Identification of Histone H2AX as a Growth Factor Secreted by an Androgen-independent Subline of Mouse Mammary Carcinoma Cells*

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Shionogi carcinoma 115 (SC 115) cells and Chiba subline 2 (CS 2) cells are clones of an androgen-responsive mouse tumor cell line and its autonomous subline, respectively. We have shown previously that CS 2 cells produce a heparin-binding growth factor that stimulates the growth of SC 115 cells as well as the growth of themselves. In this study, a growth factor was purified from serum-free conditioned media of CS 2 cells cultured without testosterone. A heparin-binding fraction showed growth-promoting activity on SC 115 cells and BALB/3T3 cells. The amino acid sequence analysis revealed that the components were identical to histones H2A.1 and H2A.X. Since histone H2A purified from bovine thymus had almost no growth-promoting activity on SC 115 cells, histone H2A.X was assumed to be a growth factor. cDNA of histone H2A.X was cloned from a library of CS 2 cells, and its sequence was confirmed. The expressed product of histone H2A.X cDNA in Escherichia coli showed remarkable stimulatory effects on growth of SC 115 cells cultured in the absence of testosterone. These results indicate that histone H2A.X is secreted from CS 2 cells cultured without testosterone and plays a role as a growth factor.

It has been reported that the growth of some hormone-responsive tumors is controlled by hormone-induced growth factors in the autocrine manner (1). For example, on MCF-7 cells, an estrogen-responsive human breast cancer cell line, transforming α-like and insulin-like growth factors are thought to mediate the estrogen-responsive growth of cancer cells (2, 3). LNCaP, an androgen-responsive human prostate cancer cell line, is thought to secrete a fibroblast growth factor (FGF)1-like peptide in response to androgen stimuli (4). It is generally accepted that hormone-responsive tumors gradually progress to hormone-unresponsive ones; however, the mechanism regulating the growth of the latter seems to be obscure.

Shionogi carcinoma 115 (SC 115) is an androgen-responsive mouse mammary tumor (5). Recently, an androgen-induced growth factor (AIGF) secreted from SC 115 cells in the presence of testosterone was purified, and its cDNA was cloned (6). The structural analysis revealed that AIGF was a novel FGF-like growth factor, which was established as the 8th one in the FGF family. An androgen-independent subline, Chiba subline 2 (CS 2), was derived from SC 115 in our laboratory (7–9), and a clone from CS 2 cells has subsequently been maintained. We have shown previously that CS 2 cells also produce a heparin-binding growth factor that stimulates the growth of SC 115 cells and CS 2 cells without testosterone (10). This factor was thought to be different from AIGF, because AIGF mRNA was not expressed in CS 2 cells (11). To shed light on growth-regulatory mechanisms of hormone-unresponsive tumor cells, the present study was undertaken on purification of the heparin-binding growth factor produced by CS 2 cells and on analysis of amino acid sequence.

**MATERIALS AND METHODS**

Cells—SC 115 cells and CS 2 cells are clones of an androgen-dependent mouse tumor cell line and its autonomous subline, respectively. The methods for cloning and culture of these cells were described previously (12). BALB/3T3 cells were donated from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and were maintained in minimum essential medium containing 10% fetal bovine serum.

Assay of Growth-promoting Activity—The assay was performed by [3H]thymidine incorporation in SC 115 cells (1 × 10⁵ cells/well) or in BALB/3T3 cells (2 × 10⁴ cells/well) as described previously (10). One unit of activity is defined as half of the maximal stimulation of thymidine incorporation induced by 2 ng/ml basic FGF (R&D Systems, Minneapolis, MN) in the SC 115 cells.

