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ANDROGEN-RESPONSIVE EXPRESSION AND MITOGENIC ACTIVITY OF SCHWANNOMA-DERIVED GROWTH FACTOR ON AN ANDROGEN-DEPENDENT SHIONOGI MOUSE MAMMARY CARCINOMA CELL LINE

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SUMMARY: We report here, using the androgen-dependent SC2G cell line derived from Shionogi mouse mammary carcinoma SC115, that cDNA encoding mouse homologue of the rat schwannoma-derived growth factor (SDGF) was isolated and its protein product was identified to be an autocrine growth factor which is expressed in response to androgen in this hormone-dependent cell line. The androgen-independent SC1G cells derived from SC115 were shown to express SDGF even in the absence of androgen, while Northern analysis probed with mouse mammary tumor virus (MMTV) DNA showed that the androgen inducible transcriptional machinery remains functional in this hormone independent cell line.

SC115 is an androgen-dependent mouse mammary tumor which offers an excellent model system, especially for studying the progression of sex hormone-dependent to -independent tumor [1-4]. In order to analyze the molecular mechanism of SC115 growth *in vitro*, we have established from SC115 solid tumor, two types of cell lines, SC1G and SC2G cells, which proliferate in an androgen-independent and dependent manner, respectively. We and others have detected mitogenic activities in the culture supernatant of SC115-derived cell lines cultivated in the presence of testosterone [5,6,7]. In our study, the serum-free growth conditioned medium of SC2G cells when cultured in the presence of 10 nM testosterone was analyzed for the mitogenic activity on SC2G cells in the absence of testosterone, and the possible growth factor produced in the conditioned medium was revealed to be a heparin-binding protein with a relative molecular mass of about 30000 [8]. In trying to identify this growth factor by screening of already known growth factors, we found that its characteristics resembled those of a recently identified rat SDGF which was purified from schwannoma cell line derived from the sheath of the sciatic nerve [9]. Polymerase chain reaction (PCR) using rat SDGF specific primers showed that the SDGF gene is expressed in SC2G cells on cultivation with

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ABBREVIATIONS: MMTV, mouse mammary tumor virus; PCR, polymerase chain reaction; SDGF, schwannoma-derived growth factor; EGF, epidermal growth factor; TGF, transforming growth factor; HB-EGF, heparin-binding epidermal growth factor; LTR, long terminal repeat

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testosterone. This paper describes the structure, androgen-dependency of expression and mitogenic activity on SC2G cells of mouse SDGF and discusses the contribution of SDGF to hormone-dependent tumor development.

MATERIALS AND METHODS

Cell Culture. Details of the isolation and characterization of SC1G and SC2G cells will be described elsewhere [7]. These cells were maintained in GIT medium (Nippon Seiyaku) containing 10 nM testosterone. GIT medium consists of DT medium (1:1 mixture of Iscove's medium and F12 medium) supplemented with insulin at 2 mg/l, transferrin at 2 mg/l, ethanolamine at 0.122 mg/l and GFS at 3 g/l. GFS is a 55 to 70% ammonium sulfate fraction of bovine serum and consists of proteins with molecular weights of 60 to 80 kD, mostly albumin.

cDNA Cloning and Sequencing. Poly(A) RNA was isolated from SC2G cells cultured in the presence of 10 nM testosterone by the standard procedure [10]. Single-stranded cDNA was synthesized from 1 μg of poly(A) RNA and 2% of the reaction mixture was used as a template in PCR. The 29-nucleotide primer, 5'-GGAAGCTTCTGTCGTGTTGCCGCAGAGAC-3', which corresponds to -45 to -26 relative to the initiation codon of rat SDGF cDNA attached to the HindIII cutting site, and the 26 nucleotide primer, 5'-CCTGCAG TGTGGCTTGGCAGTGACTC-3', complementary to 757 to 776 of rat SDGF attached to the PstI site were synthesized and utilized for PCR. The 850 bp fragment synthesized by 30 cycles of PCR under standard conditions (1 minute at 94℃, 1 minute at 50℃ and 1 minute at 72℃ in each cycle) was cloned into the HindIII-PstI site of plasmid CDM8 [11] and the inserts of four clones were sequenced by the dideoxynucleotide chain termination method [12]. Each of the four clones had one to three mismatched sequence compared to the sequences of the other three clones possibly because of the misincorporation of the nucleotides by Taq polymerase. The appropriate restriction enzyme-digested fragments with no misincorporation were obtained from some clones and ligated to generate the plasmid CDM-SDGF, which is used for further analysis.

