Epithelins 1 and 2: Isolation and characterization of two cysteine-rich growth-modulating proteins

(cancer/differentiation/growth factor/growth inhibitor/cytokine)

MOHAMMED SHOYAB*, VICKI L. MCDONALD, CHRISTINE BYLES, GEORGE J. TODARO, AND GREGORY D. PLOWMAN

Oncogen, 3005 First Avenue, Seattle, WA 98121

Contributed by George J. Todaro, July 30, 1990

ABSTRACT Two proteins, termed epithelin 1 and epithelin 2, that inhibit the growth of A431 cells, derived from a human epidermal carcinoma of the vulva, have been purified from rat kidney. Epithelin 1 stimulates the proliferation of murine keratinocytes, whereas epithelin 2 inhibits the epithelin 1-elicited growth of these cells. Thus epithelin 1 and 2 behave as agonist and antagonist, respectively, for normal epithelial cells. Epithelins are low molecular mass (=6 kDa), acid- and heat-stable, single-chain proteins containing ~20% cysteine. Some of these cysteines form disulfide linkage(s) that are essential for biological activity. The amino-terminal amino acid sequences of epithelin 1 and epithelin 2 have been determined. The two proteins showed no substantial sequence homology with other proteins. However, a significant homology was seen between the amino-terminal sequences of epithelin 1 and epithelin 2. Epithelins 1 and 2, therefore, appear to represent members of a distinct family of growth regulators.

The complex process of development and adult homeostasis is apparently mediated and regulated by a multiplicity of positive and negative regulators of cellular proliferation, differentiation, or both (1–20). The breakdown or alteration of the cellular homeostasis mechanism is a basic cause of cancer and other growth-related maladies (1–20). Continuing efforts in our laboratory have focused on discovering molecules that modulate the growth and differentiation of mammalian cells. By screening conditioned medium from mammalian cells and tissues and cell extracts for growth-modulatory activities, we have identified several molecules exhibiting growth-regulatory effects that discriminate between normal and neoplastic cells. Two such molecules, oncostatin M and amphiregulin, which inhibit the proliferation of human tumor cells but promote the growth of normal fibroblasts, have been reported (21, 22). Similar screening programs have now resulted in the isolation and characterization of two additional growth-modulating proteins, termed epithelin 1 and 2. These two distinct cysteine-rich growth regulators are structurally related but exert opposite functional effects on the growth of normal epithelial cells. In this report we describe the isolation and characterization of epithelins.

MATERIALS AND METHODS

Cell Lines. All cell lines used were obtained from The American Type Culture Collection or from our own cell banks. The murine keratinocyte BALB/MK cell line (23) was provided by S. A. Aaronson (National Cancer Institute, Bethesda, MD).

Growth-Modulatory Assay with 125I-Labeled Deoxyuridine Incorporation into DNA. A431 cells (clone A3) derived from a human epidermoid carcinoma of the vulva were used as test cells for growth-inhibitory activity (GIA). A total of 3.5 × 10⁴ cells in 50 µl of Dulbecco’s modified Eagle’s medium, supplemented with 5% (vol/vol) heat-inactivated fetal bovine serum, penicillin (60.6 µg/ml), streptomycin (100 µg/ml), and 4mM L-glutamine (test medium), was placed in all wells of a 96-well plate except peripheral wells. Three hours later, 50 µl of the test sample in test medium was added to each well; control wells received only 50 µl of test medium. Three wells were used for each concentration of test sample. Plates were incubated at 37°C for 2–3 days. After this, 100 µl of solution of 125I-labeled deoxyuridine (Amersham) [4 Ci/mg; 0.5 Ci/ml (2 µl/ml in test medium); 1 Ci = 37 GBq] was added to each well and plates were incubated at 37°C. After 4–6 hr, samples were processed as described (22).

Growth-Modulatory Assay with Murine Keratinocytes. BALB/MK cells were plated at 1 × 10⁶ cells per well in 1 ml of low-calcium medium (23) in 24-well Costar plates (area, ~2 cm² per well) and incubated overnight at 37°C. Then medium was removed and replaced with 1 ml of medium containing various concentrations of factor(s) in triplicate. The control wells received only medium without any factor. Plates were incubated at 37°C for 4 days, then medium was removed, wells were rinsed twice with 1 ml of isotonic phosphate-buffered saline, and the cells were detached with trypsin/EDTA and counted.

Other Assays. The cell growth modulatory assay using other cells, soft agar colony assay, and radioreceptor assay for epidermal growth factor (EGF) were carried out as reported (24).