Preparation of Serum-free Conditioned Media and Purification of Growth Factor—CS 2 cells (5 × 10⁶ cells/100-mm dish) were plated and cultured as described previously (10). Serum-free culture media from SC 2 cells were filtered through a nylon membrane (0.22 μm pore size; Costar Corp., Cambridge, MA) as soon as they were obtained. The filtrate was concentrated (up to 20-fold) by ultrafiltration with M₇₀,000 cut-off membrane discs (PM 10; Amicon Inc., Beverly, MA) and dialyzed against 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS. The concentrated and dialyzed culture media were applied to a 10-ml heparin-Ultrogel column (IBF Biotechnics, Villeneuve-la-Garenne, France) equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS. The column was washed with 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS extensively until the absorbance returned to base line, and then adsorbed proteins were eluted with a 0.1–3.0 M NaCl gradient in 10 mM Tris-HCl buffer (pH 7.5) containing 0.1% CHAPS. The fractions around 1.8 M NaCl were pooled, dialyzed against 0.1 mM acetic acid, and concentrated (up to 20-fold) by ultrafiltration with M₁₀,₀₀₀ cut-off membrane discs (PM 10; Amicon Inc., Beverly, MA) as soon as they were obtained. The filtrate was concentrated (up to 20-fold) by ultrafiltration with M₇₀,₀₀₀ cut-off membrane discs (PM 10; Amicon Inc., Beverly, MA) as soon as they were obtained. The filtrate was concentrated (up to 20-fold) by ultrafiltration with M₇₀,₀₀₀ cut-off membrane discs (PM 10; Amicon Inc., Beverly, MA) as soon as they were obtained. The filtrate was concentrated (up to 20-fold) by ultrafiltration with M₇₀,₀₀₀ cut-off membrane discs (PM 10; Amicon Inc., Beverly, MA) as soon as they were obtained.

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‡ The abbreviations used are: FGF, fibroblast growth factor; SC 115, Shionogi carcinoma 115; CS 2, Chiba subline 2; AIGF, androgen-induced growth factor; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate; RP-HPLC, reverse-phase high performance liquid chromatography; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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RESULTS

Purification of Growth Factor from CS 2 Cells—Ten liters of serum-free conditioned media obtained from CS 2 cells were used for purification of growth factor. The substances with growth-promoting activity were bound to heparin-Ultrogel and were eluted with the buffer containing 1.0 mM NaCl. After the heparin-Ultrogel chromatography, bioactive fractions were subjected to RP-HPLC (Fig. 1A). The bioactive fractions obtained from the RP-HPLC showed a single major band with relative molecular masses of 17 kDa on SDS-PAGE under reducing conditions (Fig. 1B). Through these purification steps, 12 μg of the purified protein was obtained from 10 liters of serum-free conditioned media, and the specific activity of fraction 37 in Fig. 1 increased up to 1.0 × 10^9 units/mg (Table I). This fraction also showed potent growth-promoting activity on BALB/3T3 cells as basic FGF (Fig. 2). Sequence analysis was performed directly with an aliquot of this fraction, but no amino-terminal amino acid was detected. Then, this fraction was digested with lysyl endopeptidase, and 13 peptides were isolated with RP-HPLC (Fig. 3). Sequence analysis of each peptide revealed that all sequences of the 13 peptides were specific sequences to both mouse H2A.1 and H2A.X and to mouse H2A.X, respectively. Hybridizations were carried out in 4 × SSC (standard saline citrate), 2 × Denhardt’s solution, 40 mM sodium phosphate buffer (pH 6.5), 0.1% SDS, 100 μg/ml sonicated and denatured salmon testis DNA, and with 1 × 10^6 cpm/ml 32P-end-labeled probes at 37°C for 16 h. The nylon membranes were sequentially washed in 2 × SSC containing 0.1% SDS at room temperature for 1 h, at 40°C for 30 min, at 45°C for 30 min, and at 50°C for 30 min before autoradiography. DNA sequencing was done by the dideoxynucleotide chain termination procedure (18) after subcloning appropriate DNA fragments into M13 mp18 and mp19 (Takara, Kyoto, Japan) (17).

Purification of Mouse H2AX Protein Synthesized in E. coli—Plasmid was constructed (pGEX-H2AX) to express the mouse H2AX protein fused with a 26-kDa glutathione S-transferase (GST) in HB101 E. coli bacteria (Toyobo, Osaka, Japan), using the EcoRI fragment (1.4-kilobase pairs) of the cloned cDNA of mouse H2AX. After digestion, the fragment was subcloned into the EcoRI site of the bacterial expression vector pGEX2T (Pharmacia). HB101 E. coli bacteria were transformed with pGEX-H2AX or with pGEX2T as control. Overnight cultures of E. coli transformed with pGEX-H2A.X or pGEX2T were diluted 1/10 with fresh medium and were incubated for 2 h before addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 10 mM and a further 10-h incubation. The cells were then pelleted and resuspended in phosphate-buffered saline containing 1% Triton X-100. The cells were lysed on ice by mild sonication and then centrifuged at 10,000 × g for 5 min at 4°C. At this stage, SDS-PAGE analysis showed that the expressed fusion protein occurred as an insoluble form in the pellet. The pellet was resuspended in 8 M urea and sonicated gently on ice. This solution was left for 1 h at room temperature and then centrifuged at 10,000 × g for 15 min at room temperature. The supernatant was sequentially dialyzed against 6 and 2 M urea and 50 mM Tris-HCl buffer (pH 8.0). Ten μg of thrombin (Boehringer Mannheim) was added to 200 μl of this solution, and the mixture was allowed to react for 1.5 h at room temperature. Then 2.8 ml of 10 mM Tris-HCl buffer (pH 7.0) was added to the mixture, and this solution was dialyzed twice against 10 mM Tris-HCl buffer (pH 7.0) for 4 h. For further purification, this solution was applied to a heparin-Ultrogel column (gel bed volume, 1 ml) equilibrated with 10 mM Tris-HCl buffer (pH 7). The adsorbed proteins to this column were eluted sequentially with 10 mM Tris-HCl buffer (pH 7.0) containing 0.2 mM NaCl, 0.5 mM NaCl, and 1.5 mM NaCl. After dialysis of each fraction against 10 mM Tris-HCl buffer (pH 7.0), the aliquot was assayed for [3H]thymidine incorporation in SC 115 cells and electrophoresed under reducing conditions on 15% polyacrylamide gel with 0.1% SDS.

