The Human Urinary Epidermal Growth Factor (EGF) Precursor

**Isolation of a Biologically Active 160-Kilodalton Heparin-Binding Pro-EGF with a Truncated Carboxyl Terminus**

In this report, we describe the isolation from human urine of a predominant 160-kDa epidermal growth factor (EGF)-immunoreactive glycoprotein that exhibits affinity for heparin. The purification procedure involved concentration and dialysis of 20–30-liter batches of fresh human urine on a high capacity ultrafiltration apparatus followed by chromatography on DEAE-Sephadex, heparin-agarose, and Sephacryl S-300. A nearly homogeneous preparation of 160-kDa protein was obtained with a yield of approximately 1 mg of 160-kDa protein from 25 liters of urine.

The amino-terminal sequence of the purified 160-kDa protein, \(\text{H-N-SAPQHXSXPETGTXA}^-\), matched residues 21–34 of the predicted sequence of human prepro-EGF and established that the 160 kDa protein (pro-EGF) is a product of the prepro-EGF gene. Characterization of the carboxyl terminus of the purified protein by digestion with carboxypeptidase B and by immunoblotting with antisera against synthetic carboxy-terminal and juxtamembrane peptides of prepro-EGF indicated that the carboxyl terminus has been truncated at an arginine residue that corresponds, most likely, to the carboxy-terminal arginine of the EGF moiety.

The intact 160-kDa pro-EGF is biologically active as evidenced by its specific binding to the EGF receptor and activation of the EGF receptor tyrosine kinase in A-431 cell membranes. Purified pro-EGF competitively inhibited the binding of \(^{125}\text{I}-\text{EGF}\) to human fibroblasts, and it stimulated the proliferation of these cells in culture. When immobilized onto culture dishes, the heparin-binding pro-EGF appeared to function both as an adhesion molecule and as a growth factor for serum-free mouse embryo cells.

Epidermal growth factor (EGF) is a 53-amino acid polypeptide originally discovered in the mouse submaxillary gland as an agent that induced precocious eyelid opening and early incisor eruption in the newborn mouse (1). EGF has been subsequently shown to elicit an array of biological responses (reviewed in Refs. 2–4) that are mediated by specific binding to an EGF receptor/tyrosine kinase located on the cell surface (reviewed in Refs. 5 and 6). EGF is structurally and functionally identical to urogastrone, an inhibitor of gastric acid secretion (7), and is a member of a family of EGF-related growth factors (reviewed in Refs. 3 and 8 and 9). The soluble growth factors appear to originate from the extracellular domain of their respective transmembrane glycoprotein precursor (reviewed in Ref. 10).

Early evidence that EGF might be derived from a larger precursor arose from the observation of high molecular weight forms of EGF in urine (11) and from the observation that urogastrone might exist as a high molecular weight glycoprotein (12). Indeed, cloning of the EGF gene from mouse (13–14) and human (15) sources revealed cDNA that encodes precursors of 1217 and 1207 amino acids, respectively. Human prepro-EGF contains an EGF sequence that is flanked on its amino terminus by a 970-residue polypeptide and on its carboxyl terminus by a 184-residue segment. The large amino-terminal polypeptide contains 8 additional EGF-like regions, the biological significance of which is unknown, and the smaller carboxy-terminal segment contains a 25-residue hydrophobic transmembrane domain that serves to anchor the protein to the cell membrane.

 NIH 3T3 cells transfected with human prepro-EGF cDNA express the precursor as a 160–170-kDa glycoprotein that exists both as an intrinsic membrane-associated form and as a soluble form found in the conditioned medium (16, 17). Partially purified EGF precursor binds to the EGF receptor on cultured fibroblasts, activates the EGF receptor tyrosine kinase, and supports the growth of EGF-dependent mouse keratinocytes (17).

The mechanism whereby prepro-EGF is processed to mature EGF is not well understood. In the salivary gland, prepro-EGF appears to be processed intracellularly to mature 6-kDa EGF that is stored as a high molecular weight complex within secretory granules (1, 18, 19). In contrast, other organs such as the kidney contain mostly unprocessed precursor with relatively low levels of mature EGF (20). A 140-kDa EGF precursor has been isolated and partially characterized from mouse kidney membranes (21). Since urine contains relatively large amounts of mature 6-kDa EGF (22), which appears to arise from the kidney (23), the renal/urinary system may be a useful model for investigating the mechanism of prepro-EGF processing. Higher molecular weight EGF-immunoreactive glycoproteins have been detected in the urine of rodents (24) and humans (25–27) and were reported to exhibit EGF-like activity.

