Purification and Characterization of a Novel Growth Factor from Human Breast Cancer Cells†

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ABSTRACT: We have purified and characterized a novel 30-kDa glycoprotein (gp30) with TGFα-like properties secreted from the estrogen receptor negative breast cancer cell line MDA-MB-231. This factor was immunoprecipitated by an anti-TGFα polyclonal antibody and also had TGFα-like biological activity, as assayed by EGF radioreceptor assay and anchorage-independent assays. In addition, the novel growth factor stimulated phosphorylation of the EGF receptor and erbB-2 receptor. However, the novel growth factor, unlike EGF and TGFα, bound to heparin–Sepharose. Purification of gp30 was obtained to apparent homogeneity by heparin affinity chromatography and subsequent reversed-phase chromatography. Tunicamycin treatment in vivo or *N*-glycanase deglycosylation in vitro revealed a putative precursor of approximately 22 kDa molecular mass in contrast to the "normal" 16-kDa precursor species for TGFα. In vitro translation of total mRNA from MDA-MB-231 cells confirmed the size of the putative precursor. Biochemical characterization of gp30 was begun by V8 protease digestion of the deglycosylated polypeptide and the translated products. Peptide mapping of V8-digested, immunoprecipitated material suggests that the amino acid sequence of this unique protein is distinct from mature TGFα and not the result of a posttranslational modification of the precursor. We conclude that this TGFα-like (gp30) polypeptide is a novel growth factor with agonistic activity for both EGF and erbB-2 receptors.

Transforming growth factors belong to a family of heat- and acid-stable polypeptides which allow cells to assume a transformed morphology and form progressively growing colonies in anchorage-independent growth assays (DeLarco & Todaro, 1978; Moses et al., 1981; Ozanne et al., 1980; Roberts et al., 1980). Transforming growth factor type α (TGFα)[1] is a polypeptide whose expression in rodent fibroblasts is selectively induced after cellular transformation by a variety of oncogenes. TGFα was first purified to homogeneity from conditioned media from the human melanoma A2058 cell line (Marquardt et al., 1982).

It has been isolated from various solid tumors (Nickell et al., 1983) and from conditioned media from a variety of human cancer cell lines including breast cancer cell lines (Todaro et al., 1980; Zwiebel et al., 1982). TGFα is a structural and functional analogue of epidermal growth factor (EGF). TGFα and EGF are small polypeptides (molecular mass of 6 kDa) encoded by separate genes (Salomon et al., 1984; Gray et al., 1983), and they share approximately 40% of their amino acid sequence (Derynck et al., 1984; Massague, 1983; Marquardt et al., 1983). Despite differences in primary structure, TGFα and EGF have similar secondary structure and bind with nearly equal affinity to the human EGF receptor (Marquadart et al., 1982). Systems used for the analysis of TGFα activity include A431 human carcinoa cells, which overexpress the EGF receptor and NRK-49F rat fibroblasts, both of which require TGFα for anchorage-independent growth (Marquardt et al., 1984). Mature TGFα is a single-chain polypeptide of 50 amino acids, and is initially synthesized as a transmembrane, palmitoylated species. Its 16–18-kDa precursor protein is biologically active and is equipotent with the mature polypeptide (Massague, 1985; Ignotz et al., 1986). TGFα is a mitogenic polypeptide which is also produced by a variety of retrovirally-, chemically-, or oncogene-transformed human and rodent cell lines [reviewed by Sporn et al. (1985)].

In addition, it is present in human mammary tumor tissue and cell lines (Solomon et al., 1984; Derynck et al., 1987a,b) and has recently been demonstrated in normal human keratinocytes (Coffey et al., 1987). There have been numerous other reports of a bioactive higher molecular weight form of TGFα found in the supernatant of cultured rat hepatocellular carcinoma cells (Luetteke et al., 1988), extracts of human milk (Zwiebel et al., 1986), alveolar macrophages (Madtes et al., 1988), and urine (Hudgins et al., 1987). The significance of the presence of TGFα in these tissues and fluids is unknown. Two recent reports have described new members of the EGF/TGFα family: the amphiregulin gene derived from MCF-7 human breast cancer cells treated with tetradecanoylphorbol acetate (Shoyab et al., 1988) and another molecule derived from human malignant gliomas (Rutka et al., 1989). The range and regulation of expression of these two genes are relatively unknown. In many cases, high molecular weight forms of TGFα constitute the predominant TGFα activity, and their estimated sizes are equal to or exceed the molecular weight predicted for the transmembrane precursor. However, their precise structural relationship with the TGFα precursor remains undefined.

The TGFα cDNA sequence encoding the 160 amino acid precursor contains potential sites for posttranslational modification of the TGFα protein in the human (Derynck et al.,

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[1] Abbreviations: TGFα, transforming growth factor α; TGFα-like, transforming growth factor α like; EGF, epidermal growth factor; HPLC, high-performance liquid chromatography; FPLC, fast protein liquid chromatography; NRK, normal rat kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; NRS, normal rabbit serum; R399, rabbit antirecombinant TGFα; RRA, radioreceptor assay; RIA, radioimmunoassay.

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1984) and rat (Lee et al., 1985), largely via glycosylation. The presence of a hydrophobic sequence in the carboxyl terminus of the protein suggests that the precursor is a transmembrane protein from which mature TGFα is released by the action of a specific protease. The release of mature TGFα from its 16–18-kDa precursor may occur in vivo by the action of an elastase-like enzyme, which cleaves at sites containing clusters of apolar amino acids, in particular near valine and alanine residues (Geneste et al., 1969). Elastase can cleave the 16–18-kDa TGFα precursor from Rat-FeSrV cells into a 6-kDa product, suggesting a high degree of specificity (Ignotz et al., 1986).

A TGFα cDNA probe encoding the 160 amino acid precursor recognizes a 4.8-kb mRNA species in a variety of breast cancer cell lines such as MCF-7, ZR 75B, MDA-MB-231, and T47D, but not HS578T, by Northern blot analysis (Derynck et al., 1984, 1985, 1987a,b). MDA-MB-231 cells secrete mature 6-kDa TGFα and also a large size TGFα-like polypeptide detected by EGF receptor binding of fractionated conditioned media (Bates et al., 1988).

Because human breast cancer cell lines and human breast tumors contain EGF/TGFα receptors, an autocrine pathway can be proposed for the TGFα. According to this hypothesis, cells secrete mitogenic polypeptides which act through their own cell-surface receptors to stimulate growth. In addition, an estrogen-regulated autocrine or paracrine growth stimulatory pathway involving TGFα in MCF-7 cells was proposed (Bates et al., 1988). Conditioned media from confluent cultures of MDA-MB-231 human breast cancer cells also stimulated proliferation of MCF-7 cells in a dose-dependent and saturable manner. The high levels of expression and secretion of a high molecular weight TGFα-related polypeptide by MDA-MB-231 cells (Bates et al., 1988) provided a suitable source for its purification and characterization.

Heparin affinity chromatography, which has been used for the isolation of endothelial cell growth factors (Shing et al., 1984; Klagsbrun et al., 1984; Gospodarowicz et al., 1984; Maciag et al., 1984) and has recently been shown to bind a high molecular weight form of an uncharacterized TGFα-like from activated alveolar macrophage (Madtes et al., 1988) and an EGF-like heparin binding polypeptide (Higashiyama et al., 1991), was used for the isolation of the high molecular weight TGFα-like polypeptide from MDA-MB-231 cells. The human breast cancer TGFα-like growth factor was isolated from conditioned media from MDA-MB-231 cells, a highly invasive and estrogen receptor-negative breast cancer cell line.

We describe a novel isolation and characterization of a unique high molecular weight growth factor. This growth factor is unique as determined by peptide mapping both of purified gp30 and of its in vitro translated p22 precursor. The novel growth factor has also been shown to activate tyrosine phosphorylation of the erbB-2 receptor (Lupu et al., 1990).

MATERIALS AND METHODS

Cell Lines. Cells from the following sources were used in these studies: MDA-MB-231 and NRK clone 49F fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Hs578T cells were received from Helene Smith (Hackett et al., 1977). A431 cells were donated by Ira Pastan (Giard et al., 1973). H8 cells, a human TGFα-transfected MCF-7 breast cancer cell line, were kindly provided by Francis G. Kern (Clarke et al., 1989). Carcinogen-immortalized normal mammary epithelial cell subline 184A1N4 and its SV40-transfected derivative 184A1N4T were kindly provided by Robin Clark (Clark et al., 1988). Rat-FeSrV-transfected cells were kindly provided by Joan Massague (Ignotz et al., 1986). All cell lines were propagated in improved modified Eagle's medium (IMEM; Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Gibco).