Expression and Assay of Growth Factor. In order to construct plasmid CDM-SDGFsol, the HindIII-EcoO104I fragment of the SDGF precursor cDNA was annealed with the synthetic oligonucleotide adaptors 5'-GACTGACTCGAGCTGCA-3' and 5'-GCTCGAGTCA-3' to generate the stop codon at the position of Leu-189, followed by cloning of the fragment into the vector CDM8. The DEAE-dextran transfection method using COS-7 cells was employed as previously described [13]. The transfected cells were cultivated in Dulbecco's modified Eagle's medium containing 10% FCS for three days to allow expression. For assays of the mitogenic activity in the supernatant, SC2G cells (2×10^4 cells / well) were seeded in 6-well cluster dishes and cultivated for 18 hours in GIT medium with 10 nM testosterone. After washing the cells with phosphate-buffered saline, the diluted COS-7 supernatant for testing was added, and the cells were cultivated for a week. Cells from duplicate plates were counted.

Northern Blot Analysis. SC1G and SC2G cells were maintained in GIT medium in the presence of 10 nM testosterone. In the case of androgen deprivation, cells were cultured in GIT medium without testosterone for one week, and used for analysis. Whole cell RNA was prepared by the guanidium thiocyanate/CsCl method [10], and 15 μg of total RNA was loaded in each lane. The blotting analysis was performed essentially as previously described [14]. The probes used were SDGF cDNA (a) and long terminal repeat (LTR) of MMTV provirus (b). Staining of RNA on the membrane was carried out as previously described [15].

RESULTS

Production of SDGF in Androgen-Dependent SC2G Cells. PCR using single-stranded cDNA prepared from SC2G cells with the primers corresponding to the sequence of the 5' and 3' untranslated regions of rat SDGF cDNA gave rise to a specific 850 base pair (bp) band in agarose gel electrophoresis (data not shown). The DNA fragment extracted from this band was cloned into the expression plasmid CDM8. The nucleotide