with 0.1% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (13). The gel was stained using a Bio-Rad silver nitrate stain kit. The bioactive fraction was lyophilized and digested with 50 ng of lysyl endopeptidase (Wako Pure Chemicals, Osaka, Japan) per 1 μg of protein in 50 mM Hepes buffer (pH 8.0) for 6 h at 37°C. The digested sample was directly loaded onto a 4.6 × 250-mm YMC C18 RP-HPLC column and developed with a linear gradient of 20–60% acetonitrile in 0.08% trifluoroacetic acid. A 1-ml fraction was collected, and the flow rate was 1 ml/min. Three μl of each fraction were assayed for [3H]thymidine incorporation in SC 115 cells. SDS-PAGE analysis of active fractions from RP-HPLC shown in panel A. 100 μl of each fraction was electrophoresed in a 15% polyacrylamide gel that was subsequently silver stained.

FIG. 1. A, RP-HPLC of the growth factor from CS 2 cells. Active fractions eluted from the heparin-Ultrogel column were loaded directly onto a 4.6 × 250-mm YMC C18 RP-HPLC column and developed with a linear gradient of 20–60% acetonitrile in 0.08% trifluoroacetic acid. A 1-ml fraction was collected, and the flow rate was 1 ml/min. Three μl of each fraction were assayed for [3H]thymidine incorporation in SC 115 cells. B, SDS-PAGE analysis of active fractions from RP-HPLC shown in panel A. 100 μl of each fraction was electrophoresed in a 15% polyacrylamide gel that was subsequently silver stained.

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Table I

| Purification steps | Protein | Total activitya | Specific activitya |
|--------------------|---------|----------------|-------------------|
| Conditioned medium | 1.5 × 10^2 | 4.7 × 10^5 | 3.0 × 10^2 |
| Ultrafiltration     | 0.5 × 10^2 | 1.4 × 10^6 | 2.6 × 10^2 |
| Retentate           |         |               |                  |
| Heparin-Ultragel 1.0 M NaCl fraction | 0.7 | 8.8 × 10^4 | 1.3 × 10^5 |
| C_4 RP-HPLC        | 1.2 × 10^2 | 1.2 × 10^4 | 1.0 × 10^6 |

a One unit of activity is defined as half of the maximal stimulation of thymidine incorporation induced by 2 ng/ml basic FGF in the SC 115 cells.

![Graph](image)

**Fig. 2.** Growth-promoting activity of the purified growth factor from CS 2 cells and of basic FGF on BALB/3T3 cells. A sample of fraction 37 in Fig. 1 (○) and basic FGF (●) was added to BALB/3T3 cells, and the growth-promoting activity was measured in terms of [3H]thymidine uptake. Each point was based on three wells. Data are shown as means ± S.E. and lie within the symbols.

