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Cloning and characterization of an androgen-induced growth factor essential for the androgen-dependent growth of mouse mammary carcinoma cells

(heparin-binding growth factors/autocrine growth/androgen/breast cancer)

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ABSTRACT **An androgen-dependent mouse mammary carcinoma cell line (SC-3) requires androgen for growth stimulation. We have shown previously that androgen acts on SC-3 cells to induce secretion of a fibroblast growth factor (FGF)-like growth factor, which in turn stimulates growth of the cells in an autocrine manner. In this study, the androgen-induced growth factor (AIGF) was purified from a conditioned medium of SC-3 cells stimulated with testosterone. cDNA cloning of AIGF by use of its partial amino acid sequence data revealed that AIGF is a distinctive FGF-like growth factor. An AIGF cDNA (pSC17) encodes a 215-amino acid protein with a putative signal peptide, which shares 30–40% homology with known members of the FGF family. The AIGF mRNA was markedly induced by 10 nM testosterone in Northern blot analysis. Expression of AIGF cDNA in mammalian cells clearly showed remarkable stimulatory effects of AIGF on growth of SC-3 cells in the absence of androgen. Thus, it is clear that the androgen-induced growth of SC-3 cells is mediated in an autocrine manner by AIGF, which is secreted by the tumor cells themselves in response to hormonal stimuli.**

Sex hormones play a critical role in carcinogenesis and mitogenesis of breast or prostate cancers (1). In the initial stages, these cancers cannot grow without specific hormonal stimuli. Among various models of cancer growth, the mechanism of autocrine growth has become one of the central concepts for cancer proliferation (2). Growth factors and growth factor receptors encoded by oncogenes have been reported (3, 4), and they seem to form an autocrine loop in cancer cells. In several hormone-responsive cancer cell lines, it has been shown that sex hormones exert action via autocrine growth factors (reviewed in ref. 5). For example, in an estrogen-responsive human breast cancer cell line, MCF-7, a transforming growth factor α (TGF-α)-like or insulin-like growth factor I (IGF-I)-like factor is thought to mediate the estrogen-responsive growth of cancer cells (6, 7). However, none of such hormone-induced autocrine factors has been structurally identified yet.

In an androgen-dependent cancer cell line (SC-3) derived from a mouse mammary carcinoma, Shionogi carcinoma 115 (SC115), we have shown that remarkable growth-stimulating activity on SC-3 cells was found in a culture medium of androgen-stimulated SC-3 cells and that the growth-stimulating molecule(s) bound tightly to a heparin-conjugated Sepharose column (8, 9). We also have reported that the androgen-induced growth of SC-3 cells was markedly inhibited by anti-basic fibroblast growth factor (FGF) polyclonal antibodies (8–11). These findings strongly suggest that one or more FGF-like growth factors are produced and secreted by the cancer cells themselves in response to androgenic stimuli and are essential for the androgen-dependent growth of SC-3 cells. However, expression of neither FGF genes nor FGF-related genes such as *hst-1* and *int-2* was found in androgen-stimulated SC-3 cells (B.S., unpublished observation). This prompted us to isolate and characterize the growth factor essential for SC-3 cells, designated as an androgen-induced growth factor (AIGF).

Here we report isolation, cDNA cloning, and functional expression of AIGF from androgen-stimulated SC-3 cells.‖ Structural analysis revealed that AIGF was a novel FGF-like growth factor. AIGF mRNA was induced by 10 nM testosterone in SC-3 cells. Expression of AIGF cDNA in mammalian cells showed that AIGF had remarkable stimulatory effects on SC-3 cells. These findings lead us to conclude that the androgen-dependent growth of SC-3 cells is mediated by AIGF through an autocrine mechanism.

MATERIALS AND METHODS

Cells. The SC-3 cell line used in the present study was derived from an androgen-responsive mouse mammary SC115 tumor. The method for cloning has been described (12).

Cell Growth Assay. The cell proliferation assay was performed by [^{3}H]thymidine incorporation in SC-3 cells as described (11).

