Biochemical and Biophysical Research Communications **261,** 113–117 (1999)
Article ID bbrc.1999.0871, available online at http://www.idealibrary.com on IDEAL®

High Mobility Group-like Protein in Bovine Milk Stimulates the Proliferation of Osteoblastic MC3T3-E1 Cells

Jun-ichi Yamamura,*[,1] Yukihiro Takada,* Masaaki Goto,†
Masayoshi Kumegawa,‡ and Seiichiro Aoe*

**Nutritional Science Laboratory, Snow Brand Milk Products Co., Ltd., 1-1-2 Minamidai, Kawagoe, Saitama 350-1165, Japan; †Research Institute of Life Science, Snow Brand Milk Products Co., Ltd., 519 Shimoishibashi, Ishibashi-machi, Shimotsuga-gun, Tochigi, 329-0512, Japan; and ‡Department of Oral Anatomy, Meikai University School of Dentistry, 1-1 Keyakidai, Sakado, Saitama 350-0248, Japan*

Received May 10, 1999

The active component in bovine milk on the proliferation of osteoblastic MC3T3-E1 cells was purified and identified. Growth-promoting activity was measured by [^{3}H]thymidine incorporation on the cell. The molecular weight of the purified protein was 10 kDa. The amino-terminal sequence of this 10-kDa protein was identical to bovine high mobility group protein (HMG) 1. This 10-kDa protein is suggested to be a basic protein and to have an HMG box, a consensus sequence motif among the HMG family. From these results, we named this protein HMG-like protein. HMG is a ubiquitous nonhistone component of chromatin and considered to be implicated in DNA replication. We found this protein in milk, and it showed a growth-promoting activity. We propose the possibility that HMG-like protein existed in milk and plays an important role for neonate in bone formation by activating osteoblasts.

Bovine milk is an abundant source of nutritional proteins. Whey is obtained from milk or defatted milk by adding acid or rennet and removing the formed coagulate. In manufacturing, whey is usually produced as a by-product of cheese or casein production. Whey protein is obtained from liquid whey by ultrafiltration, reverse osmosis, chromatography, and dialysis used to remove lactose and other components (1). One hundred milliliters of milk contains 0.55 g of whey protein. Recently, some of these highly active molecules have been isolated from whey: epidermal growth factor (2), colony-stimulating factor (3), transforming growth factor α (4), transforming growth factor β (5), insulin-like growth factor (6), and fibroblast growth factor (7). It was also found that the milk and colostrum of various species contained several factors that are able to stimulate the growth and migration of different cultured cells (8–10). We recently found that whey protein was effective in increasing the bone strength and content of hydroxyproline, collagen-specific amino acids, in ovariectomized rats (11, 12). Further experiments showed that whey protein stimulated the proliferation and differentiation of murine osteoblastic MC3T3-E1 cells *in vitro* (13). The purpose of this study is to purify and identify the active component of whey protein on the proliferation of osteoblastic cells.

[1] To whom correspondence should be addressed. Fax: +81-492-42-8696. E-mail: KYW06173@nifty.ne.jp.

MATERIALS AND METHODS

Materials. Alpha-modified essential medium (αMEM) was purchased from Flow Laboratories (Mclean, VA), and fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY). Whey protein was prepared from the whey protein concentrate (WPC) by dialysis. The WPC was purchased from New Zealand Dairy Board (Wellington, New Zealand). S-Sepharose Fast Flow, Mono S HR 10/10, and Mono Q HR 5/5 columns were purchased from Pharmacia (Uppsala, Sweden). HPLC column, Tomsorb-100 Phenyl-5u was from Tom & Sic (Tokyo, Japan). Radioactive material, [*methyl*-^{3}H]thymidine (NET-027X, NEN) was from Daiichi Chemicals (Tokyo, Japan). 3-((3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate (Chaps), polyoxyethylene sorbitan monooleate (Tween 80), and other chemicals were purchased from Wako Pure Chemicals (Osaka, Japan).