Conditioned Media Preparation, Collection, and Concentration. Conditioned media collections were carried out as previously described (Bates et al., 1986). The media were concentrated 100-fold in an Amicon ultrafiltration cell (YM5 membrane) (Amicon, Danvers, MA). Once clarified and concentrated, the media were stored at −20 °C while consecutive collections were made during the following days. The concentrated media were dialyzed using Spectrapor 3 tubing (Spectral Medical Industries, Los Angeles, CA) against 100 volumes of 0.1 M acetic acid over a 2-day period at 4 °C. The material that precipitated during dialysis was removed by centrifugation at 4000 rpm for 30 min at 4 °C; protease inhibitors were added.

Metabolic Labeling and Immunoprecipitation. Cells were grown to 80% confluence in IMEM. Cell monolayers were washed 3 times with PBS and incubated for 2 h in serum-free IMEM which lacked methionine and cysteine and was supplemented with glutamine (2.9 g/L) (Biofluids, Rockville, MD). This medium was then removed and replaced with serum-free IMEM without methionine and cysteine containing 2.5 mCi/mL [^{35}S]cysteine and methionine (Amersham, Arlington Heights, IL; 1175 Ci/mmol). A total of 2.5 mL of this meidum was used for a 5-cm dish. The medium was harvested from the culture after 16 h at 37 °C and clarified by centrifugation. Cells were washed once with PBS, harvested by scraping, and lysed in 1 mL of RIPA buffer (300 mM NaCl/100 mM Tris-HCl containing 2% Triton X-100, 2% sodium deoxycholate, 0.2% SDS, 0.4% BSA, and 2 mM PMSF). Following an incubation of 30 min on ice, the lysate was clarified by centrifugation (30 min at 4000 rpm) and used immediately or was stored at −70 °C. ^{35}S-Labeled proteins released into the conditioned media by the different cell lines were immunoprecipitated with 10 μg (specific or nonspecific) of antibody partially purified by 45% ammonium sulfate precipitation as previously described (Kessler, 1975). After solubilization, the immunoprecipitates were analyzed by 15% SDS–PAGE (Laemmli, 1970) and subsequent fluorography. Prestained molecular weight markers (Bio-Rad, Richmond, CA) were run in parallel lanes.

Tunicamycin Treatment. Tunicamycin (Sigma, St. Louis, MO) was dissolved in 50 mM sodium carbonate (pH 10.0) and filter-sterilized with a 0.22-μm filter. Confluent monolayers of MDA-MB-231, MCF-7, and Hs578T cells were grown in IMEM in the presence of 20 μg/mL tunicamycin (unless otherwise specified) (Bringman et al., 1987) for 4 h prior to metabolic labeling. Metabolic labeling was then performed as described previously.

Samples containing TGFα-like activity were incubated with 20 μg of porcine pancreatic elastase (Sigma) dissolved in 50 mM glycylglycine, pH 7.9, for 1 h at 22 °C. The samples were then subjected to immunoprecipitation and SDS–PAGE analysis.

Polyclonal and Monoclonal Antibodies. (A) Polyclonal Antibodies. Antiserum against human TGFα was obtained by immunization of a rabbit on day 0 with 400 μg of recombinant TGFα synthesized in *Escherichia coli*, kindly provided by R. Derynck, Genentech Corp. (Derynck et al., 1984). The immunogen was first conjugated to keyhole limpet hemocyanin (KLH) (Sutcliffe et al., 1980) and was emulsified in complete Freund's adjuvant and was injected intrader-

mally at multiple sites. Additional injections were given as follows: day 60, 175 μg of TGFα; days 90, 150, 180, and 210, 100 μg of TGFα. The booster injections were given subcutaneously at multiple sites in incomplete Freund's adjuvant. The rabbit serum was assayed for antibody titer by ELISA at 10–14 days following each injection. The antiserum collected at day 180, designated R399, was used for immunoprecipitation and radioimmunoassay.

(B) Monoclonal Antibodies. A monoclonal antibody against recombinant TGFα was provided by R. Derynck (Derynck et al., 1984).

Measurement of Anti-TGFα Antibody (R399) Levels by ELISA. Micro-Elisa plates (Dynatech–Immunolon II, Dynatech Laboratories, Inc., Chantilly, VA) were coated for 16 h at 4 °C with 500 ng/mL recombinant TGFα in 50 mM sodium carbonate buffer (pH 9.6). The samples to be assayed (antibody) were serially diluted 1:1000–1:64 000 with 0.15 M NaCl, 0.05 M Tris-HCl (pH 7.4), 2 mM EDTA, 5 mg/mL bovine serum albumin, and 0.05% Tween 20 (TBS–BSA–Tween) and were incubated in the wells for 2 h at 37 °C. The plates were washed 5 times with PBS–Tween and then incubated for 1 h at 37 °C with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin in TBS–BSA–Tween. The plates were then washed 5 times with PBS–Tween and incubated for 4 h at 22 °C with 100 μL per well of 0.1 mg/mL *o*-phenylenediamine/0.012% H_2O_2 in 0.1 M phosphate/citrate buffer (pH 5.0). The reaction was stopped by the addition of 50 μL/well of 2.5 N H_2SO_4, and the absorbance was measured at 492 nm using a UR 700 microplate reader (Dynatech Laboratories).

Radioimmunoassay (RIA): TGFα RIA. The presence of peptides immunologically related to TGFα was determined using a RIA kit with a polyclonal anti-rat TGFα and rat [^{125}I]TGFα (Biotope, Inc., Seattle, WA). This antibody does not cross-react with human EGF. Aliquots of conditioned media were reduced with 40 mM dithiothreitol and denatured by immersion for 1 min in a boiling water bath. Assays were done in duplicate according to the manufacturer's protocol, and each collection of conditioned media was assayed at least twice.

N-Glycanase Digestion. The purified gp30 was subjected to digestion with *N*-glycanase. Samples equivalent to 100 ng were incubated with 50 μL of 0.2 M sodium phosphate (pH 8.6)/1.25% NP40, and 2–6 μg of *N*-glycanase (Genzyme Corp., Boston, MA) was subsequently added to each sample and incubated at 37 °C for 16 h; 50 μL of 3-fold-concentrated loading buffer was added before electrophoretic analysis, performed as outlined above. The gel was silver-stained.

EGF Radioreceptor Assay. A431 membranes were prepared according to the method of Kimball and Warner (1984). A431 cells were disrupted under nitrogen and the nuclei and organelles pelleted by low-speed centrifugation. The membranes were then pelleted by centrifugation at 35 000 rpm for 1 h and resuspended in 20 mM HEPES buffer, pH 7.4. Membranes (2.5 μg/mL) were plated into 96-well plates and allowed to dry overnight at 37 °C before use. Standard binding competition studies were performed using [^{125}I]EGF (ICN, Costa Mesa, CA; specific activity 100 μCi/μg, about 50 000 cpm/well). A standard curve was constructed with 0.075–10 ng of unlabeled hEGF (receptor-grade, Collaborative Research). The different fractions to be analyzed were lyophilized and reconstituted in PBS (0.5 mL/500 mL of conditioned media). After incubation of the labeled EGF and 10 μL of the samples for 2 h at 37 °C in 200 μL of binding buffer (IMEM containing 50 mM HEPES and 0.1% BSA, pH 7.7), the wells were washed, cut from the plate, and counted. EGF-competing activity was computed using a Hewlett Packard RIA program (Aitken et al., 1977).

Anchorage-Independent Growth Assay. Soft agar cloning assays were carried out as previously described (Bates et al., 1988) using a 1-mL bottom layer of IMEM containing 0.6% Bacto-agar (Difco, Detroit, MI), 10% FBS, and 2 mM glutamine in 35-mm tissue dishes (Costar, Cambridge, MA). A 0.8-mL top layer of IMEM containing the test samples, 0.36% agar, 10% FBS, and 3×10^4 NRK cells was added after solidification of the bottom layer. Each sample was plated in triplicate. All samples were sterilized by filtration using a 0.22-μm Millex CU Millipore filter before plating. Plates were incubated in a humidified, 5% CO_2 atmosphere at 37 °C and were counted after 12-days incubation with a Bausch and Lomb stem cell colony counter (Artex Systems Corp., Farmingdale, NY).