Preparation of Serum-Free Conditioned Medium and Purification of AIGF. SC-3 cells (10^6 cells per 100-mm dish) were plated and cultured as described (11). Four liters of serum-free conditioned medium from culture of testosterone (10 nM)-stimulated SC-3 cells was concentrated (up to 10-fold) and loaded onto a heparin-Sepharose column (gel-bed volume, 5 ml) equilibrated with 10 mM Tris·HCl buffer (pH 7.0) containing 0.6 M NaCl, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 mM phenylmethylsulfonyl fluoride, 2 mM EDTA, and leupeptin at 500 μg/ml. The column was washed with the equilibration buffer, and adsorbed proteins were eluted with 10 gel-bed volumes of the equilibration buffer containing 2 M NaCl. The eluted fraction was diluted with the equilibration buffer and refractionated on a second heparin-Sepharose column (gel-bed

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Abbreviations: FGF, fibroblast growth factor; SC115, Shionogi carcinoma 115; MMTV, mouse mammary tumor virus; AIGF, androgen-induced growth factor.

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‖The AIGF sequences reported in this paper have been deposited in the GenBank data base [accession nos. D12482 (pSC17), D12483 (pSC15)].

volume, 0.5 ml) in the same manner. The eluted fraction was then loaded onto a 4.6 × 50 mm Cosmosil C_4 reverse-phase column and developed with a linear gradient of 0–60% acetonitrile in 0.1% CF_3COOH over 30 min at a flow rate of 1 ml/min. Each fraction was assayed for [^{3}H]thymidine incorporation in SC-3 cells. Fractions with biological activity were lyophilized and electrophoresed in two contiguous lanes under nonreducing conditions on a 10–20% gradient polyacrylamide gel with 0.1% SDS. The gel of one lane was sliced into 38 equal fractions, and the other was silver-stained. The proteins were eluted from the gel slices by shaking at 4°C overnight in 10 mM Tris·HCl, pH 7.5/0.1% CHAPS. The eluates were assayed for [^{3}H]thymidine incorporation in SC-3 cells. The purified AIGF was denatured with 70% formic acid for 2 hr at room temperature and digested with 500 ng of lysylendopeptidase (Wako Pure Chemicals, Osaka) in 50 mM Hepes buffer (pH 7.6) for 5 hr at 37°C. The digested sample was directly loaded onto a 3.9 × 150 mm μBondasphere C_{18} reverse-phase column. Peptides were separated with a linear gradient of 0–60% acetonitrile over 60 min at a flow rate of 1 ml/min, and distinct peaks were collected. Sequence analysis was carried out on an ABI 477A protein sequencer equipped with an on-line ABI 120A phenythiohydantoin analyzer (Applied Biosystems).

RNAs. Subconfluent SC-3 cells (about 4 × 10^6 cells per dish) cultured in Eagle's minimal essential medium (MEM) containing 2% (vol/vol) fetal calf serum treated with dextran-coated charcoal and 10 nM testosterone were scraped, and total cellular RNA was prepared by the acid guanidium isothiocyanate–phenol–chloroform extraction method (13). Poly(A)$^+$ RNA was isolated by using oligo(dT)-latex (Nippon Roche, Tokyo).

PCR Analysis. Randomly primed single-stranded cDNA was synthesized from total RNA of SC-3 cells cultured in the presence of 10 nM testosterone by using a reverse transcriptase (Superscript, Bethesda Research Laboratories, Gaithersburg, MD). Oligonucleotide primers were synthesized according to the partial amino acid sequences of AIGF. Among several primer sets, a set of 5′ > ATHAAYGCI-ATGGCIGARGAYGG < 3′ and 5′ > GCCATRTACCAIC-CYTCRTA < 3′, where H is A, C, or T, R is A or G, and Y is C or T, gave a single DNA band after PCR. PCR was cycled 30 times at 94°C for 90 sec, at 37°C for 90 sec, and at 72°C for 180 sec with 10 ng of single-stranded cDNA, 200 ng of synthesized oligonucleotides, and recombinant *Taq* DNA polymerase (Perkin–Elmer/Cetus). Nucleotide sequence of the amplified cDNA was determined by a dideoxynucleotide chain-termination method (14).