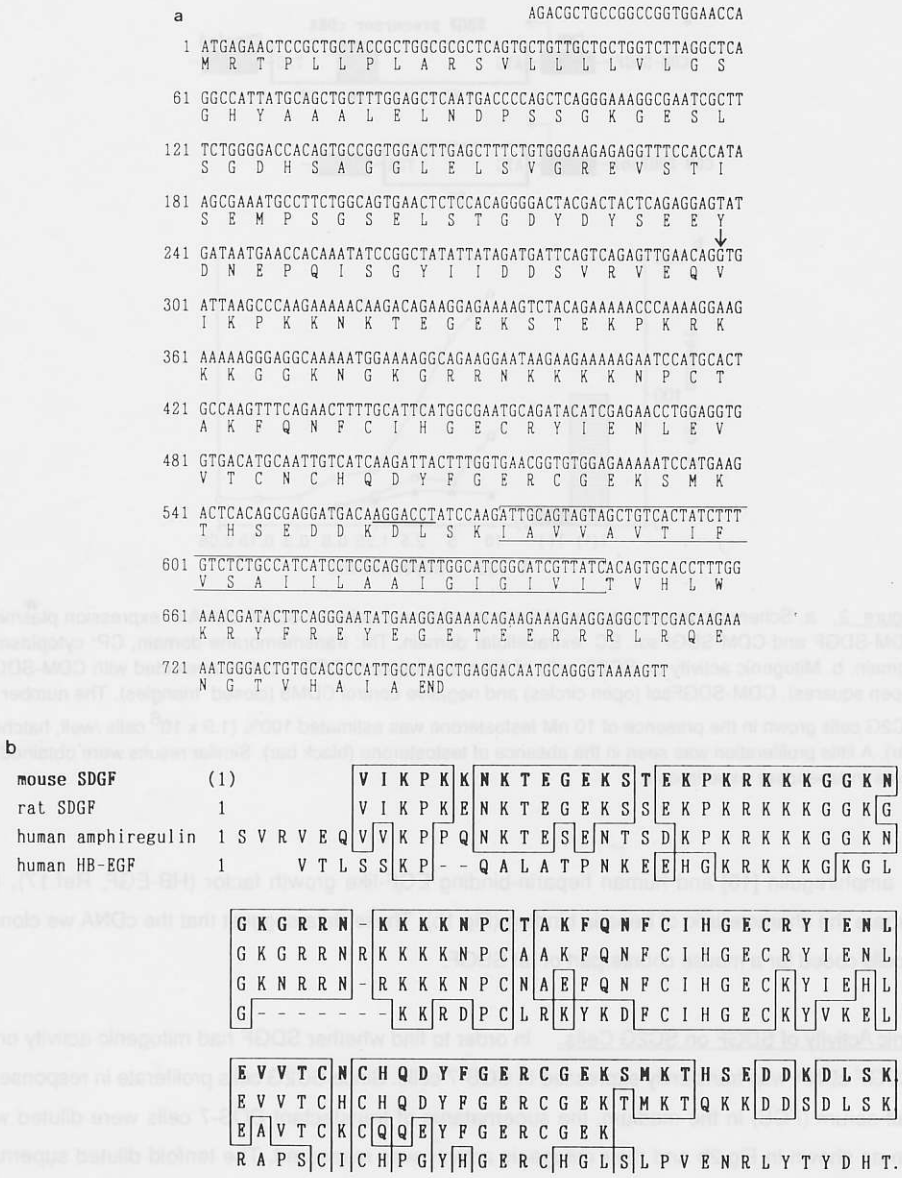

Figure 1. Structure of mouse SDGF. a. Nucleotide sequence and deduced amino acid sequence of mouse SDGF. The sequence of the primers used in PCR is not included. The valine residue from which the mature form of rat SDGF starts is conserved in mouse SDGF (arrow). The putative transmembrane domain which consists of the hydrophobic amino acids is boxed. The cutting site for restriction enzyme Eco0109I which was utilized in construction of the soluble form of SDGF (Fig.2a) is underlined. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number D01182. b. Comparison of the predicted amino acid sequence of mouse SDGF with the mature forms of rat SDGF, human amphiregulin, human HB-EGF. The number 1 at the left of the first column shows the first amino acid of each mature polypeptide. The amino acids which are identical between mouse SDGF and each of the others is boxed.

sequence of the inserted cDNA had a 744 bp open reading frame (Fig.1a) with 84% homology to rat SDGF cDNA. The deduced amino acid sequence also had the strongest homology with rat SDGF among all the members of the epidermal growth factor (EGF) family to which SDGF belongs, and higher homology to

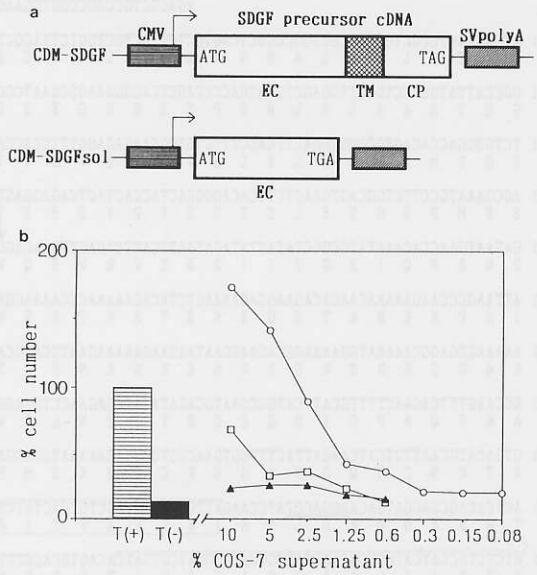

Figure 2. a. Schematic representation of the transcriptional units for SDGF cDNA in expression plasmids CDM-SDGF and CDM-SDGFsol. EC: extracellular domain, TM: transmembrane domain, CP: cytoplasmic domain. b. Mitogenic activity on SC2G cells of the supernatant of COS-7 cells transfected with CDM-SDGF (open squares), CDM-SDGFsol (open circles) and negative control CDM8 (closed triangles). The number of SC2G cells grown in the presence of 10 nM testosterone was estimated 100% (1.9×10^6 cells /well, hatched bar). A little proliferation was seen in the absence of testosterone (black bar). Similar results were obtained in three independent experiments.

human amphiregulin [16] and human heparin-binding EGF-like growth factor (HB-EGF, Ref.17), both of which share the characteristic of heparin binding (Fig.1b). These data suggest that the cDNA we cloned from SC2G cells coded for a mouse counterpart of rat SDGF.