Anchorage-Dependent Growth Assay. NRK and 184-NIA4T cells were grown in IMEM containing 5% FCS. Upon confluence, cells were detached using trypsin–versene (Biofluids, Rockville, MD) and passed at 1:20–1:50 dilutions. Cells were seeded in 12-well plates at 4000–10 000 cells/well, depending on the cell type (MDA-MB-231, 8000 cells/well in serum-free IMEM). After 24 h, the medium was changed, and the cells were treated with EGF, TGFα, or gp30 harvested at 1, 2, and 4 days, respectively, using trypsin–versene. The cells were counted using a Coulter counter.

Heparin Affinity Chromatography. Media conditioned by MDA-MB-231 cells were clarified by centrifugation for 20 min at 2000 rpm at 4 °C. The supernatant was collected and stored at −70 °C. After the heparin–Sepharose (Pharmacia) was allowed to expand in PBS, 2 mL of gel was loaded on an Econo column (Bio-Rad) and washed with about 100 bead volumes of PBS. Conditioned media were run through the beads by gravity (flow rate 20–50 mL/h). The gel was then washed with 5 volumes of PBS and eluted stepwise with an increasing gradient of NaCl in 10 mM Tris-HCl, pH 7.0 (elution buffer). Gradient steps of 0.4 to 3.0 M NaCl were used in the elution buffer until the 280-nm absorption during each step returned to base line (usually 3–5 column bed volumes). The eluate was desalted on G-25 columns (Pharmacia) and filter-sterilized before use in the different bioassays. Pooled fractions containing active materials were also desalted on PD10 columns (Pharmacia) before running through HPLC and FPLC.

Reversed-Phase High-Pressure Liquid Chromatography (HPLC). (A) Steep Acetonitrile Gradient. Steep acetonitrile gradient and all other HPLC steps were carried out at room temperature after equilibration of the C3 reversed-phase column with 0.05% TFA (trifluoroacetic acid) in water (HPLC-grade). The samples were loaded and fractions eluted with a linear gradient (0–45% acetonitrile in 0.05% TFA) at a flow rate of 1 mL/min over a 30-min period. Absorbance was monitored at 280 nm. One-milliliter fractions were collected and lyophilized before analysis for EGF receptor-competing activity.

(B) Shallow Acetonitrile Gradient. The pool of active fractions from the previous HPLC step was rechromatographed over the same column. Elution was performed with a 0–18% acetonitrile gradient in 0.05% TFA over a 5-min period followed by a linear 18–45% acetonitrile gradient in 0.05% TFA over a 30-min period. The flow rate was 1.0 mL/min, and 1-mL fractions were collected. Human TGFα-like factor was eluted at a 30–32% acetonitrile concentration as a single peak detectable by RRA.

Electrophoretic Elution of Radiolabeled Protein from Gels. After fluorography of an SDS–PAGE, bands of interest were excised, and the protein was eluted by electrophoresis into dialysis tubing over 16 h at 120 V. The contents of the dialysis bag were cooled at 4 °C and then precipitated by the addition of trichloroacetic acid to a final concentration of 20%. The precipitates were pelleted by centrifugation, washed twice with ethyl ether, and resuspended in loading buffer.

Digestion Procedure for Purified Eluted Proteins. Electroeluted proteins were dissolved at approximately 0.5 mg/mL in loading buffer which contained 0.125 M Tris-HCl (pH 6.8), 0.5% SDS, 10% glycerol, and 0.001% Bromophenol Blue. The samples were then heated at 100 °C for 5 min. Proteolytic digestion was carried out at 37 °C for 30 min by the addition of *Staphylococcus aureus* V8 protease (Sigma) to a final concentration of 25 μg/mL according to published methods (Cleveland et al., 1977). β-Mercaptoethanol and SDS were subsequently added to final concentrations of 20% and 2%, respectively. Proteolysis was stopped by boiling for 2 min. The samples were then injected on a C18 reversed-phase HPLC column (see above).

Phosphorylation of the EGF Receptor. Subconfluent A431 cells were cultured in IMEM for 10–12 h. The cells were treated with 10–30 nM TGFα, EGF, or gp30 for 30 min at 37 °C. Cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP40, 1 mM EDTA, 2 mM PMSF, and 42 mM leupeptin and immunoprecipitated as described above using monoclonal antibody 225 directed against the EGF receptor (Oncogene Science, Manhasset, NY). The immunoprecipitates were washed 3 times with RIPA buffer and resuspended in 40 μL of TNE (0.01 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 1 mM EDTA). Five microcuries of [γ-^{32}P]-ATP was added to the immunoprecipitates, and the total ATP concentration was adjusted to 15 mM (final) in a volume of 60 μL. The reaction mixture was incubated for 5 min on ice before addition of 20 μL of 3× sample buffer. The samples were boiled for 5 min and analyzed by denaturing 7.5% SDS–PAGE.

RNA Extraction. Total cellular RNA was extracted from cells by homogenizing in guanidine isothiocyanate followed by centrifugation over a cesium chloride cushion. Poly(A)$^+$ mRNA was eluted in 10 mM Tris after total cellular RNA was passed over an oligo(dt)–cellulose column (Pharmacia) equilibrated with 10 mM Tris/0.5 M NaCl, pH 8.0. After precipitation in ethanol (66% v/v) and 0.1 M acetic acid, both total and poly(A)$^+$-selected RNAs were resuspended in 10 M Tris/1 mM EDTA buffer and separated on 1% agarose/6% formaldehyde gels. Electrophoresis was carried out at 20 V over 14–16 h in 5 mM NaOAc, 1 mM EDTA, and 20 mM 3-(*N*-morpholino)propanesulfonic acid, pH 7.0 (MOPS, Sigma). The gels were stained with 2.0 g/mL ethidium bromide to allow inspection of the quality and quantity of RNA. In vitro translation assays were performed using wheat germ kit according to the manufacturer's instructions (Promega).

RESULTS

Identification of a TGFα-like Polypeptide in MDA-MB-231 Human Breast Cancer Cells. Earlier experiments using gel filtration chromatography suggested the presence of a gp30 TGFα-like related peptide in conditioned media from MDA-MB-231 cells. To determine whether this protein was recognized by antibodies developed against mature 6-kDa TGFα, MDA-MB-231 cells were metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine. Metabolically labeled

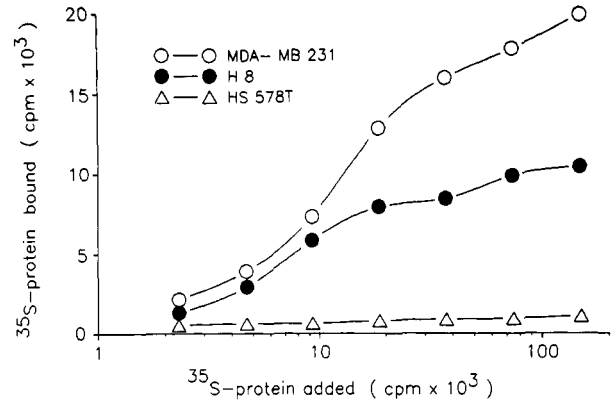

FIGURE 1: TGFα radioimmunoassay of metabolically labeled conditioned media from MDA-MB-231, Hs578T, and H8 cells. Wells of 96-well plates were coated with 10 μg/mL polyclonal antibody R399 for 2 h at 37 °C. Free binding sites were blocked with 1.0% BSA (RIA-grade) for 1 h at 22 °C. Serial dilutions of metabolically labeled conditioned media were added and incubated for 2 h at 37 °C. After a wash with 0.1% Tween 20 in PBS, radioactivity retained in the wells was counted. Data points represent the mean of duplicate values.

conditioned media from MDA-MB-231, TGFα-transfected MCF-7 (H8), and HS578T cells were tested by solid-phase RIA for immunoreactivity with a polyclonal antibody (R399) and a monoclonal antibody raised against recombinant 6-kDa TGFα. Metabolically labeled TGFα-like material from MDA-MB-231 cells reacted *only* with the polyclonal antibody. In contrast, both the polyclonal and the monoclonal antibodies cross-reacted with metabolically labeled material derived from H8 cells. No immunoreactivity was observed when preimmune serum was used (NRS or NMS). Furthermore, no cross-reactivity was observed when metabolically labeled conditioned medium from HS578T breast carcinosarcoma cells was used (Figure 1). HS578T cells do not produce TGFα mRNA (Bates et al., 1988). Specificity of the assay was demonstrated using a competition RIA with unlabeled recombinant TGFα (data not shown).