In the present report, we describe a procedure for the isolation of a heparin-binding 160-kDa EGF-immunoreactive glycoprotein from 20–30-liter batches of fresh human urine. Char-
aceration of the purified protein indicated that it is a pro-EGF and that it is derived from a membrane-anchored proprepro-EGF by amino-terminal cleavage of a 20-residue signal peptide and by carboxyl-terminal truncation at an arginine residue between the EGF moiety and the transmembrane domain. The intact 160-kDa pre-pro-EGF bound specifically to the EGF receptor and activated the EGF receptor tyrosine kinase in A-431 cell membranes, and it stimulated the proliferation of cultured human fibroblasts. When immobilized onto culture dishes, the heparin-binding pro-EGF appeared to function both as an adhesion molecule and as a growth factor for serum-free mouse embryo cells.

EXPERIMENTAL PROCEDURES

Materials—Sources of selected materials were as follows. Purified recombinant human EGF, Wakunaga Pharmaceutical Co.; WGL-Sepharose 6MB and heparin (type I) agarose; Sigma; DEAE-Sephalose and Sepharyl gel filtration media, Pharmacia Biotech Inc.; Extracti-Gel D and Sequanal grade N-ethylmorpholine, Pierce Chemical Co.; bovine pancreas phenylmethylsulfonyl fluoride-treated carbamoyl-

C3b, Separation of the purified protein indicated that it is a pro-

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EGF by amino-terminal cleavage of a 20-residue signal peptide and by

carboxyl-terminal truncation at an arginine residue between the EGF moiety and the transmembrane domain. The intact 160-kDa pre-pro-EGF bound specifically to the EGF receptor and activated the EGF receptor tyrosine kinase in A-431 cell membranes, and it stimulated the proliferation of cultured human fibroblasts. When immobilized onto culture dishes, the heparin-binding pro-EGF appeared to function both as an adhesion molecule and as a growth factor for serum-free mouse embryo cells.

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dase B, Worthington Biochemical Co.;[125I]-labeled Protein A, ICN Biomedicals; hors eradish peroxidase-conjugated Protein A, Boehringer Mannheim Co.; Pellicon cassette ultrafiltration apparatus, Millipore Co.; and YM-30 ultrafiltration discs and Centricon concentrators, Amicon Co. Commercial preparations of human urinary proteins were from Sigma (product U-8126) and Ortho Diagnostics (see Ref. 22). Human prepro-EGF C2 peptide (residues 1058–1081) and carboxy-
terminal peptide (residues 1188–1207) were custom synthesized by BioSynthesis, Inc.

Antisera—Polyclonal rabbit anti-recombinant human EGF serum was prepared in this laboratory as described (17). Polyclonal antiserum against human prepro-EGF C2 and carboxy-terminal peptides were prepared commercially by immunization of rabbits with keyhole limpet hemocyanin-conjugated peptide. Polyclonal rabbit anti-rat EGF serum was a gift from Dr. R. Savage, Temple University.

Cell Culture—Human fibroblasts (SFME cells) were grown as described by Loo et al. (29); for maintenance of stock SFME cultures, growth medium included 0.25% charcoal-treated and heat-inactivated calf serum.

Purification of the 160-kDa EGF Precursor from Human Urine—Freshly voided morning urine (20–30 liters) from random adult male donors was collected into plastic jugs. The pooled urine was concentrated to 2.2 ml by Amicon ultrafiltration (YM-30 membrane), diluted with 5 mimidazole, pH 7.0, to reduce the NaCl concentration to 15 m, and further concentrated to 500 μl. SDS and β-ME were added to give final concentrations of 2 and 5%, respectively. The sample was boiled for 5 min and then loaded onto a 1.5 × 30 cm column of Sephacyr S-300 equilibrated in 0.1 mM NaCl, 0.1% thioglycerol, 0.1% SDS, 5 mM imidazole, pH 7.0. Chromatography was carried out at room temperature with the same buffer. Fractions (1 ml) were collected, and 5-μl aliquots were assayed by SDS-PAGE and Coomassie Blue staining.