Purification of Epithelins from Rat Kidney. Acid-ethanol extraction. Rat kidneys were obtained from Pel-Freez Biologicals. Frozen rat kidneys [430 g (wet weight)] were suspended in 2370 ml of extraction buffer consisting of 2348 ml of ethanol (98%), 19 ml of concentrated HCl, 81.5 mg of phenylmethlysulfonyl fluoride, and 2.8 ml of aprotinin [23 trypsin-inhibitory units/ml from bovine lung (Sigma)]. The tissue was allowed to thaw at 4°C for 4–6 hr and the mixture was homogenized in a Waring Blender. The mixture was stirred at 4°C overnight and centrifuged at 9000 rpm in a Sorvall GS-3 rotor for 40 min, and the supernatant was carefully removed (2200 ml). Chloroform (2200 ml) and 220 ml of acidified water (375 ml of water plus 7.5 ml of concentrated HCl) were added to the supernatant; the mixture was stirred vigorously for approximately an hour and allowed to stand at room temperature to separate into two phases. The upper aqueous phase was carefully removed and dialyzed against 17 liters of 0.1 M acetic acid at 4°C in Spectra/por dialysis tubing (no. 3; molecular weight cut off, ~3000).

Abbreviations: GIA, growth-inhibitory activity; EGF, epidermal growth factor; rp, reversed phase. *To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
dialysis buffer was changed three times over a 2-day period. The retenate was lyophilized and the lyophilized material (4.55 g), termed crude extract, was stored at −20°C until further use.

**Preparative gel-permeation chromatography.** A Bio-Sil TSK-250 column (21.5 × 600 mm) (Bio-Rad) was attached to a high performance liquid chromatography (HPLC) system (Waters). The crude extract (25 mg/ml) was dissolved in 50% (vol/vol) acetonitrile/H2O with 0.1% trifluoroacetic acid. A 3-ml sample of the mixture was injected, and elution was performed isocratically with a mobile phase of 50% acetonitrile/H2O with 0.1% trifluoroacetic acid. The flow rate was 4 ml/min, and 6-ml fractions were collected. A portion of each fraction was evaporated and assayed in triplicate for GIA on A431 human epidermoid carcinoma cells (Fig. 1).

The late-eluting minor peak (fractions 25–28, as indicated) contained the activities of interest. Fractions 25–28 from 57 similar runs were pooled, concentrated, and lyophilized. The lyophilized material weighed 473 mg and contained a total of 1.1 × 10^6 GIA units.

**Reversed-phase (rp) HPLC of preparative TSK-250 fractions.** The lyophilized fractions were dissolved in 240 ml of 0.1% trifluoroacetic acid in H2O; the mixture was centrifuged and supernatants were carefully removed. The final volume was ≈250 ml. Then 125 ml of this mixture was isocratically injected onto a preparative Partisil 10 ODS-3 column (Whatman, 10 μm, 2.2 × 25 cm). The flow rate was set at 4 ml/min. Once the sample had passed onto the column, the column was washed with 150 ml of 0.1% trifluoroacetic acid in H2O. A linear gradient was generated between the primary solvent, 0.1% trifluoroacetic acid in H2O, and the secondary solvent, acetonitrile containing 0.1% trifluoroacetic acid. The gradient conditions were 0–45% in 270 min and 45–100% in 45 min. Fractions (14 ml) were collected, and aliquots of each fraction were assayed for GIA. Four broad peaks of activity were seen (Fig. 2). A second run was performed as described above. Two early eluting peaks, peak a and peak b, contained epithelin 2 and epithelin 1, respectively, and they were further purified and characterized. The late-eluting GIA peaks, peak c and peak d, contained transforming growth factors β1 and β2, respectively, in addition to other activities.

**Further purification of epithelin 1 by rp and gel-permeation HPLCs.** Fractions 55–59 (Fig. 2, peak b) from two runs were pooled and diluted 1:2 with 0.1% trifluoroacetic acid in H2O. The mixture was isocratically injected onto a semipreparative μBondapak C18 column (7.8 × 300 mm, Waters) at a flow rate of 2 ml/min at room temperature. The linear gradient conditions between primary solvent, H2O with 0.1% trifluoroacetic acid, and the secondary solvent, acetonitrile with 0.1% trifluoroacetic acid, were 0–18% in 1.8 min, 18–18% in 20 min, 18–34% in 240 min, and 34–100% in 10 min. The flow rate was 2 ml/min throughout the gradient and 7-ml fractions were collected. Aliquots were taken and assayed for GIA. Two peaks of activity were observed eluting at acetonitrile concentrations of ≈24% and ≈25%, respectively.