Cell culture. Osteoblastic MC3T3-E1 cells were kindly provided by Dr. H. Kodama (Tohoku Dental College, Koriyama, Fukushima, Japan). The cells were maintained in plastic dishes containing αMEM supplemented with 10% (v/v) FBS in a CO_2 incubator (5% CO_2, 95% air) at 37°C and subcultured every three days at a dilution of 1:5 by using 0.001% pronase (Actinase E, Kaken Chemical, Tokyo, Japan) plus 0.02% EDTA in Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (PBS), as previously described (13).

Proliferation assays. The cells were suspended (2×10^4 per milliliter) in αMEM supplemented with 10% (v/v) FBS, seeded into each well of 96-well plates (100 μl per well), and cultured for one day. The media were then changed to fresh αMEM. Two microliters of samples were added to the media in triplicate and cultured for 16 h. One

0006-291X/99 $30.00

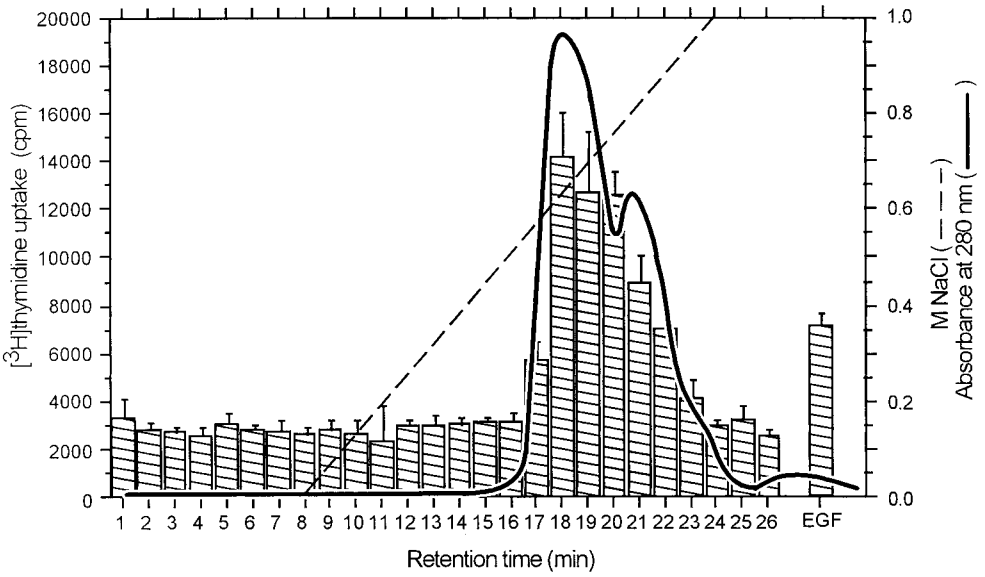

FIG. 1. Cation-exchange column chromatography of basic whey proteins from bovine milk. Whey proteins were treated with acid and heated; then the supernatant was loaded onto an S-Sepharose column. The bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl (dash line) in sodium phosphate buffer. The eluted proteins were monitored by absorbance at 280 nm, and growth-promoting activities were measured. Bars represent means and standard deviations.

microgram per milliliter of recombinant human epidermal growth factor (Seikagaku Kogyo, Tokyo, Japan) was used as a positive control of proliferation. The cells were then labeled for two hours with [^{3}H]thymidine (0.2 MBq/ml media). At the end of the labeling period, the medium was removed and the cells washed with PBS, then treated with 0.01% pronase plus 0.2% EDTA in PBS for 5 min. The cells were collected with a cell harvester (PHD cell harvester, Cambridge Technology Inc., Watertown, MA) and trapped on glass filters (Cambridge Technology Inc.). After the filter was dried, the radioactivity was counted in a liquid scintillation counter.