Amino-terminal Sequence Analysis—A 1.5-ml aliquot of heparin-

agarose-purified EGF precursor was concentrated to 120 μl by Centricon-

30, heated at 100 °C for 3 min with Laemml sample buffer, and then subjected to SDS-PAGE (7% acrylamide) using the MZE 3328.1J gel system as outlined by Moos et al. (30); gels were pre-electrophoresed in gel buffer that contained 0.1 mM thioglycolic acid. Resolved proteins were electro transferred (300 mA for 2.5 h) to polyvinylidene difluoride membranes in 10 mM CAPS buffer, pH 11. Protein bands were visualized with Coomassie Blue as described (31). The 160-kDa protein band was excised with a razor blade, and the membrane fragments were loaded onto a sample cartridge for sequence analysis. Automated Edman degradation was performed on an Applied Biosystems Model 470A gas-phase sequencer connected in-line with a Model 120A phenylthio-

hydantoin amino acid analyzer.

Carboxyl-terminal Amino Acid Analysis by Carboxypeptidase Digestion—The 160-kDa EGF precursor purified by Sephacyr S-300 chromatography (under denaturing conditions) was used as substrate for carboxypeptidase digestion; a 2.4-ml aliquot, equivalent to pooled fraction 21 contained ~20 μg of protein as estimated by SDS-PAGE and Coomassie Blue staining. The sample was mixed with 115 μl of Extracti-Gel D in a mini-column, incubated for 2 h at room temperature to remove SDS, and the flow-through fraction was concentrated to 100 μl in a Centricon-30. The sample buffer was changed to sequence grade N-ethylmorpholine (0.1 mM, pH 8.4) by repeated dilution and reconcentration. A 3-μl aliquot of phenylmethylsulfonyl fluoride-treated carboxypeptidase B (430 ng) was added, the mixture was incubated for 2 h at 37 °C, and the sample was ultrafiltered in the Centriprep to collect the liberated amino acids. Control samples that contained only enzyme or only substrate were processed in an identical manner. Amino acid content of the filtrate fraction was determined by phenylisothiocyanate derivatization followed by analysis of phenylthio-
carbamoylmethylamino acids by reverse phase high performance liquid chromatography (PicoTag system, Waters).

Induction of hEGF19 Cells and Preparation of Cell Membranes—Confluent cultures of hEGF19 cells in 100-mm dishes were rinsed with PBS and then incubated overnight in 4 ml of serum-free Dulbecco’s modified Eagle’s medium either with or without 5 mM sodium butyrate to induce the expression of prepro-EGF. To prepare membranes, cells were scraped from the dish in 0.5 ml of ice-cold PBS and then disrupted by 10 passages through a 22-gauge hypodermic needle. The homogenate was centrifuged at 800 rpm for 5 min, and the turbid supernatant fraction was centrifuged at 150,000 × g for 1 h (4 °C). The membrane pellet was resuspended in 20 ml Hepes, pH 7.4, to give a total protein concentration of ~10 mg/ml.

Other Procedures—SDS-PAGE (32) and immunoblotting procedures (21) were as described. For some immunoblots, detection was by ECL using horseradish peroxidase-conjugated protein A according to the manufacturer’s protocol. Autophosphorylation of the EGF receptor in A-431 membrane vesicles was determined essentially as described previously (33). EGF receptor binding was assayed by competitive inhibition of [125I]-EGF binding to cultured human fibroblasts essentially as described (28); the concentration of [125I]-EGF was 10 ng/1.5 ml of medium (34).