Labeled material from MDA-MB-231, H8, and Rat-FeSrV cells was immunoprecipitated with the anti-TGFα polyclonal antibody. Detection of an immunoreactive species of approximately gp30 size verified the secretion of a high molecular weight TGFα-like polypeptide in MDA-MB-231 cells. H8 cells, which overexpress classical TGFα, yielded a 6-kDa product. The expected 18-kDa precursor of the classical 6-kDa TGFα was precipitated from Rat-FeSrV cells, which are known to secrete the TGFα precursor. The intensity of the bands diminished when the immunoprecipitation was performed in the presence of excess unlabeled TGFα (Figure 2). None of these bands were immunoprecipitated by preimmune rabbit serum.

Deglycosylation and Elastase Cleavage of the Larger TGFα-like Polypeptide. The apparent heterogeneity in size of the authentic TGFα species described in the literature and its potential for N-linked glycosylation at Asn-25 (Bringman et al., 1987) led us to question whether the 30-kDa TGFα-like polypeptide secreted from MDA-MB-231 cells was a glycosylated form of TGFα. When MDA-MB-231 cells were incubated with tunicamycin, an inhibitor of cotranslational N-linked glycosylation, and the media were immunoprecipitated with the anti-TGFα polyclonal antibody, a species of 22 kDa substituted for that previously observed at 30 kDa (Figure 3A). Tunicamycin treatment did not significantly affect the levels of secreted TGFα activity as determined by both RIA and EGF receptor binding assays (data not shown).

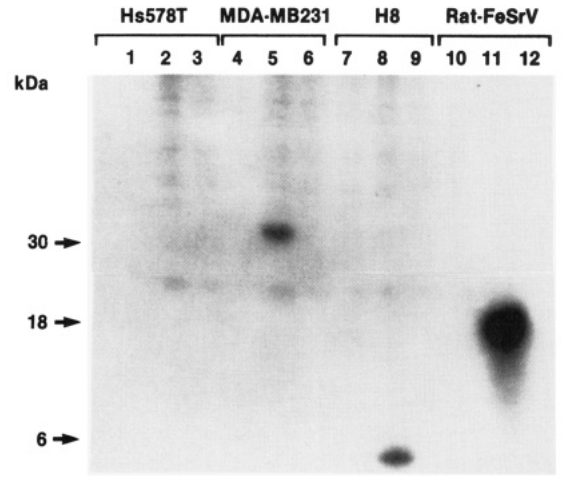

FIGURE 2: Immunoprecipitation of TGFα species from metabolically labeled media of MDA-MB-231, H8, and Rat-FeSrV cells. Immunoprecipitation was carried out with polyclonal antibody R399 or with preimmune serum (normal rabbit serum, NRS). The precipitates were analyzed by 15% SDS–PAGE under reducing conditions and fluorographed. Hs578T (lanes 2 and 3), MDA-MB-231 (lanes 5 and 6), H8 (lanes 8 and 9), and Rat-FeSrV (lanes 11 and 12) media were precipitated with polyclonal antibody R399 or preimmune serum NRS (lanes 1, 4, 7, and 10). Precipitation was also performed in the presence of 500 ng of recombinant TGFα (lanes 3, 6, 9, and 12). Molecular masses are indicated by arrows (kDa, kilodaltons).

We next addressed whether this deglycosylated species was a precursor form of mature TGFα. The 22-kDa polypeptide was treated with elastase, an enzyme known to cleave off 6-kDa mature TGFα from several TGFα precursors of different sizes (Luetteke et al., 1988; Madtes et al., 1988). An 11-kDa product was obtained (Figure 3B). This was in contrast to the 6-kDa product that was obtained when the deglycosylated 16-kDa TGFα precursor secreted by Rat-FeSrV cells was treated with elastase (Figure 3B). Shorter exposure of the gel showed clearly a precipitated band near the 11-kDa molecular mass. Furthermore, when purified gp30 was treated with *N*-glycanase, a 22-kDa product was detected by silver staining (Figure 3C). The biological activity of the purified gp30 was determined by its ability to stimulate NRK colony formation and EGF receptor phosphorylation, as described in Figures 9 and 10. The absence of cleavage of the purified gp30 after *O*-glycanase treatment (data not shown) suggests that no O-glycosylation occurs in this system.

Purification of the TGFα-like Polypeptide. TGFα-like material was isolated from serum-free conditioned media of MDA-MB-231 cells. Levels of TGFα-like polypeptide were quantified by three independent assays: capacity to induce anchorage-independent growth of NRK fibroblasts in soft agar; ability to compete with [^{125}I]EGF for EGF receptor binding on A431 human carcinoma cell membranes; and cross-reactivity with polyclonal antibodies raised against mature TGFα. To determine the approximate molecular weight of the MDA-MB-231-derived TGFα-like polypeptide, 5 mL of 100-fold-concentrated, dialyzed conditioned medium was chromatographed by gel filtration using Sephadex G-100. Elution was performed with 1.0 M acetic acid, and fractions were characterized for protein content. TGFα-like activities were eluted from the column in a single broad peak. The relative amounts of receptor binding activity and immunoreactivity present in these fractions, however, appeared to differ. Further analysis of the TGFα-like polypeptide from MDA-MB-231 cells was carried out using heparin–Sepharose affinity chromatography. In all experiments, less than 20% of the TGFα activity loaded onto the column was recovered in the unabsorbed fractions. A sharp peak of EGF receptor binding activity was eluted by heparin–Sepharose chromatography at a concentration of 0.4–0.6 M NaCl (Figure 4A). The major activity which retained 70–80% of the loaded activity is shown in Figure 4B. In contrast, neither EGF, TGFα, nor their precursors bind to heparin (data not shown). The heparin binding TGF-like polypeptide (HP-TGFα-like) was further purified by reversed-phase chromatography (HPLC) in two steps. A pool of fractions containing EGF receptor-competing activity from heparin–Sepharose chromatography was reconstituted in 0.05% TFA in water and then chromatographed on a μBondapak C_3 column. A steep acetonitrile gradient (0–100%) was used in this step. The elution pattern is shown in Figure 5 (top). The TGFα-like polypeptide elutes as a sharp peak in 30% acetonitrile and is separated from the bulk of the contaminating proteins. The capacity of the individual fractions to compete for EGF receptor binding and to stimulate the growth of NRK cells in soft agar was determined (Figure 5, bottom). A pool of the active fractions (indicated with an horizontal bar) was rechromatographed on the same column. Fractions were eluted with a 0–20% acetonitrile gradient in 0.05% TFA for 5 min followed by a linear 20–40% acetonitrile gradient. The TGFα-like polypeptide activity was eluted at 25–30% acetonitrile and effectively separated from other contaminant proteins. We used size-exclusion chromatography under acidic conditions to verify the size of the TGFα-like peptide. The active fractions for EGF receptor-competing activity (Figure 6A) were approximately 30 kDa in size. The purified 30-kDa TGFα-like polypeptide was analyzed by SDS–PAGE. An example of the apparent homogeneity of the active material is shown in Figure 6B [see also Lupu et al. (1991)]. At this point, we cannot conclude that this purified polypeptide precisely comigrates with the ^{35}S metabolically labeled material described earlier, although both migrate at approximately 30 kDa. In other experiments, when traces of metabolically labeled MDA-MB-231-conditioned media were used as a tracer, comigration of ^{35}S-labeled material with 30-kDa protein was observed (data not shown).

A summary of the steps leading to the isolation and purification of TGFα-like polypeptide is presented in Table I. A 27% recovery of activity and approximate 5400-fold purification were achieved.