cDNA Cloning. Oligo(dT)-primed double-stranded cDNA was synthesized by a cDNA synthesis system (Bethesda Research Laboratories) and ligated into a plasmid vector pcDLSRα296 (15) by using *Bst*XI linkers. Hybridization was performed by using a ^{32}P-labeled 240-base-pair (bp) probe obtained by the PCR at 65°C in 1.5× SSPE (1× SSPE = 0.15 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA) containing 1.0% SDS and 0.5% Blotto. Subsequent washing was in 2× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7) containing 0.1% SDS at 50°C for 30 min and 0.1 × SSC containing 0.5% SDS at 37°C for 30 min. Twenty positive clones were subjected to transfection to COS-7 cells. COS-7 cells were transfected with 10 μg of cDNA by the calcium phosphate precipitation method. Forty-eight hours after transfection, the medium was collected and assayed for [^{3}H]thymidine incorporation in SC-3 cells. The vector plasmid alone was also transfected to COS-7 cells, and the conditioned medium was also checked for biological activity as control.

Northern Blot Analysis. SC-3 cells (8 × 10^5 cells per 100-mm dish) were plated in 10 ml of MEM containing 2% fetal calf serum treated with dextran-coated charcoal in the absence of testosterone. On the following day, the cells were washed with phosphate-buffered saline and were further incubated either in the absence or presence of 10 nM testosterone in MEM containing 2% fetal calf serum. At indicated hours, poly(A)$^+$ RNAs were isolated as described above; 2 μg of poly(A)$^+$ RNAs were electrophoresed in 1% agarose gel containing 0.66 M formaldehyde and transferred onto nylon membrane. Hybridization was performed in 1.5× SSPE containing 1.0% SDS and 0.5% Blotto at 65°C for 18 hr. Blots were subsequently washed with 2× SSC containing 0.1% SDS at 50°C for 30 min and in 0.1× SSC containing 0.5% SDS at 37°C for 30 min. Autoradiography was carried out at −70°C for 48 hr.

RESULTS

Purification of AIGF. Serum-free conditioned medium obtained from testosterone-stimulated SC-3 cells had remarkable growth-stimulatory effects on SC-3 cells. Material with growth-stimulatory activity was bound to heparin-Sepharose and was eluted with the buffer containing 2 M NaCl. After repeated heparin-Sepharose chromatography, bioactive fractions were subjected to a reverse-phase HPLC (Fig. 1 *Top*). Bioactive fractions obtained from the reverse-phase HPLC showed two major proteins on SDS/PAGE at relative molecular masses of 32 kDa and 28 kDa, respectively, under both nonreducing (Fig. 1 *Middle*) and reducing conditions (data not shown). Both 32-kDa and 28-kDa proteins showed similar biological potencies on SC-3 cells (Fig. 1 *Middle*). Although N termini of both proteins were blocked, peptide mapping after lysylendopeptidase digestion on a reverse-phase HPLC revealed that most peptides derived from the two proteins were eluted at identical positions (Fig. 1 *Bottom*). Amino acid sequences of the peptide fragments were determined and are shown in Fig. 2 *Upper* (underlined).

cDNA Cloning of AIGF. According to the sequence data of the peptides, putative degenerated oligonucleotide primers were synthesized, and PCR was performed. Several primer sets were used for the reaction, one of which gave a 240-bp DNA band involving several sequences of the peptide fragments obtained from lysylendopeptidase digestion of AIGF. The amplified DNA fragment was used as a probe to screen a cDNA library of testosterone-stimulated SC-3 cells ligated into a mammalian expression plasmid vector, pcDLSRα296. Screening of 4 × 10^4 clones yielded 40 positive clones, 20 of which were subjected to transfection to COS-7 cells. Eight clones gave remarkable proliferative effects on SC-3 cells. All of the bioactive clones contained 1.0- to 1.2-kilobase (kb) inserts.