RESULTS

Identification of a 160-kDa EGF-immunoreactive Glycoprotein in Human Urine—We have confirmed the presence of high molecular weight EGF-immunoreactive glycoproteins in human urine by SDS-PAGE and immunoblotting with a polyclonal antiserum against recombinant human EGF. Using WGL affinity chromatography to isolate glycoproteins from fresh urine, immunoblotting revealed a predominant EGF-immunoreactive protein of ~160 kDa and a lesser amount of an ~70-kDa protein (Fig. 1A, lane 1). (The apparent M, of both immunoreactive species was slightly lower, and the level of immunoreactivity somewhat greater, if the proteins were not reduced with β-mercaptoethanol prior to SDS-PAGE and im-
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Fig. 1. Identification of EGF-immunoreactive proteins in human urine. A, fresh urine samples (10 ml) were incubated overnight at 4°C with 100 μl of WGL-Sepharose 6MB. Adsorbed proteins were eluted by boiling in Laemmli sample buffer, resolved by SDS-PAGE (7%), electrotransferred to nitrocellulose, and immunoblotted with rabbit polyclonal antiserum against human EGF, 1:30 dilution. In lane 2, a 33-μl aliquot of antiserum was preincubated with 10 μg of recombinant human EGF overnight at 4°C prior to immunoblotting. B, fresh human urine (50 ml) was dialyzed against water at 4°C using 2-kDa molecular mass cutoff tubing, lyophilized, and reconstituted with 0.5 ml of water. A 40-μl aliquot was boiled for 3 min in Laemmli sample buffer, subjected to SDS-PAGE (6–12% gradient gel), electrotransferred, and blotted with human EGF antiserum, 1:500 dilution. DF, dye front.

The 160-kDa EGF-immunoreactive protein in dialyzed urine was similar to that in an equivalent amount of urine adsorbed onto WGL-Sepharose and suggests that nearly all of the urinary 160-kDa EGF-immunoreactive protein is glycosylated. Similar immunoblots were obtained with a commercially available (Sigma) preparation of dialyzed and lyophilized human urine, and with an acetone powder of human pregnancy urine (22) that was utilized previously for the isolation of mature 6-kDa EGF.

In contrast to the relatively large amounts of high molecular weight EGF-immunoreactive proteins in fresh urine or urine powders, only trace amounts of mature EGF were detected by immunoblotting. Similar findings were reported by Lakshmanan et al. (26) and were ascribed to the relative insensitivity of the immunoblotting procedure to detect mature EGF.

Comparison of fresh urine samples from 8 adult donors (7 male and 1 female) revealed the presence of the 160-kDa EGF-immunoreactive protein in each specimen with only minor variations in the total amount of immunoreactive material.

Purification of the 160-kDa EGF-immunoreactive Protein from Human Urine—To define more firmly the chemical and biological properties of the urinary 160-kDa EGF-immunoreactive protein, we attempted its isolation. For rapid concentration of large volumes of urine, we utilized a high capacity Pellicon cassette ultrafiltration apparatus equipped with a 30-kDa cut-off low protein binding membrane. A 100-fold concentration of 20 liters of urine was achieved in ~1 h, thereby minimizing protein degradation as well as the formation of precipitates that may occur when urine stands for prolonged periods. During dialysis of the retentate fraction, it was essential that the buffer contained at least 0.1 M NaCl because lower salt concentrations caused the 160-kDa EGF-immunoreactive protein to aggregate.

Ultracentrifugation of the dialyzed retentate at 180,000 × g for 4 h produced a loose gel-like pellet; greater than 90% of the 160-kDa EGF-immunoreactive protein remained in the supernatant fraction as determined by immunoblotting. Analysis of the pellet fraction by SDS-PAGE and Coomassie Blue staining revealed that it contained a relatively large amount of an 80–100-kDa protein. This protein comigrated on SDS-PAGE with a purified sample of Tamm-Horsfall glycoprotein (uromodulin), an abundant urinary protein with EGF-like sequences that forms high molecular weight aggregates in solution (34).

After ultracentrifugation, the supernatant fraction was chromatographed on DEAE-Sephaloc. Immunoblot analysis revealed that the 160-kDa EGF-immunoreactive protein was retained by the DEAE-Sephaloc with little or no material in the flow-through fraction (Fig. 2A, lanes 1 and 2). The bulk of the immunoreactive material was eluted with 0.5 M NaCl (lane 3). On Coomassie Blue staining, a 160-kDa protein band was detected in the original retentate fraction (Fig. 2B, lane 1) and in the DEAE-eluate (lane 3), but not in the flow-through fraction (lane 2). Based on Coomassie Blue staining, the recovery of 160-kDa protein from DEAE-Sephaloc was greater than 75%.