Fractions 30–34 were pooled, and 45 ml of 0.1% trifluoroacetic acid in water was added to the pooled fraction. The mixture was isocratically applied onto a μBondapak-CN column (3.9 × 300 mm, Waters) at a flow rate of 1 ml/min at room temperature. The gradient conditions were 0–10% in 1 min, 10–10% in 19 min, 10–30% in 200 min, and 30–100% in 7 min. The flow rate was 0.5 ml/min and 1.5-ml fractions were collected. Most of the activity emerged from the column at an acetonitrile concentration of ≈21.5%.

Fractions 36–43 were pooled and diluted with 0.1% trifluoroacetic acid/H2O to a final volume of 115 ml and chromatographed exactly as described above. Most of the activity eluted from the column in two peaks eluting at ≈22.5% and ≈23.5% acetonitrile (Fig. 3A).

Fractions 51 and 52 (first cyano-column run) were individually concentrated, using a Speed-Vac concentrator (Savant), to ≈70 μl, to which was added an equal volume of acetonitrile containing 0.1% trifluoroacetic acid. This 140-μl sample was injected onto two Bio-Sil TSK-250 columns (7.5 × 300 mm, Bio-Rad) arranged in tandem. The elution was performed isocratically with a mobile phase of 50% acetonitrile/H2O with 0.1% trifluoroacetic acid at room temperature. The flow rate was 0.4 ml/min; 0.4-ml fractions were collected and aliquots were assayed for GIA.

Fractions 44 and 45 (Fig. 3A) were individually concentrated to 70 μl and then subjected to gel-permeation chromatography as described above. The chromatographic profile of fraction 44 is given in Fig. 3B.

**Further purification of epithelin 2 by rp and gel-permeation HPLCs.** Fractions 50–54 (Fig. 2, peak a) from two runs were pooled and diluted 1:2 with 0.1% trifluoroacetic acid/H2O. The mixture was applied onto a semipreparative μBondapak
Results and Discussion

Initial Characterization. Epithelin 1 and epithelin 2 were resistant to treatment with 1 M acetic acid, 1 M ammonium hydroxide, 6 M urea, 0.01 M sodium metaperiodate, to heating at 56°C for 30 min, and to treatment with various glycosidases or lipases. However, activity was sensitive to reduction, to reduction and treatment with 4-vinylpyrididine, and to digestion with proteinases such as trypsin, endoproteinase Lys-C, and endoproteinase Glu-C (V8). Amino acid composition analysis revealed that both epithelins contain ~20% cysteine residues. These results suggest that these factors are cysteine-rich proteins containing some cysteines in disulfide linkage(s) that are essential for biological activity but do not contain oligosaccharides or lipid moieties that are obligatory for biological activities.

Purification of Epithelin 1 and Epithelin 2 and Certain Physical Properties. Summaries of the purification of epithelin 1 and epithelin 2 are presented in Tables 1 and 2, respectively. Both factors were purified to apparent homogeneity by a similar six-step protocol. The early-step fractions contain

Table 1. Summary of purification of epithelin 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein, µg</th>
<th>GIA units</th>
<th>Specific activity, units/mg</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>4,550,000</td>
<td>1,283,100</td>
<td>282</td>
<td>—</td>
</tr>
<tr>
<td>Prep. TSK-250</td>
<td>473,000</td>
<td>112,200</td>
<td>237</td>
<td>—</td>
</tr>
<tr>
<td>Prep. ODS (b)</td>
<td>17,600</td>
<td>14,100</td>
<td>801</td>
<td>100</td>
</tr>
<tr>
<td>Semiprep. C18</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1b</td>
<td>1,510</td>
<td>2,080</td>
<td>1,377</td>
<td>14.8</td>
</tr>
<tr>
<td>Peak 2b</td>
<td>3,460</td>
<td>5,460</td>
<td>1,578</td>
<td>38.7</td>
</tr>
<tr>
<td>Anal. cyano</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1b</td>
<td>60</td>
<td>713</td>
<td>11,889</td>
<td>3.4</td>
</tr>
<tr>
<td>Peak 2b</td>
<td>73</td>
<td>1,190</td>
<td>16,301</td>
<td>8.4</td>
</tr>
<tr>
<td>Anal. TSK-250</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peak 1b</td>
<td>41</td>
<td>845</td>
<td>20,609</td>
<td>6.0</td>
</tr>
<tr>
<td>Peak 2b</td>
<td>62</td>
<td>1,305</td>
<td>21,048</td>
<td>9.3</td>
</tr>
</tbody>
</table>

*Prep. TSK-250, preparative Bio-Sil TSK-250 column; prep. ODS, preparative Partisil 10 ODS-3 column; semiprep. C18, semipreparative µBondapak C18 column; anal. cyano, analytical µBondapak-CN column; anal. TSK-250, analytical Bio-Sil TSK-250 column.