Structure of AIGF. Nucleotide sequencing of one of the clones (pSC17) revealed an open reading frame encoding putative 215 amino acid residues with a 3′-untranslated sequence (Fig. 2 *Upper*). The ATG initiation codon is located at nucleotide position 174, followed by a putative signal peptide of hydrophobic residues. A stop codon is present at position 819. All peptides except peak 7 obtained from lysylendopeptidase digestion are involved in the sequence (Fig. 2 *Upper*). However, nucleotide sequencing of another clone (pSC15) revealed that codons for 10 amino acids in pSC17 clone (residues 25–34 in Fig. 2 *Upper*) were replaced with codons for 63 amino acid residues (Fig. 2 *Upper*). These clones seem to be products of alternatively spliced transcripts of AIGF gene because both pSC17 and pSC15 clones were identical except for the short substitution. One peptide sequence, peptide 7, is encoded by pSC15 but not by pSC17 (Fig. 2 *Upper*). One potential N-glycosylation site is present in both pSC17 and pSC15 (Fig. 2 *Upper*). The N terminus of AIGF was found to be blocked, and one possible candidate for the N terminus is Gln-23, which may result in the blockage of the N terminus due to pyroglutamination and fulfills the

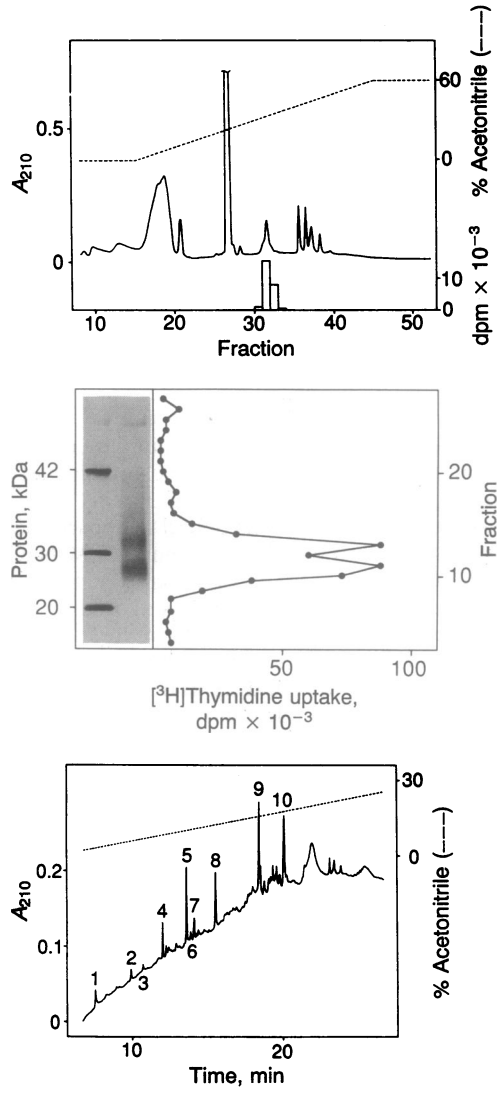

FIG. 1. Purification steps of AIGF. (*Top*) Elution profile of AIGF on reverse-phase HPLC. Eluted fractions obtained from the heparin-Sepharose column were separated by a C_4 reverse-phase column. Each fraction was assayed for [^{3}H]thymidine incorporation in SC-3 cells. (*Middle*) SDS/PAGE profile of AIGF obtained by reverse-phase HPLC. Active fractions obtained by reverse-phase HPLC were analyzed by SDS/PAGE under nonreducing conditions and visualized by the silver-staining method. Eluates from gel slices were assayed for [^{3}H]thymidine incorporation in SC-3 cells. (*Bottom*) Peptide mapping after lysylendopeptidase digestion of AIGF. Peptides obtained by lysylendopeptidase digestion of AIGF were separated by C_{18} reverse-phase column. Distinct peaks (nos. 1–10) were analyzed by the protein sequencer.