The 160-kDa protein in the DEAE-eluate exhibited salt-dependent binding to heparin-agarose, and this interaction was utilized for further purification. The DEAE-eluate was desalted to 90 mM NaCl, adsorbed onto heparin-agarose, and the 160-kDa protein eluted with 0.5 M NaCl. Immunoblot analysis of the heparin-agarose fractions revealed the presence of the 160-kDa immunoreactive protein in the eluate (Fig. 2A, lane 5) with little or no immunoreactive material in the flow-through (lane 4). Coomassie Blue staining of the heparin-agarose fractions indicated that the 160-kDa protein bound selectively to heparin-agarose since the bulk of the protein was present in the flow-through fraction (Fig. 2B, lanes 4). In the heparin-agarose
eluate (lane 5), the 160-kDa protein corresponded exactly to the 160-kDa EGF-immunoreactive band detected on immunoblots and represented 40–60% of the total protein. The overall recovery at this stage, as estimated by Coomassie Blue staining, was ~1 mg of 160-kDa protein from 25 liters of fresh urine.

Gel Filtration Chromatography of the Heparin-Agarose-purified EGF Precursor—The heparin-agarose-purified 160-kDa protein was analyzed by gel filtration chromatography on Sephacryl S-200. Under native conditions, the 160-kDa protein eluted near the void volume with little or no separation from other lower molecular mass proteins as detected by SDS-PAGE and Coomassie Blue staining of a denatured sample of each column fraction (data not shown). Similar results were obtained when gel filtration was performed in the presence of 1 M NaCl or 0.1% thioglycerol, suggesting that in the heparin-agarose eluate the 160-kDa protein may be a component of a heterogeneous high molecular weight complex.

To purify further the 160-kDa protein, an aliquot of heparin-agarose eluate was denatured by boiling in SDS (2%) and β-mercaptoethanol (5%) and then subjected to gel filtration chromatography on Sephacryl S-300 in buffer that contained 0.1% SDS and 0.1% thioglycerol. Under these conditions, the 160-kDa protein eluted with significant resolution from lower molecular mass proteins as detected by SDS-PAGE and Coomassie Blue staining of column fractions (Fig. 3). Fractions 25 and 26 contained nearly homogeneous 160-kDa protein.

Amino-terminal Sequence Analysis of the Urinary 160-kDa EGF Precursor—To confirm that the urinary 160-kDa EGF-immunoreactive protein is a product of the human prepro-EGF gene, we determined a partial amino acid sequence of the purified protein. Heparin-agarose-purified material was resolved by SDS-PAGE and electrotransferred to polyvinylidene difluoride, and the 160-kDa band was excised and subjected to automated Edman degradation. Sequencer runs A and B represent the entire 160-kDa band from the first polyvinylidene difluoride sheet and approximately one-third of the band from the second sheet, respectively. The yield of Ser-PTH from cycle 1 (runs A and B combined) was calculated to be 36% of the initial gel load of 125 pmol of 160-kDa protein.

| Cycle number | Amino acid recovered | Yield of PTH-derivative |
|--------------|----------------------|------------------------|
|              |          | Run A | Run B |
| 1            | Ser      | 17.3  | 9.4   |
| 2            | Ala      | 23.1  | 5.8   |
| 3            | Pro      | 10.3  | 2.6   |
| 4            | Gln      | 16.4  | 3.3   |
| 5            | His      | 1.3   | ND*   |
| 6            | Xb       |       |       |
| 7            | Ser      | 3.3   | 1.7   |
| 8            | Xb       |       |       |
| 9            | Pro      | 7.0   | 1.8   |
| 10           | Glu      | 2.0   | ND*   |
| 11           | Gly      | ND    | 3.4   |
| 12           | Thr      | 4.6   | 1.9   |
| 13           | Xb       |       |       |
| 14           | Ala      | 2.0   | ND*   |

*Total picomoles of recovered PTH-derivative corrected for background.