Biological Characterization of the TGFα-like Material. The EGF receptor binding activity of the gp30 TGFα-like protein was compared with that of EGF in a radioreceptor assay. Both growth factors competed with [^{125}I]EGF for EGF receptor sites on A431 membranes. The specific EGF-competing activity of the purified TGFα-like polypeptide was found to be (1–1.5) × 10^6 units/mg; 1.1 ng/mL TGFα-like polypeptide was required to inhibit EGF binding by 50%. TGFα-like polypeptide was as effective as EGF in receptor binding. Furthermore, the purified gp30 TGFα-like polypeptide stimulated the growth of serum-deprived NRK fibroblasts (Figure 7A) and induced colony formation of these cells in soft agar (Figure 7B). The bioactivity of the purified TGFα-like polypeptide was also tested by anchorage-dependent growth assays of the carcinogen-immortalized human mammary epithelial cells 184A1N4 (data not shown) and anchorage-independent growth assays of 184A1N4-derived cells partially transformed by SV40 T antigen, 184A1N4T (data not shown). The biological activity of the purified gp30 TGFα-like factor was further assessed by examining its ability to induce autophosphorylation of the EGF receptor. A431 cells, which overexpress the EGF receptor, were incubated with various concentrations of EGF, TGFα, or HB-TGFα-like growth factors. Each of the three peptides similarly

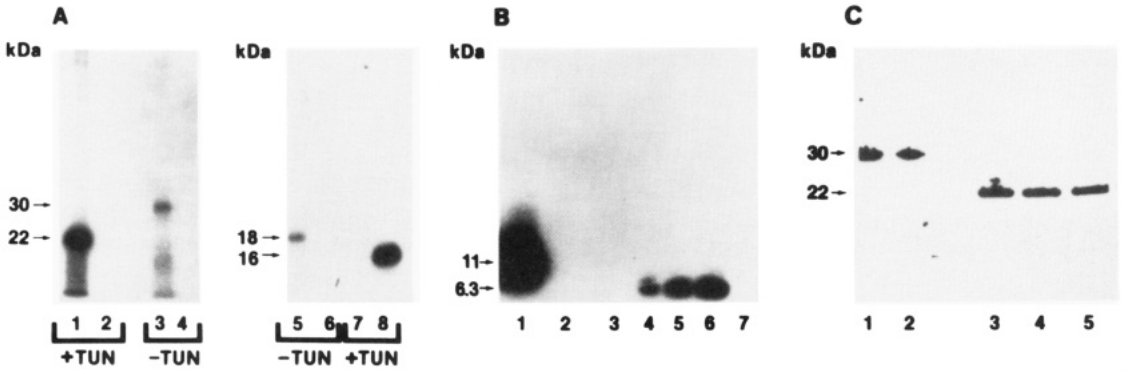

FIGURE 3: Inhibition of glycosylation, enzymatic deglycosylation, and specific cleavage of the TGFα-related species. (A) Metabolic labeling of MDA-MB-231 (lanes 1–4) and Rat-FeSrV (lanes 5–8) was carried out in the absence or presence of tunicamycin (20 μg/mL). Immunoprecipitations were performed with polyclonal antibody R399 (lanes 1, 3, 5, and 8) or preimmune serum (lanes 2, 4, 6, and 7) followed by SDS–PAGE analysis. (B) Metabolic labeling of conditioned media derived from MDA-MB-231 and FeSrV cells was carried out in the presence of tunicamycin as described above, followed by specific cleavage with 25 μg of elastase. Immunoprecipitation was then performed with R399 antibody (lanes 1, 5, and 6) and NRS (lanes 2, 3, and 7). Lanes 1 and 2 represent precipitated material derived from MDA-MB-231 cells, lanes 5–7 represent precipitated material derived from FeSrV cells, and lane 4 represents immunoprecipitation of mature TGFα. Samples were analyzed by 15% SDS–PAGE. Molecular mass markers are indicated by arrows (kDa, kilodaltons). (C) Samples equivalent to 100 ng of purified gp30 derived from MDA-MB-231 cells (lane 1) were incubated as described under Materials and Methods with buffer alone (lane 2) or with 2 (lane 3), 4 (lane 4), and 6 μg (lane 5) of *N*-glycanase followed by silver staining of a 15% SDS–PAGE.

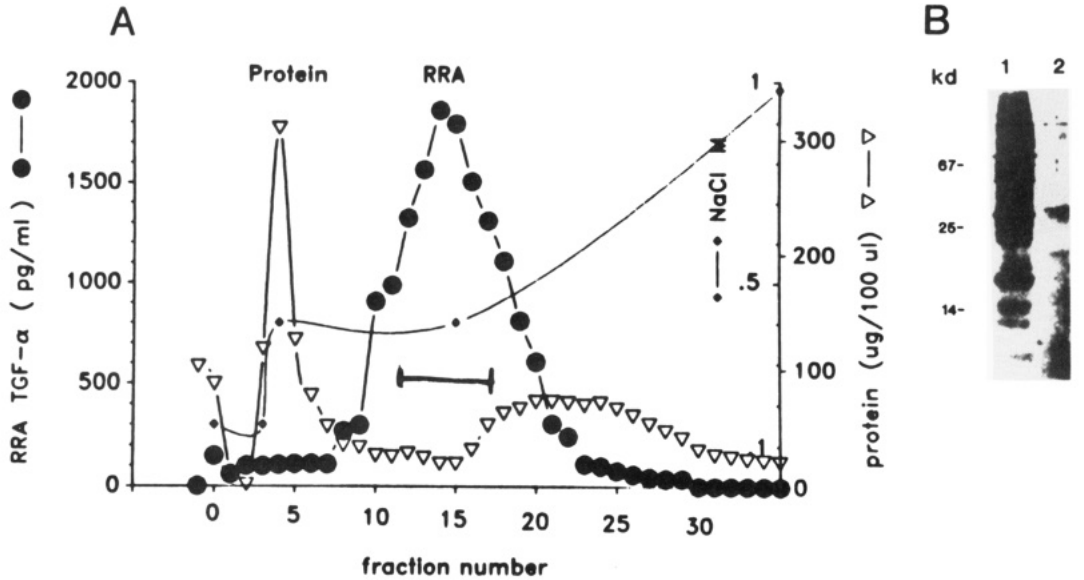

FIGURE 4: Heparin–Sepharose affinity chromatography of TGFα-like activity from MDA-MB 231 cells. (A) Affinity chromatography of 1 L of unconcentrated conditioned media was performed on a heparin–Sepharose column with elution by a steep gradient from 0.4 to 3.0 M NaCl. Absorbance was monitored at 280 nm (open triangles). Three-milliliter fractions were collected and lyophilized after desalting. The lyophilized fractions were reconstituted in 500 μL. Aliquots from the resulting 500-μL fractions were analyzed for EGF receptor binding activity (closed circles). The horizontal bar indicates the fractions pooled for reverse-phase C_3 chromatography (see Figure 6). (B) An aliquot of 30 ng from the active fraction was analyzed by 15% SDS–PAGE followed by silver staining. Lane 1 represents unconcentrated media and lane 2 the active fraction after chromatorgraphy.

stimulated phosphorylation of the EGF receptor (Figure 8).

In Vitro Translations. In order to determine if HB-TGFα-like is a product different than a posttranslational modification of the TGFα precursor, we performed in vitro translations of mRNA isolated from MDA-MB-231 cells. As a control, mRNA derived from H8 cells was used. Immunoprecipitation of the specific translated products was performed after treatment with elastase. The precipitated 22-kDa polypeptide for MDA-MB-231 mRNA (Figure 9A, lane 1) and 16-kDa polypeptide for H8 mRNA (Figure 9A, lane 7) were subjected to elastase cleavage, resulting in peptides of 11 kDa for MDA-MB-231 cells (Figure 9A, lane 3, and Figure 9B, lane B) and 6 kDa for H8 cells (Figure 9A, lane 11, and Figure 9B, lane A). These in vitro translated proteins corresponded to the sizes observed earlier for the deglycosylated 22-kDa polypeptide from MDA-MB-231 cells and the unglycosylated 16-kDa polypeptide from Rat-FeSrV cells. The in vitro translated HB-TGF-like products are summarized in Table IIB. These results indicated that the 11-kDa peptide derived from MDA-MB-231 cells is probably not a result of posttranslational modifications.