Purification of HMG-like protein with column chromatography. Fifty grams of whey protein concentrate was dissolved in one liter of deionized water and acidified with hydrochloric acid to pH 4.0. Whey protein solution was then heated at 80°C for 10 min and centrifuged at 5000*g*, 4°C, for 30 min. Ethanol was added to the supernatant to give a final concentration of 30% and centrifuged. The supernatant was concentrated with a rotary evaporator. The condensate was dissolved in 6.67 mM sodium phosphate buffer (pH 7.0). It was then loaded onto an S-Sepharose column (16 × 300 mm), which was equilibrated with the same buffer at a flow rate of 4 ml/min. The bound proteins were washed with the same buffer and eluted with a linear gradient of 0 to 1.0 M NaCl in that buffer. Eluates were collected into 40 tubes. The total protein was measured by absorbance at 280 nm. The growth-promoting activity of osteoblasts was measured by [^{3}H]thymidine incorporation assay, using MC3T3-E1 cells. The aliquot of eluates was diluted 1:10 with water and added into a serum-free medium of osteoblasts (2 μl in 100 μl media). The growth-promoting fractions were pooled, diluted with 10 vol of 6.67 mM sodium phosphate buffer (pH 7.0), and loaded onto a Mono S 10/10 column, equilibrated with the same buffer at a flow rate of 2 ml/min. The bound proteins were washed, eluted by linear gradient of 0 to 1.0 M NaCl, and fractionated into 20 tubes. The growth-promoting activities were measured in all tubes. The active fractions were pooled and diluted with 10 volumes of 6.67 mM sodium phosphate buffer (pH 8.5) and loaded onto a Mono Q 5/5 column, equilibrated with the same buffer at a flow rate of 1.5 ml/min. The bound proteins were washed, eluted by linear gradient of 0 to 1.0 M NaCl, and fractionated into 20 tubes; they were then assayed for growth-promoting activities. The active fractions were pooled and subjected to HPLC purification.

Purification of HMG-like protein with HPLC. Chaps was added to the active fraction at a final concentration of 0.1% (w/v) and trifluoroacetic acid of 0.2% (v/v). The active fraction was then loaded to a phenyl reversed-phase HPLC column (4.6 × 250 mm), which was equilibrated with 0.1% trifluoroacetic acid in water at a flow rate of 0.5 ml/min. The bound proteins were washed and eluted by a linear gradient of 0 to 80% of acetonitrile, then collected into 70 tubes containing Chaps. The fractions were lyophilized and dissolved in 50 μl of water, and the growth-promoting activities were measured.

Polyacrylamide gel electrophoresis. The fraction with biological activity was electrophoresed under reducing conditions on a 16% polyacrylamide gel with 0.1% SDS in Tris–Tricine system (14), using a Peptide–PAGE mini precast gel (TEFCO, Tokyo, Japan). The gel was silver-stained. Molecular weight was estimated with a M_r standard, Mark 12 (TEFCO).

Amino acid sequence analysis. The purified protein solution was subjected to amino acid sequence analysis on a gas-phase protein sequencer (Model 494, Applied Biosystems Perkin–Elmer, U.S.A.).

RESULTS

When an acid- and heat-treated bovine whey protein was loaded on a cation exchanger S-Sepharose column, the bound proteins were eluted with a linear gradient of NaCl. The eluted pattern and osteoblastic cell growth-promoting activities (represented by [^{3}H]thymidine uptake) are shown in Fig. 1. Osteoblast growth-promoting fractions were eluted with about 0.6 M NaCl, corresponding to fraction numbers 18 to 20. These fractions were pooled and diluted with 10 vol of 6.67 mM sodium phosphate buffer (pH 7.0), then loaded onto a Mono S cation exchange column. The