To define the site of carboxyl-terminal modification, we have prepared a second polyclonal antiserum against a synthetic peptide (designated C2) that corresponds to a cytoplasmic region of prepro-EGF (residues 1058–1081) immediately adjacent to the transmembrane domain. In hEGF19 cells, where the expression of prepro-EGF is induced by sodium butyrate (16), this antiserum blotted a 160-kDa protein in membranes isolated from butyrate-induced, but not uninduced, cells (Fig. 4, lanes 3 and 4); blotting of the 160-kDa protein was blocked by preincubation of the antiserum with excess C2 peptide, and preimmune serum was nonreactive (data not shown). In contrast, the antiserum exhibited no detectable immunoreactivity toward the heparin-agarose-purified EGF precursor (lane 2). These findings suggest that the 160-kDa urinary EGF precursor contains a truncated carboxyl terminus, with the processing site located between the EGF moiety and the juxtamembrane C2 region.

To establish more precisely the site of carboxyl-terminal processing, we have subjected the urinary EGF precursor to carboxypeptidase digestion in order to determine the carboxyl-terminal residue. Since proteolysis of the precursor by a presumed arginine esterase would result in a carboxyl-terminal arginine, digestion was carried out with carboxypeptidase B, which preferentially cleaves arginine or lysine residues. When
Sephacryl S-300-purified 160-kDa EGF precursor (125 pmol) was incubated with 0.43 μg of carboxypeptidase B for 2 h at 37°C, approximately 110 pmol of arginine was recovered; little or no arginine was recovered from reaction mixtures that lacked either enzyme or substrate. Also recovered was a small amount of tyrosine, which is of uncertain significance. The results suggest that arginine is the carboxyl-terminal residue of the urinary 160-kDa pro-EGF.

Binding of Intact Urinary Pro-EGF to the EGF Receptor and Stimulation of EGF Receptor Autophosphorylation—To ascertain whether the intact urinary pro-EGF binds to the EGF receptor, its interaction with the EGF receptor in A-431 cell membranes was examined. Urinary pro-EGF was incubated with A-431 membrane vesicles at 0°C and, after pelleting the membranes by centrifugation, the presence of pro-EGF in the soluble and membrane fractions was determined by immunoblotting with EGF antigen. As shown in Fig. 5, incubation of pro-EGF with A-431 membrane vesicles resulted in a loss of the 160-kDa protein from the supernatant fraction (lanes 1 versus 2) and a concomitant appearance of the protein in the membrane pellet (lane 4 versus 5). Binding of the 160-kDa pro-EGF to the membrane vesicles represented specific binding to the EGF receptor since binding was completely blocked by preincubation of the membranes with an excess (10 μg) of purified EGF. We conclude that the intact 160-kDa pro-EGF binds to the EGF receptor in A-431 cell membranes.

To assess further the interaction of purified urinary pro-EGF with the EGF receptor, its ability to stimulate EGF receptor autophosphorylation was examined. In a standard phosphorylation reaction mixture that contained A-431 membrane vesicles and 32P-labeled ATP, addition of 50 μl (0.5 μg) of purified urinary pro-EGF resulted in a 2- to 3-fold increase in 32P incorporation into the 170-kDa EGF receptor (data not shown). The degree of stimulation of EGF receptor phosphorylation was similar to that obtained with 6 ng of pure recombinant human EGF. Since the phosphorylation reaction was carried out at 0°C, a temperature where little or no processing of the EGF precursor would be expected, the results again suggest that the intact urinary pro-EGF binds to the EGF receptor and, in the presence of ATP, stimulates receptor autophosphorylation.

Biological Activity of Purified Urinary Pro-EGF in Cultured Human Fibroblasts—To assess the biological activity of urinary pro-EGF toward intact cells, its interaction with cultured human fibroblasts was examined. In a standard competitive inhibition binding assay, heparin-agarose-purified pro-EGF inhibited the binding of 125I-EGF to human fibroblasts; a 25-μl aliquot (0.5 μg of 160-kDa protein) was equivalent to ~8 ng of pure EGF (Table II). Similar results were obtained with an aliquot of heparin-agarose-purified pro-EGF that was chromatographed on Sephacryl S-200 to remove any traces of mature EGF.