Mitogenic Activity of SDGF on SC2G Cells. In order to find whether SDGF had mitogenic activity on SC2G cells, SDGF cDNA was transiently expressed in COS-7 cells. Since SC2G cells proliferate in response to 10% fetal calf serum (FCS) in the medium, the supernatants of transfectant COS-7 cells were diluted with GIT medium as shown in Fig.2b and their mitogenic activity was measured. The tenfold diluted supernatant of COS-7 cells transfected with CDM-SDGF, the expression vector for intact SDGF cDNA (Fig.2a), showed 68% of the growth-promoting activity on SC2G cells compared to that of 10 nM testosterone. As shown in Fig.1a, the SDGF molecule has a putative trans-membrane domain consisting of hydrophobic amino acids in its carboxyl terminal half, and the mature SDGF might be given rise to by proteolytic cleavage just outside the transmembrane portion and then be secreted into the culture medium. In fact, mature polypeptides of EGF and TGF α are known to be generated and secreted from such precursor molecules [18-21]. Since it is likely that the lower level of growth-promoting activity in COS-7 transfectants was due to the incomplete ability of the processing mechanism to generate a mature form of SDGF protein, we artificially constructed the cDNA encoding secreted form of SDGF by mutating the codon CTA (Leu-189, the third amino acid outside the transmembrane domain) to the termination codon and removing the transmembrane and cytoplasmic domain. The expression vector for this cDNA, CDM-SDGFsol (Fig.2a), was transfected into COS-7 cells, and

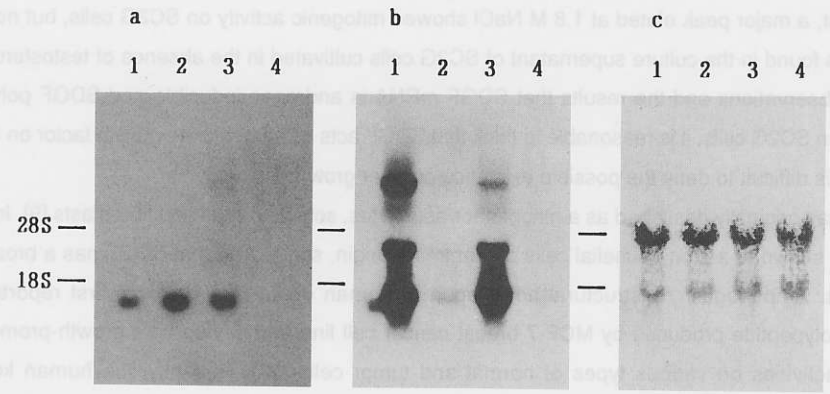

Figure 3. Northern blot analysis for the expression of SDGF mRNA (a) and MMTV-related RNA (b). The data in a, b and c were obtained using the same nitrocellulose filter. Lane 1, 2, total RNA isolated from SC1G cells cultured in the presence or absence of 10 nM testosterone, respectively. Lane 3, 4, total RNA from SC2G cells cultured in the presence or absence of 10 nM testosterone, respectively. c. Methylene blue staining of the nitrocellulose membrane used in a and b.

mitogenic activity of more than 170% of that of 10 nM testosterone was observed in the tenfold diluted supernatant (Fig.2b). Therefore, the presence of the secreted form of SDGF seems adequate for full proliferation of SC2G cells.