Peptide Mapping. In order to determine the degree of homology between gp30 and mature TGFα, peptide mapping was performed using the method of Cleveland. Immunoprecipitation of metabolically labeled conditioned media from MDA-MB-231, H8, and Rat-FeSrV cells was carried out with the R399 anti-TGFα polyclonal antibody. Precipitates were analyzed by SDS–PAGE, and the specific bands were electroeluted (gp30 from MDA-MB-231 cells, 6 kDa from H8 cells, and 18 kDa from Rat-FeSrV cells). These proteins were subjected to enzymatic treatment with *N*-glycanase and elastase. The sizes of the precipitated bands are summarized in Table IIA. The products were then subjected to peptide digestion using 25 μg/mL V8 protease. After complete

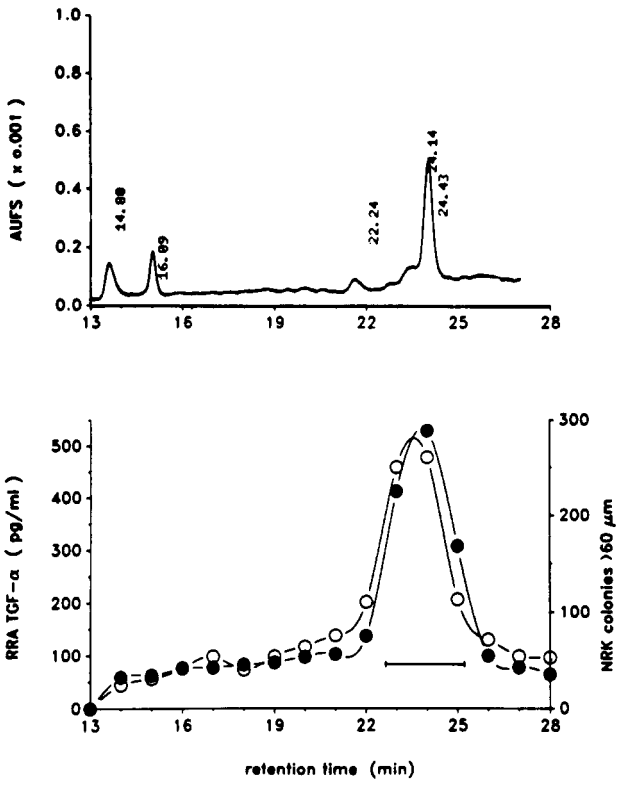

FIGURE 5: Reversed-phase C_3 chromatography of conditioned media from MDA-MB-231 cells after heparin affinity chromatography. A sample of 1.5 mg of total protein from the active fraction eluted from heparin–Sepharose was loaded on a μBondapak C_3 column in 0.05% TFA. Elution was achieved with a linear gradient of acetonitrile (0–100%). As described under Materials and Methods, 1 mL/min fractions were collected, lyophilized, and reconstituted in 200 μL of H_2O. The absorbance at A_{280nm} is indicated in the top panel. Fractions were assayed for EGF-competing activity (closed circles) using a 100-μL aliquot or for NRK colony formation (open circles) using a 50-μL aliquot.

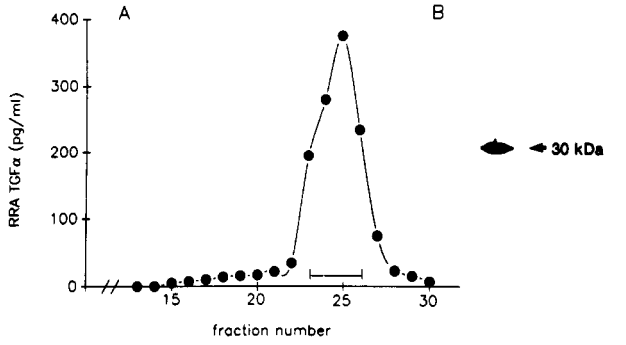

FIGURE 6: Size-exclusion rechromatography of TGFα-like material. (A) Pooled fractions from the previous step were rechromatographed by acidic size-exclusion chromatography (Suprose 12, Pharmacia, NJ) to verify the size of the growth factor. The elution was performed with 1 M acetic acid; 1-mL fractions were collected and then lyophilized. After reconstitution with 100 μL of Tris-HCl (pH 7.5), the fractions were analyzed for EGF-competing activity by radioreceptor assay. (B) Homogeneity of proteins contained in the pooled fractions from the final purification step were determined by silver staining of a 15% SDS–PAGE. This reproduces Figure 3C, lane 2.

digestion, the samples were analyzed by C18 reversed-phase chromatography. Three major peptide peaks for each cell line eluted at different acetonitrile concentrations by reversed-phase chromatography. However, the concentrations at which the peptides isolated from MDA-MB-231 cells eluted were different from the peptides isolated from H8 and FeSrV cells as described in Table III and Figure 10. The peptide elution patterns of the 6-kDa TGFα derived from H8 cells and RatFeSrV cells were essential identical. Similar results were obtained with 40 μg of V8 protease, indicating that the concentration of the enzyme was not responsible for the differential peptide cleavage.

To further explore the identity of the two apparently distinct protein products, V8 protease digestion was performed on electroeluted polypeptides after immunoprecipitations of in vitro translated products. The resulting peptides were analyzed by C18 reversed-phase chromatography under identical acetonitrile gradients. Peptide mapping of the translated products had a very similar profile as the purified gp30 factor after treatment with *N*-glycanase and elastase as shown in Table III. These experiments were done 3 times for each V8 protease concentration, and the results were reproducible.

DISCUSSION

General Significance. On the basis of in vitro studies, TGFα has been postulated to be a autocrine mediator of tumor growth (Sporn et al., 1980). Studies suggest that TGFα activity can be detected in body fluids of cancer patients. Its presence may provide important information concerning the biology of a patient's tumor (Stromberg et al., 1986a,b; Twardzick et al., 1982; Sherwin et al., 1983). In effusions from breast cancer patients, the presence of TGFα was correlated with the absence of estrogen and progesterone receptors in primary breast tumors (Artega et al., 1988). We have shown that hybridization of MDA-MB-231 mRNA to a TGFα cDNA probe yielded an mRNA of the predicted size of 4.8 kb as well as a novel 1.6-kb species (Bates et al., 1988). The significance of the 1.6-kb mRNA is unknown, but it was also seen in poly-$(A)^+$-enriched RNA from human primary breast cancers. Expression of the 4.8- and 1.6-kb transcripts in MDA-MB-231 cells is associated with the presence of the high molecular weight form of TGFα secreted by MDA-MB-231 cells as detected by RIA using antibodies against mature TGFα. However, the presence of the 1.6-kb transcript is not in all cases associated with the 30-kDa polypeptide (Susan Bates, personal communication). The extent of production of this TGFα-like polypeptide in breast cancer cell lines remains to be seen.

In the present study, we have shown that a heparin binding TGFα-like growth factor is produced by the estrogen receptor-negative human breast adenocarcinoma cell line MDA-MB-231 and is released into the media. We purified this polypeptide to apparent homogeneity and partially characterized it.

Glycosylation and Secretion. Multiple polypeptide species with TGFα-like activity have been described with sizes ranging from 6 to 68 kDa. These forms are most likely processing products derived from a common glycosylated and palmitoylated precursor. It has been suggested that the 18-kDa species corresponds to the entire TGFα precursor while the 6-kDa and larger forms represent N-glycosylated forms of TGFα released after proteolytic cleavage of this same transmembrane TGFα precursor. None of these species has a size similar to the gp30 with TGFα-like activities describe in this study. Evidence for N-glycosylation of the 22-kDa TGFα-like precursor form derived from MDA-MB-231 cells is shown by a reduction in molecular weight after tunicamycin treatment. Likewise, *N*-glycanase treatment of the purified gp30 yielded a protein of the same molecular weight. The presence of tunicamycin had little effect on the secretion of gp30 into the medium. It is therefore unlikely that N-glycosylation plays an important role in secretion or cell-surface transport as observed for other membrane-anchored forms of growth factors.

Table I: Purification of TGFα-like Activity from Conditioned Medium from MDA-MB-231 Cells[e]

purification step	protein recovered[a] (mg)	EGF competing act. (units/mg of TGFα)[b]	rel sp act. (units/mg of protein)	degree of purification (x-fold)	recovery (% act.)
conditioned medium	98	450	4.6	1	100
acid-soluble supernatant	82	419	5.1	1	93
gel filtration	2.95	209	70.8	15.3	46
(1) heparin–Sepharose	1.54	230	149	32.3	51
(2) reverse phase[c]	0.03	173	5766	1253	38
(3) reverse phase[d]	0.005	124	24800	5400	27

[a] Total protein was determined using BSA as a standard. The quantitation of step 6 was based on extrapolation from standard values. The absolute specific activity of a companion aliquot was found to be 1 million units/mg. [b] One unit of EGF competing activity is defined as the amount of protein that inhibits the binding of [^{125}I]EGF to the receptor by 50%. [c] Steep acetonitrile gradient. [d] Shallow acetonitrile gradient. [e] 1–3 are subsequent purification steps. Each value represents the mean of 4–6 experiments, and they were reproducible within 10%.

