, hydrophobic peptide that can form cation-selective transmembrane channels in model as well as in biological membranes [for review, see Andersen (1984) and Urry (1985a)]. It is produced by *Bacillus brevis* as a mixture of gramicidins A, B, and C in a molar ratio of about 80/5/15. The structure of gramicidin A is HCO-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-NHCH$_2$CH$_2$OH. In gramicidins B and C, the tryptophan at position 11 is replaced by phenylalanine and tyrosine, respectively (Sarges & Wilkop, 1965a–c; Gross & Witkop, 1965).

In addition to its channel properties, the hydrophobic pentadecapeptide gramicidin also has a dramatic effect on lipid structure [for a review, see Killian and De Kruijff (1986)]. In bilayer-forming lipids, such as diacylphosphatidylcholines, gramicidin can induce H_{II} phase formation, provided that the lipid acyl chain length is longer than 16 carbon atoms (Van Echteld et al., 1982). For this effect, the tryptophan residues

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‡ Present address: Department of Biochemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

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