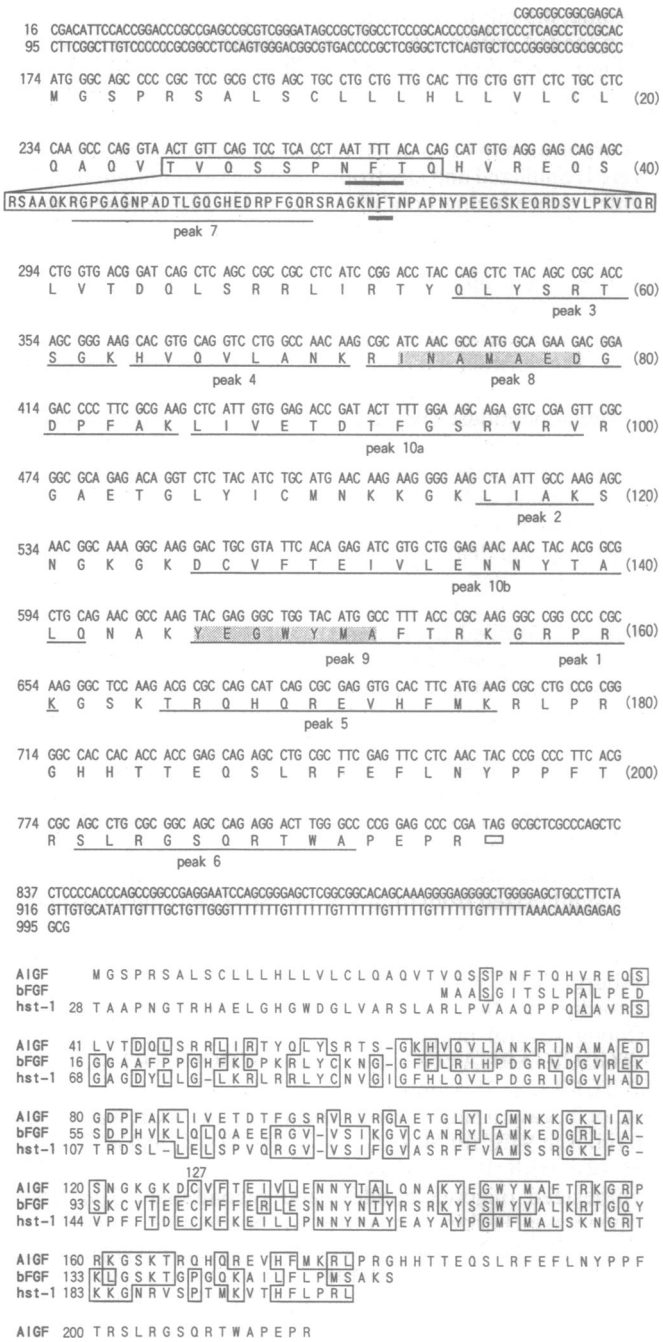

FIG. 2. Structure of AIGF. (*Upper*) Nucleotide sequence and deduced amino acid sequence of AIGF. AIGF cDNA (clone pSC17) encodes a protein of 215 amino acids. Peptide sequences obtained by lysylendopeptidase digestion (nos. 1–10 in Fig. 1 *Bottom*) are underlined. Two peptides were included in peak 10 in Fig. 1 *Bottom*. Two primer sites used in PCR are shaded. Substituted amino acid residues between two independent clones, pSC17 and pSC15, are boxed. Potential N-glycosylation sites are also underlined. (*Lower*) Comparison of amino acid sequences of AIGF with basic FGF (bFGF) (16) and *hst-1* oncogene products (17). Homologous sequences are boxed. Gaps, shown by dashes, are introduced to maximize homology.

rules of von Heijne (18). Molecular weights of the mature proteins would be 22,390 and 28,098, deduced from the sequences of pSC17 and pSC15, respectively. The differences between the two major proteins of 32 kDa and 28 kDa on SDS/PAGE and the calculated molecular weights are probably due to glycosylation at possible glycosylation sites of the alternatively spliced transcripts.