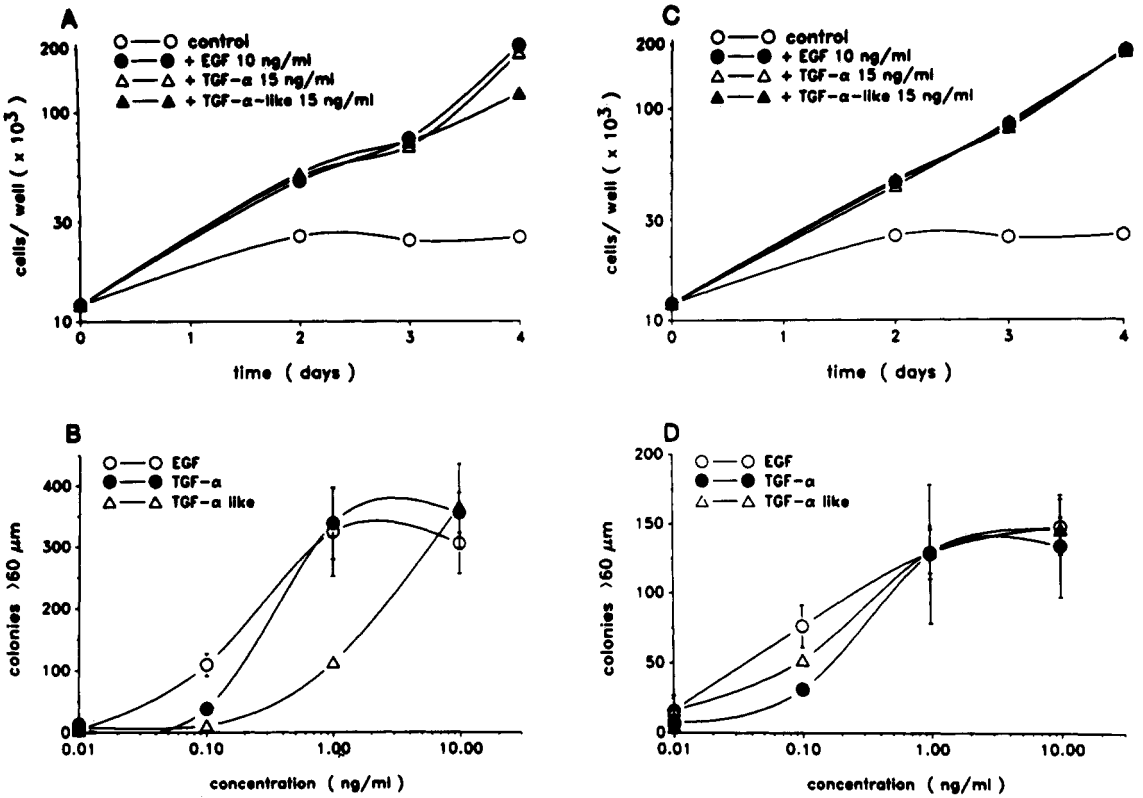

FIGURE 7: Effect of TGFα-like material on anchorage-dependent and anchorage-independent growth of NRK and A1N4T cells. Anchorage-dependent: The effect of purified gp30 TGFα-like protein on anchorage-dependent growth was tested during a 4-day period using NRK fibroblasts (A). The effect of the TGFα-like growth factor was compared to the effects of EGF and mature TGFα. Anchorage-independent: NRK cells (B) were tested for colony formation after addition of the quantities of active protein indicated.

Previous studies (Ignotz et al., 1986) and our results with the Rat-FeSrV cells have shown that elastase can cleave a 6-kDa product from the TGFα precursor (18 kDa). Here we provide evidence that cleavage with elastase of the 22-kDa precursor, the deglycosylated form of gp30, yielded an 11-kDa product. Elastase concentrations 5-fold higher than those required for complete conversion of the 22-kDa precursor species into an 11-kDa product did not cause any further digestion, indicating that enzymatic cleavage by elastase is saturable. After elastase treatment, we observed an increased degree of immunoreactivity, suggesting that immunoreactive epitopes were more favorably exposed in the 11-kDa product than in the 22-kDa deglycosylated precursor or the gp30 derived from MDA-MB-231 cells. Processing gp30 is summarized in Table IIA,B.

Heparin Binding Properties. EGF and TGFα (precursors or mature forms) can be separated from heparin binding growth factors, such as PDGF (56, 57) and FGF (58), by heparin affinity chromatography; EGF and TGFα can be recovered in the nonabsorbed fraction (22). Heparin affinity chromatography is a conventional method for the isolation of endothelial cell growth factors such as bFGF. A high molecular weight protein with TGFα-like activity from activated human alveolar macrophage that binds to heparin–Sepharose was reported by Madtes (1988). Two peaks of activity at approximately 0.7 and 1.5 M NaCl were indicated. Further processing of these molecules has not been reported. Recently, a novel heparin binding EGF polypeptide was described by Higashiyama (1990). This molecule, in contrast to gp30, has a higher affinity binding to heparin, and its molecular mass is about 22 kDa. During the preparation of this paper, Yarden et al. (1991) described an activity similar to our 30-kDa activity secreted from rat-1 cells. Further studies and the protein sequence will determine similarities between the two proteins.

In the present study, we have demonstrated that a gp30 TGFα-like protein from human breast cancer epithelial cells has the capability to bind to heparin and be eluted from the column at a concentration of 0.5–0.6 M NaCl. Furthermore, the 22-kDa product obtained after deglycosylation of the gp30 TGFα-like glycoprotein also had the ability to bind to heparin, showing that heparin binding of gp30 was due to the protein backbone. Thus, the heparin binding portion of the

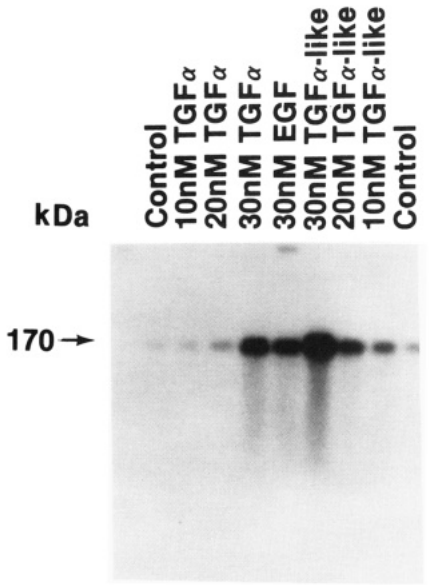

FIGURE 8: Induction of tyrosine autophosphorylation of EGF/TGFα receptors by gp30 TGFα-like activity. A431 cells were incubated for 30 min at 37 °C with different concentrations of HEGF, recombinant TGFα, and TGFα-like protein as described under Materials and Methods. After cell lysis, EGF receptors were immunoprecipitated with monoclonal antibody 225. Proteins were separated by SDS–PAGE. Phosphorylation reactions were carried out in the presence of [γ-^{32}P]ATP.

EGF receptor-competing activity produced by MDA-MB-231 cells represents a species distinct from EGF and TGFα.

Reversed-Phase and Gel Filtration Chromatography Purification. Previous studies (Stromberg et al., 1986a,b; Shimo Kado et al., 1985; Martinet et al., 1986; Baird et al., 1985; Perroteau et al., 1986) using binary solvent systems consisting of TFA and a limiting organic solvent have clearly shown the high resolution of a large molecular weight polypeptide by reversed-phase chromatography. Biological activity of the samples was tested by EGF receptor-competing activity and by the ability to stimulate the growth of NRK cells. The activity appeared as a sharp peak at 30% acetonitrile and was separated from the bulk of contaminating proteins. The purification increased the specific EGF receptor-competing activity of the sample, with minimal loss of growth stimulation activities. Further purification was achieved with a shallow acetonitrile gradient. A sharp peak of EGF receptor-competing activity was generated with 30% loss of previous activity. This sharp peak was analyzed by acidic size-exclusion chromatography, and the purity of the final TGFα-like glycoprotein was established by SDS–PAGE after silver staining. The purified high molecular weight TGFα derived from MDA-MB-231 showed remarkable EGF receptor-competing activity. Approximately equimolar concentrations of this growth factor and EGF were required to inhibit EGF binding activity by 50%, suggesting a close structural relationship between these two polypeptides. The half-maximal response to TGFα-like polypeptide in soft agar was reached at approximately 60 EGF receptor-competitive units.