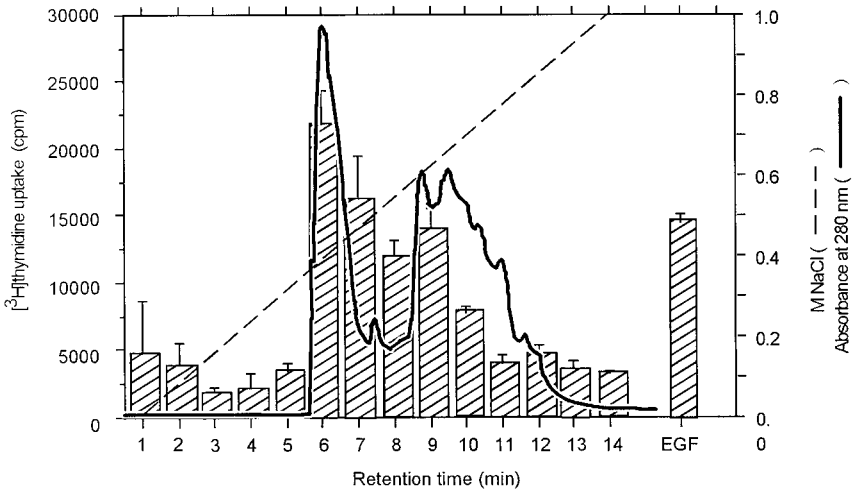

FIG. 2. Cation-exchange column chromatography of the growth-promoting fractions eluted from S-Sepharose column. The active fractions were pooled and loaded onto a Mono S column. The bound proteins were eluted with a linear gradient of 0 to 1.0 M NaCl (dash line) in sodium phosphate buffer. The eluted proteins were monitored by absorbance at 280 nm, and growth-promoting activities were measured. Bars represent means and standard deviations.

eluted pattern and activities are shown in Fig. 2. Osteoblast growth-promoting fractions were eluted with about 0.4 M and 0.6 M NaCl, corresponding to fraction numbers 6 and 9, respectively. The former fraction (number 6) was further purified and identified as a fragment of kininogen (Yamamura *et al.*, in submission). The latter fraction (number 9) was diluted with 10 vol of 6.67 mM sodium phosphate buffer (pH 8.5) containing Tween 80 and loaded onto a Mono Q anion-exchange column. Osteoblast growth-promoting fractions were eluted with about 0.1 M NaCl. The fraction was subjected to HPLC purification. The HPLC was performed with a phenyl reversed-phase column (Fig. 3). The active fraction was eluted with a single peak. This fraction was then electrophoresed and subjected to amino acid sequence analysis. The purification was summarized in Table 1.

Electrophoretic analysis revealed that the purified protein migrated as a single band that corresponded to 10 kDa (Fig. 4). To characterize this 10-kDa active protein, the amino-terminal 33 amino acids were identified. The identified sequence was analyzed by a homology search using SWISS PROT protein sequence data base. The homologous sequence was the amino terminal sequence of bovine high mobility group protein (HMG) 1. These sequences were aligned on Fig. 5. According to the amino-terminal sequence and the molecular weight, we named the purified protein "HMG-like protein."

DISCUSSION

Our previous report demonstrated that there were some active components for the proliferation of osteoblast in the whey protein. It was suggested that the active components were heat-stable and ethanol-stable (up to 30% ethanol) (12). In the present study, we purified the proteins that stimulate the proliferation of osteoblastic MC3T3-E1 cells from bovine milk. The use of an ion exchanger column was effective for the first separation of crude sample because the basic proteins bound to cation exchanger and the other major proteins in whey were removed. In Mono S cation exchange chromatography, the proliferative activity was divided into two peaks. It was reproducible in the same chromatography. In the present study, one protein was further purified with an anion exchanger Mono Q column and a reversed phase HPLC. Concerning purified

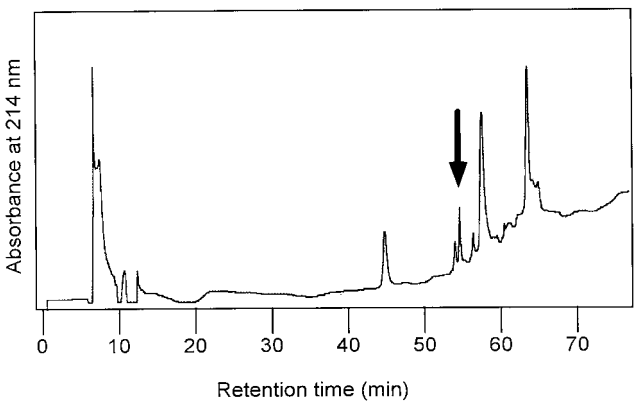

FIG. 3. Reversed-phase column chromatography of the growth-promoting fractions eluted from a Mono Q column. The active fractions were pooled and loaded onto a Tomsorb-100 Phenyl-5u column on HPLC. The eluted proteins were monitored by absorbance at 214 nm, and growth-promoting activities were measured. The arrow indicates the highest activity.