†One unit of GIA is the amount of factor required to inhibit 125I-labeled deoxyuridine incorporation into A431 cells by 50%.

Other GIAs are also present in these fractions. These values include all activities. Less than 1% of the activity in crude preparations is due to epithelin 1.
multiple GIAs detected with A431 cells (Figs. 1 and 2). More than 99% of the tumor cell inhibitory activities detected in the crude extract (Tables 1 and 2) are due to the presence of transforming growth factor β and other growth-inhibitory molecules. Epithelins 1 and 2 constitute only a minor fraction (<1%) of the total activity in early fractions. The above facts make it difficult to quantitate the specific activities of the factors at early stages of purification. The real specific activity of epithelins 1 and 2 in the crude fraction is probably <2 GIA units/mg protein. The specific activity of purified epithelin 1 was 2.1 x 10^4 units/mg of protein, whereas epithelin 2 had a much lower specific activity of 3.8 x 10^3 units/mg of protein.

The molecular weights of epithelin 1 and epithelin 2, as determined by gel-permeation chromatography on TSK-250 columns, were ≈5000 and ≈4000, respectively (Fig. 3 B and D). S-pyridylethylated epithelin 1 or 2 exhibited a molecular weight of ≈13,000 by the similar gel-permeation chromatography. These results suggest that both epithelins are very compact proteins, and reduction and pyridylethylation of cysteine residues alter this compactness.

Fig. 4 shows an analysis of epithelin 1 and epithelin 2 in an 18% polyacrylamide gel under reducing conditions. Epithelin 1 and epithelin 2 migrated in the gel as single bands with median relative molecular weights of ≈5500 and ≈6000, respectively. Similar results were obtained when the proteins were electrophoresed under nonreducing conditions (data not shown). Thus epithelins are single-chain low molecular weight proteins.

**Table 2. Summary of purification of epithelin 2**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein, µg</th>
<th>GIA units*</th>
<th>Specific activity, units/mg</th>
<th>Yield, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude</td>
<td>4,550,000</td>
<td>1,283,100*</td>
<td>282</td>
<td>—</td>
</tr>
<tr>
<td>Prep. TSK-250</td>
<td>473,000</td>
<td>112,200*</td>
<td>237</td>
<td>160</td>
</tr>
<tr>
<td>Prep. ODS (peak a)</td>
<td>24,500</td>
<td>9,567</td>
<td>432</td>
<td>100</td>
</tr>
<tr>
<td>Semiprep. C18</td>
<td>4,750</td>
<td>1,190</td>
<td>250</td>
<td>12.4</td>
</tr>
<tr>
<td>Anal. cyano</td>
<td>169</td>
<td>460</td>
<td>2741</td>
<td>4.8</td>
</tr>
<tr>
<td>Anal. TSK-250</td>
<td>37</td>
<td>141</td>
<td>3810</td>
<td>1.5</td>
</tr>
</tbody>
</table>

Abbreviations as are in Table 1.
*One unit of GIA is the amount of factor required to inhibit 125I-labeled deoxyuridine incorporation into A431 cells by 50%.
*Other GIAs are also present in these fractions. These values include all activities. Less than 1% of the activity in the crude preparations is due to epithelin 2.

Amino-Terminal Amino Acid Sequences of Epithelin 1 and Epithelin 2. Automated degradation of S-pyridylethylated epithelin 1 and epithelin 2 was performed with ≈300 pmol of protein. The partial amino-terminal amino acid sequences of epithelins 1 and 2 are as follows:

**Epithelin 1:** VKC-DLEVSCPDPYGTYCSSRNLPG
**Epithelin 2:** VVCPDASKQCPDDSTCCELPTG

The partial amino acid sequences of epithelin 1 and epithelin 2 were compared with all proteins in the National Biomedical Research Foundation data base (release 23), GenBank (release 63), and the European Molecular Biology Laboratory data base (release 22). The amino-terminal amino acid sequences of epithelin 1 and epithelin 2 did not reveal any substantial sequence homologies with any other known sequence. However, a significant homology (≈50%) was seen between amino-terminal amino acid sequences of epithelin 1 and epithelin 2, thus indicating that these two proteins are structurally related to each other. Of the first 22 amino acids sequenced in epithelin 2, 12, including the four cysteines, are identical to those found in epithelin 1.

