Identification of fibroblast growth factors in bovine cheese whey

BY MARY-LOUISE ROGERS*, DAVID A. BELFORD, GEOFFREY L. FRANCIS AND F. JOHN BALLARD

Cooperative Research Centre for Tissue Growth and Repair, PO Box 10065, Gouger Street, Adelaide, SA 5000, Australia

(Received 15 September 1994 and accepted for publication 17 December 1994)

SUMMARY. Acidic and basic fibroblast growth factors (FGF) were identified in bovine cheese whey after partial purification using a two step procedure. Cation-exchange chromatography produced a mitogen-rich extract which was loaded on to a heparin-sepharose column and eluted stepwise with 0.8, 1.2 and 2.0 M-NH₄HCO₃. Mitogenic activity was found in all three fractions by cell growth assays using Balb/c-3T3 fibroblasts. Immunoblotting identified acidic FGF in the 1.2 M-eluate and basic FGF in the 2.0 M-eluate, but neither acidic nor basic FGF was detected in the 0.8 M-fraction. Quantitative radioreceptor assays indicated 5.8 ng of acidic FGF-like activity and 19.8 ng of basic FGF-like activity per 1 whey in the appropriate eluates. This study represents the first direct demonstration of FGF in milk.

Heparin-affinity chromatography has been widely used as a method for purifying and characterizing growth factors. Classically, the purification of acidic and basic fibroblast growth factors (aFGF, bFGF) from a wide variety of tissues of mesodermal and ectodermal origin has been facilitated by the efficiency of this technique (Gospodarowicz et al. 1987; Klagsbrun, 1989; Baird & Bohlen, 1990). A number of other growth factors have been isolated from bovine milk and colostrum, including insulin-like growth factor-I and II (Francis et al. 1988), platelet-derived growth factor (Brown & Blakeley, 1984) and transforming growth factor-β (Jin et al. 1991), although epidermal growth factor has not been convincingly identified in bovine milk (Read et al. 1984; Carpenter & Wahl, 1990). More recently, two heparin-binding growth factors of 19 and 6 kDa were recovered from bovine prepartum (milk-like) secretions (Sandowski et al. 1993), but no direct evidence for aFGF or bFGF in milk has yet been obtained.

Bovine cheese whey is a convenient and essentially inexhaustible source of the growth factor component of bovine milk. In this study, we have used a combination of cation-exchange chromatography and heparin-affinity chromatography to isolate heparin-binding growth factors from whey. Elutions from heparin-affinity chromatography of a whey extract were found to stimulate the growth of Balb/c-3T3 fibroblasts in cell assays. The presence of aFGF and bFGF in two of these fractions was confirmed by immunoblotting and radioreceptor assays.

* For correspondence.
Recombinant human bFGF and aFGF were supplied by Austral Biologicals (San Ramon, CA 94583, USA). **I** was from Amersham Australia Pty Ltd (North Ryde, NSW 2113). All other reagents used were analytical grade.

**Production of whey extract**

The initial stages of the whey fractionation were kindly carried out by Drs G. Regester and G. Smithers at the CSIRO Dairy Research Laboratory (Highett, VIC 3190). Pasteurized whey obtained as an end product of cheese manufacture was passed through a 0.8 μm ceramic filter to remove solids. The ultrafiltrate was adjusted to pH 6.5 and applied to a column of S-Sepharose Fast Flow cation-exchange resin (AMRAD Pharmacia Biotech, North Ryde, NSW 2113) that had been equilibrated with 50 mM-sodium citrate buffer, pH 6.5. After washing the column with the same buffer, the adsorbed material was eluted with 0.5 M-NaCl. This eluate was diafiltered against water, concentrated by ultrafiltration (molecular mass cut-off, 3 kDa) and freeze dried.

**Heparin-sepharose chromatography**

Chromatography was performed using a Bio-Rad column (50 mm x 50 mm i.d.) containing Heparin-Sepharose CL-6B (AMRAD Pharmacia Biotech) coupled to an FPLC system (AMRAD Pharmacia Biotech). The column was pre-equilibrated with 0.6 M-NaCl–10 mM-Tris, pH 7.0. Freeze-dried whey extract (1 g) was dissolved in this equilibration buffer at a concentration of 25 mg/ml and loaded onto the column, washed and eluted at 140 mm/h (Lobb *et al.* 1986) with equilibration buffer and with two column volumes of 0.1 M-NH₄HCO₃. The column was then stepwise eluted with 100 ml of 0.8, 1.2 and 2.0 M-NH₄HCO₃. Protein was measured in each pooled eluate using bovine serum albumin as a reference (RIA grade; Sigma-Aldrich Pty Ltd, Castle Hill, NSW 2154). The fractions were then freeze dried.