The purified pro-EGF had a stimulatory effect on the growth of cultured human fibroblasts. When cells were incubated over a 10-day period with 100 μl of heparin-agarose-purified pro-EGF/ml of medium, there was a 1.6-fold increase in cell number as compared to control cultures (Table II). The stimulation by pro-EGF was slightly less than the 1.9-fold stimulation observed with 10 ng/ml pure EGF.

Based on the molar concentration of 160-kDa protein in the heparin-agarose eluate, as estimated by SDS-PAGE and Coomassie Blue staining, the urinary pro-EGF was calculated to possess ~50% of the activity of mature EGF as assayed by competitive inhibition of 125I-EGF binding to cultured fibroblasts and by stimulation of EGF receptor autophosphorylation in A-431 cell membranes.

Stimulation of Serum-free Mouse Embryo (SFME) Cell Adhesion and Growth by Immobilized Pro-EGF—To examine further the biological activities of urinary pro-EGF, we utilized cultured SFME cells that require EGF for growth and also depend on polylysine or fibronectin for efficient cell attachment (29). We have confirmed these properties of SFME cells and, in preliminary experiments, have observed that purified pro-EGF effectively substituted for EGF in the stimulation of SFME cell growth; in the absence of supplemental EGF or pro-EGF, the cells detached from the dish and lost viability within 1–2 days (data not shown).

To examine the possibility that the heparin-binding pro-EGF
The results suggest two possible molecular structures for the urinary 160-kDa EGF (Fig. 7, A and B). Form B, terminating at Arg-1023, is the more likely since the 160-kDa pro-EGF in urine is a soluble protein and would be expected to lack the transmembrane domain (residues 1033–1057). It is unlikely that the truncation site would be at an arginine further upstream from Arg-1023, e.g. at Arg-1011 or -1015 (see Ref. 15), since this would truncate the EGF sequence and compromise biological activity. A human EGF analog, hEGF<sub>1–46</sub>, that lacks the 7 carboxyl-terminal residues of full-length hEGF, is reported to possess less than 0.5% of the biological activity of intact hEGF (36).

A specific proteolytic enzyme that cleaves the carboxyl terminus of the membrane-anchored EGF precursor, with release of soluble pro-EGF from the transmembrane domain, has not been described. However, there is some evidence to suggest the involvement of a membrane-associated endoprotease. Using hEGF<sub>19</sub> membranes, autolysis for 1 h at 37 °C resulted in the formation of a soluble EGF precursor that was of similar molecular mass (on SDS-PAGE) to the EGF precursor extracted from the membranes with detergent (17). Prolonged autolysis (24 h at 37 °C) of rat kidney membranes released two forms of soluble EGF-immunoreactive material, and this process was inhibited by aprotinin (37).

Further processing of the 160-kDa pro-EGF to lower molecular mass species may occur within the lumen of the renal tubule and/or in the urine per se. Kallikreins and other endopeptidases have been described in urine (38), and some of these may be active toward urinary pro-EGF. Purified mouse submaxillary gland EGF binding protein (18), also known as mouse glandular kallikrein-9, was shown to process mouse kidney EGF precursor to a 97-kDa species (21), and rat submaxillary gland kallikreins K1, K7, and K10 were also shown to process a rat urinary 45-kDa EGF precursor to an approximately 6-kDa EGF species (39). In the present study, it was of interest that the native pro-EGF appeared to be a component of a high molecular weight complex, as estimated by gel filtration chromatography of the heparin-agarose eluate. It is possible that association of pro-EGF with specific urinary proteins could act both as a growth stimulant and as an adhesion molecule, we precoated a portion of a standard culture dish with heparin-agarose-purified pro-EGF prior to plating the dish with SFME cells (in medium without exogenous serum or EGF). This treatment resulted in cell attachment and growth only in the area coated with pro-EGF; a typical culture dish with heparin-stained cells is shown (Fig. 6). We conclude that, when immobilized on a culture dish, the heparin-binding pro-EGF functions both as an adhesion molecule and as a growth stimulant for SFME cells.

**DISCUSSION**

The production of peptide growth factors from the extracellular domain of a membrane-anchored precursor is a common theme in cell biology (reviewed in Refs. 10 and 35). While details of precursor processing have been elucidated for several growth factors, relatively little is known about the mechanism of processing of pro-prepro-EGF to mature EGF. Since prepro-EGF and mature EGF are relatively abundant in kidney and urine, respectively, the renal/urinary system may serve as a useful model for understanding the mechanism of prepro-EGF processing.