Biological Properties of Epithelin 1 and Epithelin 2. The inhibition of 125I-Labeled deoxyuridine incorporation into DNA of A431 cells by various concentrations of purified epithelin 1 and epithelin 2 is given in Fig. 5A. A 50% inhibition of DNA synthesis was seen with epithelin 1 at 12.8 ng per well and with epithelin 2 at 450 ng per well. Thus, a 50% DNA synthesis inhibition in A431 human epithelial carcinoma cells was seen at =21 nM epithelin 1 and =0.75 µM epithelin 2. Epithelin 1 is about 36 times more potent in this assay. However, it should be noted that the GIA of epithelins 1 and 2 depends on experimental conditions such as number of cells per well (cell density), time of factor application, duration of treatment, serum concentration, and other variables.

The effect of epithelin on the incorporation of 125I-labeled deoxyuridine into DNA of various tumor and nontumor human cell lines as well as several nonhuman cell lines was investigated. Epithelin 1 (20 ng/ml, maximum dose tested) slightly inhibited the growth of human colon carcinoma cell line HCT116, while epithelin 2 (270 ng/ml, maximum dose tested) did not show any effect on this cell line. Both proteins significantly inhibited the 125I-labeled deoxyuridine incorporation into DNA of mink lung CCL 64 cells and monkey kidney COS1 cells. Neither protein exhibited any significant effect on human fibroblasts and several other human tumor cells at the maximum dose of the proteins tested (20 ng/ml for epithelin 1 and 270 ng/ml for epithelin 2).

The continued growth of a murine keratinocyte cell line, BALB/MK, is dependent on EGF, transforming growth factor α, or amphiurelin (23, 24, 27). These cells proliferated...
Fig. 5. (A) Dose–response curve of epithelins 1 and 2 on the inhibition of 125I-labeled deoxyuridine incorporation into DNA of A431 cells. ●, Epithelin 1; ○, epithelin 2. (B) Effect of epithelin 1, epithelin 2, and EGF on the growth of murine keratinocytes BALB/MK cells. △, EGF; ●, epithelin 1; ○, epithelin 2. (C) Dose–response curve of epithelin 2 on the inhibition of epithelin 1 (20 ng/ml)-induced growth of murine keratinocytes. ●, Epithelin 2; dashed line, number of cells in the absence of any epithelin 2.

quite well in the presence of epithelin 1 (Fig. 5B). Epithelin 2, however, did not exhibit any significant effect on the growth of these cells. In fact, as shown in Fig. 5C, epithelin 2 inhibited the epithelin 1-induced growth of BALB/MK cells in a dose-dependent manner. A 50% inhibition is seen at ≈7 nM of epithelin 2. Thus, epithelin 2 acts as an antagonist of the agonist epithelin 1, in this system.

EGF or transforming growth factor α induces anchorage-independent growth of rat kidney cells NRK-SA6 in the presence of transforming growth factor β (24). Like EGF, epithelin 1-induced anchorage-independent growth of normal rat kidney cells in a dose-dependent manner, whereas epithelin 2 was found to be a noninducer of colony formation in soft agar of normal rat kidney cells. Again, epithelin 2 at ≈85 nM inhibited ≈50% of the epithelin 1-induced colony formation in soft agar.

Neither epithelin 1 nor epithelin 2 significantly affected the binding of 125I-labeled EGF to its receptors, thus suggesting that these growth regulators do not mediate their biological effects through the EGF receptor system. However, receptors specific for epithelins may exist.

In conclusion, we have isolated two distinct structurally related growth regulators that act through an unidentified membrane receptor system. Further studies on the cloning, structure, topology, expression, and regulation of the epithelin gene on the distribution of epithelin 1 and epithelin 2 during various pathophysiological conditions, on the nature of a specific receptor, and on the mechanism of signal transduction may shed light on the physiological function of epithelins. Other growth-modulatory peptides are multifunctional (11, 16, 19–22); we would expect the same to be the case for epithelins.

We thank Ms. Connie Spooner for her editorial assistance and Ms. Cynthia Hagen for her assistance in preparation of this manuscript.