**Cell growth assays**

The cell proliferation assay used Balb/c-3T3 fibroblasts cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing fetal calf serum (100 ml/l) at 37 °C in 50 ml CO₂/l. Cells were plated in 96 well microtitre plates at 2 x 10⁴ cells per well and allowed to attach overnight. After a 2 h wash in DMEM, dilutions of column fractions were added in a final volume of 100 μl and incubated for a further 48 h. The indicated concentrations of recombinant aFGF and bFGF were included on each plate as controls. Cell growth was quantified using an automated methylene blue staining method (Oliver *et al.* 1989) and results were expressed as percentages of the response to DMEM containing fetal calf serum (200 ml/l).

**Immunoblots**

Portions of the 1.2 and 2.0 M-NH₄HCO₃ eluates were subjected to SDS-PAGE under reducing conditions (Wadzinski *et al.* 1987) and electroblotted on to nitrocellulose sheets (Schleicher and Schuell Pty Ltd, D-3354 Dassel, Germany) using a Novablot system (AMRAD Pharmacia Biotech). Recombinant aFGF and bFGF were included as positive controls. Nitrocellulose sheets were probed with polyclonal antibodies specific for bovine bFGF (Sigma-Aldrich) or aFGF (a gift from Dr I. Hendry, John Curtin School of Medical Research, Australian National
Fibroblast growth factors in bovine whey

University, Acton, ACT 2601). Bound antibody was detected using alkaline phosphatase conjugated to rabbit IgG and developed with a 5-bromo-4-chloro-3-indolyl phosphate–nitro blue tetrazolium substrate (Sigma-Aldrich).

Radioreceptor assays

Radioreceptor assays were performed using confluent monolayers of BHK-21 (ATCC CCL 10) cells grown in DMEM containing fetal calf serum (100 ml/l) in 24 well microtitre plates. \(^{[125]I}\)bFGF was prepared using the chloramine-T method (Van Obberghen-Schilling & Pouyssegur, 1983) and separated from free \(^{125}\)I by gel filtration (specific activity of 54 µCi/µg). Dilutions of protein fractions from the heparin–sepharose column or unlabelled aFGF or bFGF were added to BHK-21 cells with \(^{[125]I}\)bFGF in a total volume of 0.5 ml, and incubated at 4 °C for 4 h. Cells were washed twice with Hanks’ salts, once with Hanks’ salts containing 2.0 M-NaCl, and dissolved in 0.5 M-NaOH containing Triton X-100 (1 ml/l) after which the bound radioactivity was counted (Moscatelli, 1987). Values were plotted and analysed using Tablecurve (Jandel Scientific, San Rafael, CA 94912-8920, USA) and binding is expressed as the percentage bound in the absence of unlabelled peptide.

RESULTS

Isolation of heparin-binding fractions

As a first step in isolating heparin-binding growth factors from bovine cheese whey, cation-exchange chromatography was used to remove the major whey proteins. This step yielded 30 g freeze-dried whey extract/tonne whey; this corresponded to 5 g whey protein/l (cation-exchange fraction). This fraction was subjected to stepwise elution as described above, and 1 g of the cation-exchange fraction yielded 2.2, 0.48 and 0.22 mg protein respectively in the 0.8, 1.2 and 2.0 M fractions. Use of the volatile salt NH\(_4\)HCO\(_3\) in the elution buffer allowed immediate freeze drying of the recovered fractions, thus minimizing further protein losses associated with desalting procedures.

Cell growth activity of heparin-binding whey fractions

To determine whether the fractions recovered from heparin-affinity chromatography were mitogenic, dilutions of the three eluates were tested on Balb/c-3T3 fibroblasts using a dye-binding assay (Oliver et al. 1989), and the growth response compared with that achieved with recombinant aFGF and bFGF standards. As shown in Fig. 1, the protein recovered in all fractions and FGF induced the growth of Balb/c-3T3 fibroblasts in a dose-dependent fashion. Maximal responses to both the 1.2 and 2.0 M fractions were achieved at ~1.2 µg/ml total protein.