Correlation of Androgen Dependency between SDGF mRNA Expression and Proliferation of SC1G and SC2G Cells. Northern blot analysis of total RNA prepared from SC1G and SC2G cells was carried out to examine the effect of androgen on the expression of SDGF mRNA in androgen-independent and -dependent cells, respectively (Fig.3a). In SC2G cells, about 1.5 kilobases band was detected in the presence of 10 nM testosterone while no detectable bands were observed in the absence of testosterone. On the other hand, in SC1G cells, SDGF mRNA was expressed constitutively even in the absence of testosterone as well as in its presence. Therefore, a notable correlation was observed between the androgen dependency of cell proliferation and that of SDGF gene expression in these cell lines. Many cases have been reported in which activation of endogenous *int* oncogenes by the insertion of MMTV into the genome is a causative event in mammary carcinoma in the mouse [see Ref.22 for review]. Some other investigators have suggested the involvement of MMTV in the acquisition of hormone unresponsibility in SC115 [23]. Northern blot analysis using a long terminal repeat of MMTV as a probe showed androgen-inducible expression of endogenous MMTV-related gene not only in SC2G but also in SC1G cells (Fig.3b). Thus, an androgen-inducible transcriptional machinery seems to still functional normally in SC1G cells and, unlike *int* oncogenes, the SDGF gene may not be expressed under the control of MMTV-LTR which might have integrated in the neighbor of SDGF gene in the chromosome.

DISCUSSION

We regard the cDNA that we cloned from SC2G cells to be a mouse counterpart of rat SDGF based on the structural similarity. In our previous study of heparin-Sepharose column chromatography of the SC2G cell

supernatant, a major peak eluted at 1.8 M NaCl showed mitogenic activity on SC2G cells, but no mitogenic activity was found in the culture supernatant of SC2G cells cultivated in the absence of testosterone. Based on these observations and the results that SDGF mRNA is androgen-inducible and SDGF polypeptide is mitogenic on SC2G cells, it is reasonable to think that SDGF acts as an autocrine growth factor on SC2G cells although it is difficult to deny the possible existence of other growth factors.

SDGF was originally described as a mitogen for astrocytes, schwann cells and fibroblasts [9]. In this work, SDGF was shown to act on epithelial cells of mammary origin, suggesting that SDGF has a broad range of target cells. Amphiregulin, a structural homologue of human origin (Fig.1b), was first reported to be a secreted polypeptide produced by MCF-7 breast cancer cell line and having both growth-promoting and -inhibiting activities on various types of normal and tumor cells [24]. Recently, the human keratinocyte autocrine growth factor has been identified to be amphiregulin [25], and a high frequency of amphiregulin mRNA expression was observed in primary or metastatic human colorectal cancers [26]. Therefore, the participation of SDGF or amphiregulin as well as EGF and TGF α in the proliferation of a variety of tumor cells in an autocrine or paracrine manner should be recognized as a crucial event in tumor development.

Highly hydrophobic region found in the amino acid sequence of murine SDGF suggests that the precursor molecules are anchored in the cytoplasmic membrane like EGF and TGF α precursors. In the case of TGF α, a multiple processing step is included in the generation of the mature soluble factor [27]. On the other hand, the membrane-bound form of proTGF α can also activate EGF receptor and transduce the growth signal into the target cells [28,29]. In our experiment, incomplete mitogenic activity of the COS cell supernatant transfected with CDM-SDGF compared to that with CDM-SDGFsol (Fig.2) indicates that a cell-type specific processing mechanism is necessary to generate mature SDGF polypeptide. Moreover, it can be speculated that, in SC2G cells, membrane-anchored SDGF precursor molecules as well as a processed form of SDGF can bind to their receptors on adjacent cells and promote their proliferation.

The androgen-independent phenotype of SC1G cells can be regarded to reflect the more malignant stage in tumor development. Therefore, it is tempting to assume that hormonally dysregulated expression of SDGF gene might be a critical event in the transition from the androgen-dependent to the -independent stage in SC115 although it is not known yet whether SC1G cells still proliferate in response to SDGF in an autocrine manner. Since conserved androgen-dependent expression of MMTV mRNA in SC1G cells indicates that the hormonal dysregulation might be a specific event for the SDGF gene, we think further study on the difference of the molecular mechanism of SDGF gene expression between SC1G and SC2G cells could lead us to understanding of the acquisition of autonomous proliferating phenotype following hormone depletion. Since the activation of *int* oncogenes by MMTV proviral insertion is found only in the case of the mouse, the MMTV-unrelated expression of SDGF gene in SC115 cells might mean that hormonal regulation and dysregulation of SDGF as an autocrine growth factor could also occur in human cancer, possibly in prostatic carcinoma.

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