Comparison of Amino Acid Sequences of AIGF to the FGF Family. A search for homology between AIGF and known proteins has revealed that AIGF has significant homology to the FGF family (Fig. 2 *Lower*). In total, AIGF shares 30–40% homology with basic FGF, keratinocyte growth factor (KGF), FGF-5, FGF-6, and *int-2*- and *hst-1*-encoded proteins (19–24). However, there is no particular sequence extensively homologous to any part of other FGFs. Even the receptor binding domain of basic FGF (25)—i.e., residues 114–123 in mouse FGF—shares only 44.4% homology. Although two cysteine residues (Cys-33 and Cys-100 in mouse basic FGF) are highly conserved in the FGF family, only one of the cysteine residues (Cys-127 in AIGF, corresponding to Cys-100 in mouse basic FGF) is conserved in AIGF (Fig. 2 *Lower*).

Induction of AIGF mRNA by Testosterone. The androgenic induction of AIGF was confirmed in the Northern blots. The mRNA of AIGF was not detectable in Northern blots in the absence of testosterone (Fig. 3). The mRNA of AIGF became detectable at 6 hr, and its level was markedly increasing up to 24 hr after the stimulation of SC-3 cells by 10 nM testosterone (Fig. 3).

Expression of AIGF cDNA in Mammalian Cells. Expression of AIGF cDNA in mammalian cells also demonstrates remarkable growth-stimulatory effects of AIGF on SC-3 cells in the absence of androgen (Fig. 4 *Upper*). Moreover, AIGF produced morphological changes in SC-3 cells that were indistinguishable from those stimulated by testosterone. SC-3 cells stimulated by AIGF showed a transformed phenotype in contrast to a normal epithelial phenotype of SC-3 cells being treated with the conditioned medium of cultured mock-transfected COS-7 cells (Fig. 4 *Lower*).

DISCUSSION

Although a number of investigators have suggested by *in vitro* studies that sex hormones may exert some of their growth-promoting effects through modulation of autocrine growth factors (reviewed in ref. 5), none of the hormone-induced autocrine growth factors has been purified and sequenced yet. AIGF is the first, as far as we know, hormone-induced autocrine growth factor identified. AIGF was purified from the conditioned medium of cultured androgen-stimulated SC-3 cells. AIGF mRNA was markedly induced by androgen. Expression of AIGF cDNA in mammalian cells demonstrated remarkable stimulatory effects on the growth of SC-3 cells with transformed morphological changes even in the absence of androgen. We have shown (11) that anti-basic FGF polyclonal antibodies inhibited growth of SC-3 cells stimulated by androgen as well as by partially purified AIGF. Cross-reactivity between the FGF antibodies and AIGF was confirmed by Western blotting (unpublished data), and AIGF was the sole FGF-like growth factor secreted by SC-3 cells (9). These results clearly demonstrate that AIGF, secreted from SC-3 cells in response to androgenic stimuli, mediates proliferative effects of androgen on SC-3 cells.

AIGF is a new member of the FGF family. *int-2* gene and *hst-1* gene, the products of which belong to the FGF family, were independently or coordinately expressed in some mouse mammary tumors induced by integration of mouse mammary tumor virus (MMTV) (26). Since DNA of MMTV was found in genomic DNA of an original SC115 tumor (27), it is speculated that stable insertion of MMTV would activate the transcription of the AIGF gene.

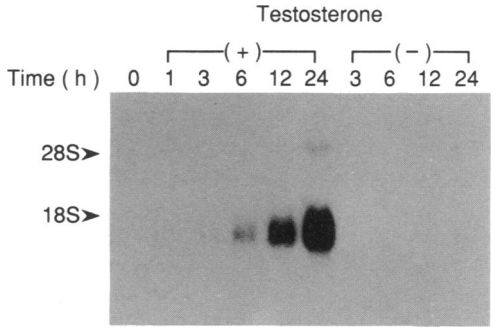

FIG. 3. Induction of AIGF mRNA by testosterone. Testosterone-induced expression of AIGF mRNA is shown by Northern blot analysis. Each lane contained 2 μg of poly(A)$^+$ RNA of SC-3 cells incubated in the presence (lanes +) or absence (lanes −) of 10 nM testosterone for the indicated time in hours. Blots were hybridized with an AIGF cDNA probe derived from clone pSC17. Migration positions of ribosomal RNAs are shown on the left.