Detection of FGF by immunoblotting

Evidence that the heparin-binding fractions recovered at 1.2 and 2.0 M-NH\(_4\)HCO\(_3\) contained aFGF and bFGF was provided by immunoblotting. The presence of aFGF in the protein eluting at 1.2 M-NH\(_4\)HCO\(_3\) was confirmed by the detection of a single broad immunoreactive band at 15 kDa, migrating slightly further than recombinant human aFGF(1-154) (Fig. 2a). Two immunoreactive bands at 14 and 17 kDa were detected in the 2.0 M eluate when probed with an antibody raised against a synthetic peptide corresponding to the first 24 amino acids of bovine bFGF (Fig. 2b); the higher molecular mass band migrated to the same position as the recombinant bFGF.
Fig. 1. Stimulation of cell growth by heparin-binding factors obtained from bovine whey. Growth responses of Balb/c-3T3 fibroblasts in the presence of dilutions of protein from heparin-sepharose chromatography fractions were compared with responses to recombinant acidic fibroblast growth factor (FGF) and basic FGF: fractions from ○, 0.8 M-; ●, 1.2 M- and △, 2.0 M-NH₄HCO₃ eluates and △, acidic FGF; □, basic FGF. For details, see Materials and Methods. Cell growth is expressed as a percentage of the response to 200 ml fetal calf serum (FCS)/l; the growth response to Dulbecco’s modified Eagle’s medium alone has been subtracted. Each point is the mean of triplicate cultures.

Molecular mass markers, kDa

(a) 21 - (b) 14 -

Fig. 2. Immunoblots of the fractions from heparin–sepharose chromatography of whey extract probed with (a) anti-bovine acidic fibroblast growth factor (FGF) and (b) anti-bovine basic FGF. Lane 1, 13 µg of the 1.2 M-NH₄HCO₃ fraction; lane 2, 220 ng acidic FGF; lane 3, 4 µg of the 2.0 M-NH₄HCO₃ fraction; lane 4, 220 ng basic FGF. The positions of 21 kDa (trypsin inhibitor) and 14 kDa (lysozyme) molecular mass markers are indicated.
Fibroblast growth factors in bovine whey

Fig. 3. Radioreceptor assays for basic fibroblast growth factor (FGF) in the 2-0 M-NH₄HCO₃ fraction and acidic FGF in the 1-2 M-NH₄HCO₃ fraction from heparin–sepharose chromatography of whey extract. A dilution series of ◆, the 2-0 M fraction and ▲, the 1-2 M fraction were compared with appropriate dilutions of ●, unlabelled basic FGF and ▲, acidic FGF. For details, see Materials and Methods. Results are expressed as the percentage bound in the absence of unlabelled peptide and are the means of three determinations.

There was no evidence of immunoreactive aFGF or bFGF in the 0-8 M-NH₄HCO₃ fraction (results not shown).

Verification of FGF activity by radioreceptor assay

Further confirmation that cell growth activity of the whey-derived heparin-binding fractions is at least partly due to the presence of FGF was provided by radioreceptor assays using BHK-21 cells. Dilutions of both the 1-2 and 2-0 M eluates were effective in competing with ¹²⁵I-labelled bFGF for receptor sites on the cell monolayer (Fig. 3). Comparison with a bFGF standard indicated that 3 ng bFGF/µg protein was found in the 2-0 M-NH₄HCO₃ fraction, corresponding to a recovery of 19.8 ng bFGF/1 whey. Quantitation of aFGF in the 1-2 M-eluate, using the same assay with reference to a recombinant aFGF standard (Neufeld & Gospodarowicz, 1986) detected 0.4 ng aFGF/µg protein or 5.8 ng/1 whey.

DISCUSSION

Although fibroblast growth factors are found in several different mammalian tissues (Gospodarowicz et al. 1987; Klagsbrun, 1989), they have not been identified in milk or colostrum. Using bovine cheese whey as an inexhaustible source of the growth factor component of milk (Klagsbrun & Neumann, 1979; Derouiche et al. 1990), we have applied a two step procedure to concentrate heparin-binding growth factors. Initial ion-exchange chromatography effectively removed major whey proteins such as β-lactoglobulin and α-lactalbumin which, owing to their anionic nature, do not bind the cation-exchange resin at pH 6.5. A similar cation-exchange
step is included in the routine isolation of aFGF and bFGF from bovine brain (Lobb et al. 1986). Heparin--sepharose chromatography of the cation-exchange fraction isolated from whey permitted identification of aFGF and bFGF. The 1-2 M elution from this heparin-affinity chromatography contained aFGF and the 2-0 M fraction bFGF, which is consistent with previous tissue-derived FGF isolation procedures (Lobb et al. 1986).