Histone H2A (H2A.1, H2A.2) and H2A.X are homologous (96–97%), each histone has a unique carboxy-terminal sequence (20). Consequently the isolated growth factors were thought to be identical to the two histones, H2A.1 and H2A.X. From the recovered amounts and the absorbance of each peptide, the ratio of the amounts of H2A.1 and H2A.X was estimated to be approximately 5 to 1. Histone H2A purified from bovine thymus (Boehringer Mannheim) was the mixture of H2A.1 and H2A.2, the amino acid sequences of which were identical to those of mouse H2A.1 and H2A.2, and did not show any growth-promoting activity on SC 115 cells (Fig. 5). From these results, histone H2A.X was assumed to be the candidate for a growth factor secreted from CS 2 cells.

cDNA Cloning of Mouse H2A.X—Cloning of mouse histone H2A.X cDNA was performed to examine the growth-promoting activity of expressed mouse histone H2A.X protein. A cDNA library was prepared from poly(A)^+ mRNA of CS 2 cells and screened with two antisense oligonucleotide probes, AX-1 and X-1. From 4 × 10^2 independent clones, 26 positive clones that were hybridized with both probes were obtained. Since their restriction enzyme maps were identical, 5 of the clones were characterized. Each of these clones was digested with EcoRI, and the resulting EcoRI fragment that was hybridized with both probes was subjected to sequence analysis. The sequence analysis revealed that all of these clones included mouse histone H2A.X cDNA.

Production of Mouse H2A.X Protein in E. coli and Its Growth-promoting Activity—To analyze the growth-promoting activity of the mouse H2A.X protein, the EcoRI DNA fragment containing mouse H2A.X cDNA was ligated into the EcoRI site of the pGEX2T expression vector. The entire mouse H2A.X gene-coding region and 56-base pair 5′-noncoding region were translated as a fusion protein with a 26-kDa GST in E. coli. The SDS-PAGE analysis showed that the expressed fusion protein occurred as aggregates or inclusion bodies after lysing the cells (data not shown). Since the inclusion bodies were in an insoluble and inactive form, they were solubilized with 8 M urea and then refolded with dialysis. Isolation of the inclusion bodies was beneficial to purification of the expressed fusion protein from other solubilized proteins derived from E. coli. The refolded fusion protein was then digested with thrombin to remove GST, and the expressed mouse H2A.X had an additional 23 amino acids at the amino terminus. Using a heparin-Ultrigel column, the expressed H2A.X was further purified (Fig. 6). The expressed mouse H2A.X protein that was eluted from the heparin-Ultrigel column with 1.5 M NaCl showed a single band with relative molecular masses of approximately 18 kDa on SDS-PAGE, which was larger than that of H2A.X purified from CS 2 cells because of an additional 23 amino acids. The 1.5 M NaCl fraction containing expressed mouse H2A.X protein showed remarkable growth-promoting activity on SC 115 cells compared with the solution obtained from E. coli transformed with a vector plasmid, pGEX2T, alone in the same manner (Fig. 5).

**DISCUSSION**

The growth of SC 115 cells is stimulated by AIGF (6), acidic and basic FGFs (10, 11), and schwannoma-derived growth factor (21). This study showed that, in addition to these growth factors, histone H2A.X was assumed to be the candidate for a growth factor secreted from CS 2 cells because of an additional 23 amino acids. The 1.5 M NaCl fraction containing expressed mouse H2A.X protein showed remarkable growth-promoting activity on SC 115 cells compared with the solution obtained from E. coli transformed with a vector plasmid, pGEX2T, alone in the same manner (Fig. 5).
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Histone H2AX is expressed as a GST fusion protein (1.5 M NaCl fraction, lane 9 in Fig. 6) and histone H2A was expressed from bovine thymus. The same fraction obtained from proteins expressed by the vector plasmid alone was also assayed as control. Four ng of each sample were added to the well of SC 115 cells.

The expression experiment confirmed (Fig. 5), histone H2A.X was assumed to be a growth factor did not show any growth-promoting activity on SC 115 cells (Figs. 1 and 5). Moreover, histone H2A.X stimulated the growth of BALB/3T3 cells. These results imply that H2A.X plays a role of not only organizing chromatin architecture during S-phase but also growth regulator through the cell cycle in these tissues and embryonic cells.

Since H2A.X has no signal sequence for secretion, it is not yet clear how H2A.X is secreted from the cells. However, hydrophobic amino acids are abundant from the middle of the molecule to the carboxyl terminus of H2A.X, and it is considered that this region is an internal signal sequence (33). It is now important to clarify the mechanism by which H2A.X acts on these histones, was identical to high mobility group-1 and was found at human hepatoma-derived cell line, HuH-7 (30). Its primary sequence shares homology with high mobility group-1 protein. It was also reported that brain heparin-binding protein (amphotericin), which enhances neurite outgrowth in cerebral neurons, was identical to high mobility group-1 and was found at relatively high levels in differentiated F9 teratocarcinoma cells. These results imply that H2A.X plays a role of not only organizing chromatin architecture during S-phase but also growth regulator through the cell cycle in these tissues and embryonic cells.

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