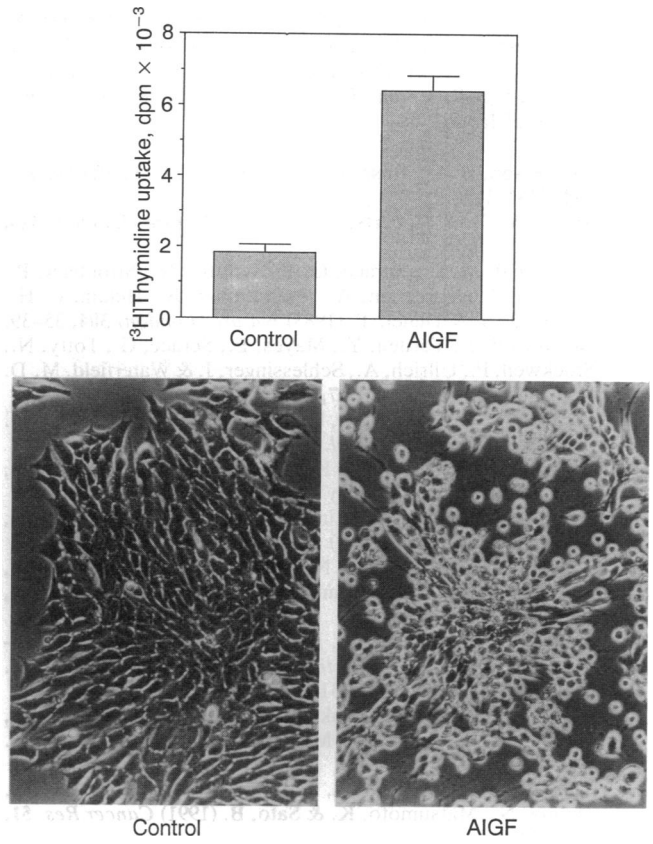

FIG. 4. Expression of AIGF cDNA in mammalian cells. (*Upper*) Stimulatory effects of culture medium of COS-7 cells transfected with AIGF cDNA on DNA synthesis of SC-3 cells. Conditioned medium of COS-7 cells transfected with vector plasmid containing AIGF cDNA (pSC17) or transfected with vector plasmid alone (control) was assayed by [^{3}H]thymidine incorporation in SC-3 cells. (*Lower*) Morphological alternations of SC-3 cells induced by AIGF. SC-3 cells stimulated by AIGF (*Right*) or nonstimulated (*Left*) were photographed through contrast optics. (×100.)

The progression of hormone-responsive cancers to hormone-unresponsive autonomous growth is often observed in human breast and prostate cancers. In an estrogen-responsive human cancer cell line, MCF-7, it has been shown that the cancer cells transfected by v-Ha-*ras* oncogene showed autonomous growth and no longer responded to exogenous estrogen (28). We have reported that other cell lines derived from SC115, which became unresponsive to androgen, secreted one or more growth factors different from AIGF (8). Indeed, AIGF mRNA was not detected in such cancer cells (unpublished data). Another growth mechanism might be involved in malignant progression.

AIGF possesses a putative signal sequence, while acidic and basic FGFs do not. All proteins encoded by FGF-related oncogenes possess N-terminal signal peptide sequences. A cDNA encoding basic FGF bearing an immunoglobulin signal peptide sequence at the N terminus can transform NIH 3T3 cells (29). Thus, the presence of a signal peptide sequence appears to be an important factor in determining the transforming potential of members of the FGF family.

In conclusion, we have demonstrated here, through identification of AIGF essential for androgen-dependent growth of SC-3 cells, that the growth factor mediates hormonal action on the growth of cancer cells. Further *in vitro* studies on AIGF should clarify the molecular mechanism of hormonal action on cell proliferation and may provide the possibility of preventing promotion of hormone-responsive cancers.

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