Although growth factors are a minor component of whey protein, the impurity of the FGF-containing fractions was surprising given the reported specificity of heparin-affinity chromatography (Gospodarowicz et al. 1987; Klagsbrun, 1989; Baird & Bohlen, 1990). Furthermore, addition of an (NH4)2SO4 precipitation step to the isolation procedure (see Lobb et al. 1986) did not significantly enhance the purity of the final product (results not shown), presumably reflecting the diversity of protein in the whey fraction. Attempts to quantify FGF in crude whey or the cation-exchange fraction using radioreceptor assays were unreliable owing to interference, rendering accurate calculation of recoveries throughout the isolation procedure impossible.

All three fractions recovered from the heparin-affinity column induced the growth of Balb/c-3T3 cells, which were also responsive to purified recombinant FGF. Although the bioactive constituents in the 0-8 M fraction were not identified, factors such as platelet-derived growth factor elute from a heparin-affinity column in 0-8 M salt (Ferrara & Henzel, 1989; Klagsbrun, 1992). Identification of aFGF in the 1-2 M fraction is consistent with previously reported protocols (Lobb et al. 1986), although total growth factor activity in the 1-2 M fraction (Fig. 1) was not accounted for by the concentration of aFGF determined by quantitative radioreceptor assay (Fig. 3). It is therefore likely that other heparin-binding factors are present in the 1-2 M eluate. Notably, maximal growth in response to the 1-2 M eluate exceeded that achieved with saturating concentrations of recombinant aFGF (Fig. 1). N-terminal heterogeneity of tissue-derived aFGF is well described (Gospodarowicz et al. 1987) and would account for the lower molecular mass of the whey-derived molecule compared with the recombinant aFGF(1-154) standard (Fig. 2a).

To date, bFGF is the only known factor that requires 2-0 M salt for elution from a heparin-affinity column (Lobb et al. 1986; Klagsbrun, 1992); the presence of bFGF in the 2-0 M whey-derived eluate was confirmed by immunoblotting (Fig. 2b). The presence of a 14 kDa band may reflect cleavage during the relatively harsh conditions of the cheesemaking process. Although multiple forms of bFGF have been reported (Brigstock et al. 1990), they are generally of higher molecular mass and consistent with alternative translation sites. The concentration of bFGF in the 2-0 M eluate was sufficient to account for the observed bioactivity in the Balb/c-3T3 cell growth assay (Fig. 2b).

The detection of aFGF and bFGF in whey adds to the list of growth factors described in milk (Brown & Blakeley, 1984; Francis et al. 1988; Jin et al. 1991) and is consistent with a previous report describing heparin-binding growth factors in bovine prepartum secretions (Sandowski et al. 1993). The source and significance of FGF in milk are unknown; assuming no losses during the isolation procedure, the concentration of bFGF in milk (19-8 ng/l) is within the physiologically active range (Gospodarowicz et al. 1987). Earlier studies (Barracough et al. 1990; Ke et al. 1993) showing that expression of FGF is a feature of mammary gland fibroblasts and myoepithelial cells, rather than the epithelial cells, would argue against a mammary gland origin. Indeed, the presence of soluble FGF is unusual (Klagsbrun, 1992) and it may be derived from lysis of the cellular component of milk during cheesemaking.
Fibroblast growth factors in bovine whey

We thank Drs G. O. Regester and G. Smithers together with the staff of the Dairy Research Laboratory of the Commonwealth and Scientific Industrial Research Organisation, Highton, Victoria for preparation of whey extract.

REFERENCES


FERARRA, N. & HENZEL, W. J. 1989 Putative follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochemical and Biophysical Research Communications 161 851-858.


MOSCATELLI, D. 1987 High and low affinity binding sites for basic fibroblast growth factor on cultured cells: absence of a role for low affinity binding in the stimulation of plasminogen activator production by bovine capillary endothelial cells. Journal of Cellular Physiology 131 123-130.

NEUFELD, G. & GOSPODAROWICZ, D. 1986 Basic and acidic fibroblast growth factors interact with the same cell surface receptors. Journal of Biological Chemistry 261 5631-5637.


Downloaded from https://www.cambridge.org/core. IP address: 54.191.40.80, on 14 Jul 2017 at 16:29:00, subject to the Cambridge Core terms of use, available at https://www.cambridge.org/core/terms. doi:10.1017/S0022029900031198