In the present study, after confirming the presence of high molecular weight EGF-related glycoproteins in human urine (25–27), the urinary 160-kDa EGF-immunoreactive protein was isolated and characterized. The results established that this protein is indeed a product of the human prepro-EGF gene, and that the amino-terminal serine residue of the 160-kDa protein corresponds to Ser-21 of the published sequence of human prepro-EGF (15). The initial 20-residue hydrophobic sequence of prepro-EGF was presumably cleaved by the action of a signal peptidase.

Characterization of the carboxyl terminus of the 160-kDa protein by carboxypeptidase digestion and by immunoblotting with antisera against synthetic carboxyl-terminal and juxtamembrane C2 peptides (prepro-EGF residues 1188–1207 and 1058–1081, respectively) indicated that the carboxyl-terminal residue is an arginine and is located amino-terminal to the juxtamembrane C2 region. The cDNA sequence of human prepro-EGF predicts two arginine residues in a potential truncation region between the EGF moiety and the C2 sequence; Arg-1023 corresponds to the carboxyl terminus of the EGF moiety itself, and Arg-1058 corresponds to the first arginine on the cytoplasmic side of the transmembrane domain. The results suggest two possible molecular structures for the
might serve to regulate its processing to mature EGF.

We have shown that the urinary 160-kDa pro-EGF is biologically active as evidenced by its specific binding to the EGF receptor, activation of the EGF receptor tyrosine kinase, and stimulation of the proliferation of cultured human fibroblasts. Our data with A-431 cell membranes demonstrate that it is the intact 160-kDa pro-EGF that binds to the EGF receptor; binding did not require conversion of pro-EGF to a lower molecular mass species. High molecular weight EGF precursors extracted from hEGF19 cells (17) and mouse kidney membranes (21) have also been shown to possess biological activity. While several EGF-related growth factors appear to be active in their membrane-bound form (10, 40), it remains to be established unequivocally that this is the case for the membrane-anchored EGF precursor with intact transmembrane and cytoplasmic domains.

The present data indicate that the urinary 160-kDa pro-EGF is a heparin-binding protein. It has been suggested that binding of growth factors to heparin-like molecules on the cell surface or extracellular matrix may serve to concentrate the soluble growth factor in the vicinity of its corresponding receptor (18) or to modulate growth factor activity (41). Mature EGF has been reported not to require heparin for EGF receptor binding or activation (41), but whether heparin affects the biological activity of urinary pro-EGF remains to be established.

Our experiments with SFME cells suggest an additional possible function for pro-EGF. When culture dishes were coated with urinary pro-EGF, the immobilized protein appeared to function as a cell adhesion molecule as well as a growth factor for SFME cells. The mechanism whereby pro-EGF promotes cell adhesion is not clear, but several possibilities exist. Immobilized heparin-binding pro-EGF may bind SFME cells via cell surface heparin-like molecules and/or EGF receptors, thereby promoting cell attachment. Alternatively, binding may occur due to other intrinsic adhesion properties of pro-EGF. It is of interest to note that the cell adhesion molecule laminin contains multiple EGF-like motifs (42). Further characterization of the adhesion properties of pro-EGF may provide insight into possible physiological roles for the EGF precursor in cell-matrix or cell-cell interactions. It is possible that in the urinary tract pro-EGF might bind to urothelial glycosaminoglycans or to heparin-like molecules on damaged mucosa and thereby serve to enhance urothelial repair.

Acknowledgments—We acknowledge Dr. David Barnes (Oregon State University) for generously providing SFME cells. G. P. gratefully acknowledges the support of Dr. W. Scott McDougal (Massachusetts General Hospital).

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The Human Urinary Epidermal Growth Factor (EGF) Precursor: ISOLATION OF A BIOLOGICALLY ACTIVE 160-KILODALTON HEPARIN-BINDING PRO-EGF WITH A TRUNCATED CARBOXYL TERMINUS

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J. Biol. Chem. 1995, 270:27954-27960.
doi: 10.1074/jbc.270.46.27954

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