Peptide Mapping. Peptide mapping is widely used to establish homology between different proteins. Partial enzymatic digestion of proteins with V8 protease in the presence of SDS is followed by analysis of the digested peptides by either SDS–PAGE or reversed-phase analysis (HPLC). The HPLC peptide profile that was obtained after V8 protease digestion of the 11-kDa product from MDA-MB-231 cells was different from that seen after V8 protease digestion of the 6-kDa polypeptide from H8 cells and the 6-kDa product from

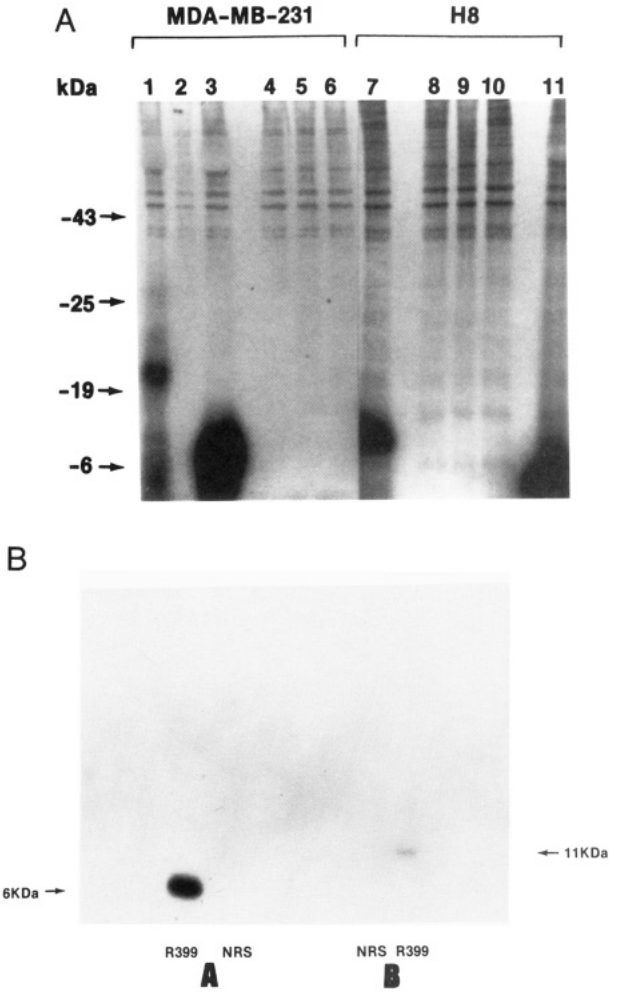

FIGURE 9: In vitro translation and immunoprecipitation of mRNA. (A) Total RNA from MDA-MB 231 cells (lanes 1–6) and H8 (lanes 7–11) was subjected to in vitro translation. After treatment with 25 μg of elastase (lanes 3, 5, 10, and 11), the samples were immunoprecipitated with anti-TGFα R399 (lanes 1, 3, 7, and 11). Lanes 2, 4–6, and 8–10 denote incubation with preimmune (NRS) serum. Precipitates were analyzed by 15% SDS–PAGE and detected by fluorography. Molecular masses in kilodaltons (kDa). (B) Total RNA from MDA-MB 231 cells (B) and H8 (A) was subjected to in vitro translation. After treatment with 25 μg of elastase, the samples were immunoprecipitated with anti-TGFα R399 or preimmune (NRS) serum. Precipitates were analyzed by 15% SDS–PAGE and detected by fluorography.

Table II: Biochemical Processing of Secreted and in Vitro Translated Polypeptides Derived from MDA-MB-231, H8, and Rat-FeSrV Cells

(A) Sizes (kDa) of Precipitated Bands	MDA-MB-231	Rat-FeSrV	H8
electroelution from SDS–PAGE	30	18	6
N-glycanase treatment	22	16	6
elastase cleavage	11	6	6

(B) Sizes (kDa) of in Vitro Translated HB-TGF-like Products	MDA-MB-231	Rat-FeSrV	H8
in vitro translated product	22	16	6
elastase cleavage	11	6	6

Rat-FeSrV cells. This suggested that the peptide sequence of the factor isolated from MDA-MB-231 cells was different from that of the other cells. Peptide mapping differences, however, cannot rule out posttranslational modifications that can occur. In order to demonstrate that gp30 is a product of a different gene, we performed elastase cleavage and V8

Table III: Peptide Mapping of the 6- and 11-kDa Polypeptides Derived from MDA-MB-231, H8, and Rat-FeSrV Cells[a]

cell type	HPLC % acetonitrile (under same acetonitrile gradient) for electroeluted polypeptide: after deglycosylation and elastase treatment	after in vitro translation and elastase treatment
MDA-MB-231 (11 kDa)	16, 18.7, 21.7	15.5, 18.3, 21.3
H8 (6 kDa)	24, 28, 32	23.7, 28.7, 32.3
Rat-FeSrV (6 kDa)	24, 29, 32.6	23.9, 29.2, 32.4

[a] A summary of the metabolically labeled and in vitro translated proteins from MDA-MB-231, H8, and FeSrV cells. The resulting products were obtained as described for Figure 10.

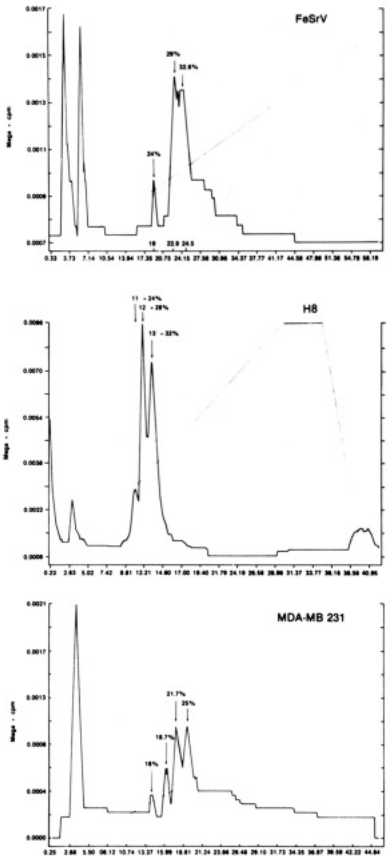

FIGURE 10: Peptide mapping of the 6- and 11-kDa polypeptides derived from MDA-MB-231, H8, and Rat-FeSrV cells. Proteins were metabolically labeled and were then immunoprecipitated with R399 polyclonal antibody. The precipitates were analyzed by 15% SDS–PAGE. Bands from the samples (30 kDa from MDA-MB-231 cells, 18 kDa from FeSrV cells, and 6 kDa from H8 cells) were excised from the gel treated with 20 μg/mL *N*-glycanase and cleaved with elastase. Then the proteins were digested with 25 μg/mL V8 protease. The products were then analyzed by reversed-phase HPLC; peaks indicate percent acetonitrile used for elution. The digestion pattern obtained with Rat-FeSrV cells is shown in the upper panel, H8 in the middle panel, and MDA-MB-231 in the lower panel.

digestion of immunoprecipitated material obtained after in vitro translation from total mRNA from MDA-MB-231 and H8 cells. The different molecular mass sizes of these polypeptide are shown in Table III. The peptide mapping pattern was identical to that seen earlier from material secreted from MDA-MB-231 cells. This observation would appear to rule out additional posttranslational modifications of the 22-kDa deglycosylated precursor of gp30 derived from MDA-MB-231 cells. We conclude that the 22-kDa precursor is not the membrane-anchored TGFα precursor described by other groups. A similar size TGFα-like form has been reported in the macrophage system. This soluble form, however, has not been further characterized.

Biological Activity. The binding of EGF and TGFα to the EGF receptor initiates a complex series of events including activation of the EGF receptor and its intrinsic tyrosine kinase activity, receptor clustering, and internalization and degradation of the ligand–receptor complex. A large body of evidence now supports the conclusion that activation of the receptor-associated tyrosine kinase is essential for receptor-mediated response (Brachmann et al., 1989). The purified gp30 TGFα-like growth factor was able to stimulate autophosphorylation of the EGF receptor more effectively than mature 6-kDa TGFα under identical conditions. Under similar conditions, we have recently shown that gp30 can also induce direct tyrosine phosphorylation of erbB-2 (Lupu et al., 1990), in contrast to EGF or TGF which do not induce tyrosine phosphorylation of erbB-2 (Lupu et al., 1990).

The biological activity of the final purified TGFα-like material was shown by its ability to induce anchorage-dependent and -independent growth of NRK cells. The immortalized human mammary epithelial cell line 184A1N4 was used to study anchorage-dependent growth of cells while the partially transformed 184A1N4T cell line was used to investigate anchorage-independent growth. In order to transform, these cells require EGF/TGF. Both of these cell lines responded to gp30 TGFα-like protein in a dose-dependent manner similar to that observed for EGF and TGFα. These results suggest a growth-stimulatory role for this gp30 polypeptide. It is possible that this novel mitogenic TGFα-like growth factor from a malignant breast cancer cell line may play a role in either the genesis or the maintenance of human breast cancer.

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