TABLE 1

Summary of the Purification Procedure for Milk HMG-like Protein

	WPC	S-Sepharose	Mono S	Mono Q	Phenyl-HPLC
Volume (ml)	5000	48	12	3	0.1
Total activity (EqEGF[a])	1.3×10^6	4.3×10^5	6.0×10^4	3.2×10^3	3.4×10^1
Recovery (%)	100	32	4.4	0.24	0.0025

[a] Equal activity to 1 ng EGF on osteoblast proliferation assay.

protein, the amino acid sequencing study revealed that this protein was identical to the high mobility group protein (HMG) 1. HMG1 is well known as a nuclear non histone chromosomal protein considered implicated in DNA replication and cellular differentiation (15).

There are both acidic and basic domains within the HMG1 molecule. One striking feature of HMG1 is the presence near the carboxyl-terminus of a 30-residue polyacidic domain that is composed entirely of glutamic and aspartic acid residues. In contrast, the amino-terminal of HMG1 contains basic domain. Therefore HMG1 is an extremely polar molecule, although its net charge is not far from neutrality. The amino-terminal amino acid sequence of our purified protein is identical to that of HMG1. This protein is considered a basic protein because it is bound to the cation exchanger at neutral pH in the purification step.

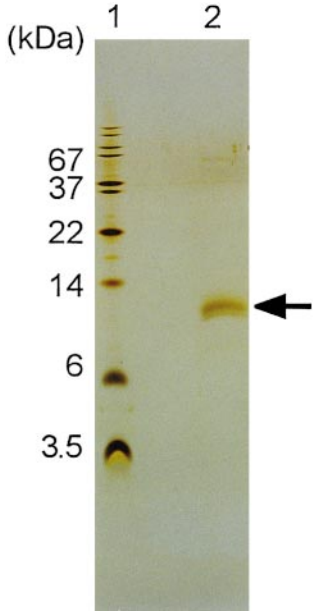

FIG. 4. Electrophoretic analysis of the HMG-like protein. The purified fraction with biological activity was electrophoresed (lane 2) under reducing conditions on a 16% polyacrylamide gel with 0.1% SDS in Tris–Tricine system. The gel was silver-stained. Molecular weight was estimated with a M_r standard (lane 1). The arrow indicates the migration of HMG-like protein.

According to the basic domain and the sequence study, this protein is considered to be an HMG-like protein in bovine milk.

Wier *et al.* (17) showed that HMG1 consists of two HMG boxes, a highly homologous, folded, basic DNA-binding domain, each of approximately 80 amino acid residues. According to the molecular weight, the HMG-like protein is considered to contain one HMG box. The HMG box is a sequence motif recognized in certain sequence-specific DNA-binding proteins, in HMG1, and in other HMG-like DNA-binding proteins, such as UBF, eukaryotic upstream binding factor (18), and SRY, a gene cloned from the sex-determining region of the human Y chromosome (19). The HMG box is defined by a set of highly conserved residues (most distinctively aromatic and basic) and appears to define a novel DNA-binding structural motif. The primary structure of HMG is highly conserved among mammals, and HMG proteins in many species comprise an HMG family. The primary structure of bovine HMG1 (16), human HMG1 (20), HMG2 (21), murine HMG1 (22), porcine HMG1 (23), HMG2 (24), and rat amphoterin (25) were reported and showed much similarity. The HMG-like protein in milk in the present study is considered to be a member of the HMG family.

Although HMG1 is implicated in DNA replication and cellular differentiation, no report has been made asserting that this protein stimulates cellular proliferation. Our present study is the first to demonstrate that a member of the HMG family existed in milk and that is showed a growth-promoting activity. This activity was specific for osteoblast, not for fibroblast Balb/3T3 and Chinese hamster V79 cells (data not shown). Therefore it is expected that the HMG-like protein in milk plays a significant role in bone formation by activating osteoblasts.

```
A: GKGXXKKPRGKMSXYAXFVQTXREEHKKXHPDASVNFXEF
   ***  ******** ** **** ****** ******** **
B: GKGDPKKPRGKMSSYAFFVQTCREEHKKKHPDASVNFSEFSK...
```

FIG. 5. Comparison of amino acid sequence between purified protein (A) and its homologous sequence (B). Amino acid sequences are described with the standard single-letter notation for amino acid residues. Amino-terminal sequences of purified protein and homologous protein are aligned, and the same residues between the two sequences are denoted by asterisks.

REFERENCES

1. Hiddink, J., de Bore, R., and Romijn, D. J. (1978) *Neth. Milk Dairy J.* **32,** 80–93.
2. Yagi, H., Suzuki, S., Noji, T., Nagashima, K., and Kuroume, T. (1986) *Acta Pedrier. Scanda.* **75,** 233–235.
3. Sinha, S., and Yunis, A. A. (1983) *Biochem. Biophys. Res. Commun.* **114,** 797–803.
4. Zweibel, J. A., Bano, M., Nexi, E., Salomon, D. S., and Kidwell, W. R. (1986) *Cancer Res.* **46,** 933–939.
5. Jin, Y., Cox, D. A., Knecht, R., Raschdorf, F., and Cerletti, N. (1991) *J. Protein Chem.* **10,** 565–575.
6. Ballard, F. J., Nield, M. K., Francis, G. L., Dahlenberg, G. W., and Wallace, J. C. (1982) *J. Cell Physiol.* **110,** 249–254.
7. Rogers, M. L., Belford, D. A., Francis, G. L., and Ballard, F. J. (1995) *J. Dairy Res.* **62,** 501–507.
8. Klagsbrun, M., and Neumann, J. (1979) *J. Supramol. Struct.* **11,** 349–359.
9. Shing, Y. W., and Klagsburn, M. (1984) *Endocrinology* **115,** 273–282.
10. Tokuyama, H., and Tokuyama, Y. (1989) *Cell Biol. Int. Rep.* **13,** 251–258.
11. Takada, Y., Kobayashi, N., Kato, K., Matsuyama, H., Yahiro, M., and Aoe, S. (1997) *J. Nutr. Sci. Vitaminol.* **43,** 199–210.
12. Takada, Y., Matsuyama, H., Kato, K., Kobayashi, N., Yamamura, J., Yahiro, M., and Aoe, S. (1997) *Nutr. Res.* **17,** 1709–1720.
13. Takada, Y., Aoe, S., and Kumegawa, M. (1996) *Biochem. Biophys. Res. Commun.* **223,** 445–449.
14. Schagger, H., and von Jagow, G. (1987) *Anal. Biochem.* **166,** 368–379.
15. Bustin, M., Lehn, D. A., and Landsman, D. (1990) *Biochim. Biophys. Acta* **1049,** 231–243.
16. Kaplan, D. J., and Duncan, C. H. (1988) *Nucleic Acid Res.* **16,** 10375–10375.
17. Wier, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R., Laue, E. D., and Thomas, J. O. (1993) *EMBO J.* **12,** 1311–1319.
18. Jantzen, H.-M., Admon, A., Bell, S. P., and Tjian, R. (1990) *Nature* **344,** 830–836.
19. Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A.-M., Lovell-Badge, R., and Goodfellow, P. N. (1990) *Nature* **346,** 240–244.
20. Wen, L., Huang, J. K., Johnson, B. H., and Reeck, G. R. (1989) *Nucleic Acids Res.* **17,** 1197–1214.
21. Shirakawa, H., and Yoshida, M. (1992) *J. Biol. Chem.* **267,** 6641–6645.
22. Tsuda, K.-I., Kikuchi, M., Mori, K., Waga, S., and Yosihda, M. (1988) *Biochemistry* **27,** 6159–6163.
23. Yotov, W. V., and St-Arnaud, R. (1992) *Nucleic Acids Res.* **20,** 3516–3516.
24. Shirakawa, H., Tsuda, K., and Yoshida, M. (1990) *Biochemistry* **29,** 4419–4423.
25. Merenmies, J., Pihlaslari, R., Laitinen, J., Wartiovaara, J., and Rauvala, H. (1991) *J. Biol. Chem.* **266,** 16722–16729.