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EXPRESSION AND PURIFICATION OF BIOLOGICALLY ACTIVE HUMAN OSF-1 IN *Escherichia coli*

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SUMMARY: OSF-1 (also known as pleiotrophin, HB-GAM, HBGF-8 or HBNF) is a heparin-binding, neurotrophic protein. Its tissue-specific expression in rats is developmentally regulated and the protein is highly conserved between species. The protein is believed to be involved in neuronal development. Previous experiments in our laboratory showed that OSF-1 is primarily expressed in brain and bone. The biological function of OSF-1 in bone is unknown. In order to overcome the limited availability of the native protein, we now report on the high-level expression of human OSF-1 in *Escherichia coli*. The protein is present in the form of inclusion bodies, which were isolated and solubilized. The partially purified protein was refolded and further purified employing heparin sepharose chromatography. N-terminal sequence determination revealed the same amino acid sequence as the natural mature protein. The isolated backfolded recombinant human OSF-1 did promote neurites outgrowth in primary cultures of cortical neurons.

Our laboratory is focussing on the identification of osteoregulatory factors. In the course of these studies, a cDNA was isolated from the murine osteoblastic cell line MC3T3-E1 encoding a protein expressed in bone and brain (1). The deduced protein is lysine-rich, basic and consists of 168 amino acids, including a 32 amino acid signal sequence. A human counterpart cDNA was isolated from a brain cDNA library (1), which revealed a single residue substitution in the mature sequence. The independent isolation of the apparently identical protein has been described from bovine (2, 3) and rat (4) brain and from bovine uterus (5) by at least four laboratories. Synonymous expressions for OSF-1 include the terms: heparin-binding neurotrophic factor (HBNF) (6, 2), 18 kDa heparin binding protein (4), later renamed as heparin-binding growth-associated molecule (HB-GAM) (7), and heparin-binding growth factor 8 (HBGF-8) (5), renamed later as pleiotrophin (PTN) (8). Cloning and analysis of the respective cDNAs revealed highly conserved proteins between

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Abbreviations: OSF-1, Osteoblast-specific factor 1; rhOSF-1, recombinant human OSF-1; DTT, dithiothreitol; MAP, methionine-aminopeptidase; SDS, Sodium dodecylsulfate, PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; bFGF, basic fibroblast growth factor; NGF, nerve growth factor.

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species, indication of an important biological function. No homology to known growth or neurotrophic factors, such as basic and acidic FGF, ciliary neurotrophic factor (CNTF) and the NGF family could be observed. However, partial sequence homology of OSF-1 with midkine (MK), a differentiation and growth regulator identified in teratocarcinoma cells induced with retinoic acid (9, 10), was observed. The studies by Li et al. (8) indicate that the expression of the protein is developmentally regulated in the rat. It has been reported that the protein has in addition to its neurotrophic activity some mitogenic activity (5, 8). There is presently no information on the biological function of OSF-1 in bone but it is tempting to speculate that OSF-1 might act as a growth or differentiation factor, possibly on osteoblasts. In order to provide sufficient amounts of OSF-1 for such studies, we wished to express the human OSF-1 cDNA in *E.coli*. Here we report that recombinant human OSF-1 can be expressed in *E. coli* at high yield and that the recombinant protein has neurite outgrowth activity.

MATERIALS AND METHODS

Construction of expression plasmid. Two PCR primer were synthesized using the automatic DNA synthesizer from Applied Biosystems. Primer A (5' AACCATGGGGAAGAAAGAGAAACCAG) encodes a newly introduced NcoI site and the initiation codon. Since the first codon of mature hOSF-1 is GGG (encoding glycine), this NcoI site (5'CC/ATGG) is fused to the mature coding sequence such that the initiation codon ATG precedes the GGG codon. Primer B (5' TTCTGCAGCTTTTAATCCAGCATCTTCTCC) encodes a newly introduced PstI site just downstream of the termination codon. Using 10 ng of human cDNA (1), about 500 ng of the fragment was isolated from the PCR reaction (11). The fragment was digested with NcoI and PstI and cloned between the respective sites into pHSG741 (unpublished) and transformed into *E. coli* HB101. Plasmids of ampicillin resistant colonies were analyzed with restriction enzymes. The promoter and OSF-1 coding regions of a correct candidate clone were sequenced with the dideoxy chain termination method (12) and the predicted DNA sequence was confirmed. This plasmid was named pOSF201.

Purification and renaturation. Single transformants were inoculated into LS medium (13) containing 100 µg/ml ampicillin and incubated overnight. 1 ml of this culture was used to inoculate 100 ml of M9-GC medium (14) containing ampicillin at the above concentration and cultured for 20 hrs. Cells were harvested by centrifugation and resuspended in 10 ml prechilled extraction buffer (50 mM Tris-HCl, pH7.3, 100 mM NaCl, 10 mM EDTA). The cells were disrupted by sonication. The lysate was centrifuged at 15,000 g for 10 min and the proteins present in sediment and supernatant fractions were analyzed by SDS-PAGE in the absence or presence of 40 mM dithiothreitol (DTT). The inclusion bodies were washed twice with 5 ml of 100 mM n-octyl-beta-glucopyranoside in TE (14) and twice with 5 ml of TE to remove the detergents. The washed inclusion bodies were suspended in 2 ml of unfolding buffer (8 M urea [deionized], 10 mM Tris-HCl, pH7.3, 20 mM DTT, 0.5 M NaCl) and incubated at 37 °C for 60 min. Remaining cell debris was removed by centrifugation. The solubilized protein was subjected to gel permeation chromatography using Sephacryl S-300 (Pharmacia), equilibrated with unfolding buffer. Pooled fractions containing partially purified rhOSF-1 was diluted with unfolding buffer to a protein concentration of 0.1 mg/ml. The protein was dialysed against refolding buffer (20 mM Na-Phosphate, pH7.3, 0.5 M NaCl) at room temperature for 70 hr with changing of the buffer every 10-15 hr. The dialysed protein was collected and centrifuged at 100,000 g for 16 hr to remove incorrectly refolded protein aggregates. The supernatant was applied to the heparin sepharose CL-6B column (Pharmacia), equilibrated with refolding buffer. The column was washed with refolding buffer and bound protein was eluted with phosphate-buffered salt solution (2.0 M NaCl). The ratio of bound protein vs. total protein was defined as the heparin binding activity. The eluted fraction was dialysed against PBS (10 mM Na-Phosphate, pH 7.3, 0.15 M NaCl) and frozen at -80 °C.

Amino acid composition. Refolded rhOSF-1 was hydrolyzed in 6 M HCl under reduced pressure at 110 °C for 21 hr. The hydrolysate was analyzed by the phenyl-n-thiocarbomyl method (15) on the PICO TAG system (Waters Millipore Corp., Milford, MA).

N-terminal sequencing. rhOSF-1 was electroblotted to the PVDF membrane after separation with SDS-PAGE under reducing conditions. The protein band corresponding to rhOSF-1 was subjected to N-terminal amino acid sequencing using the Applied Biosystems model 477A/120A pulse liquid gas phase automatic microsequencer.

Neurite outgrowth assay. For rat embryonic cortical neurons the assay was performed as described by Rauvala and Piklaskari (16) and Boehlen et al. (2). Briefly brains from 17 day fetal rats were removed. The brains were dispersed to single cells in DMEM supplemented with 10 % FCS and antibiotics using a sterile 10 ml pipette followed by repeated aspiration through a 1 ml pipettman sterile tip. The cell suspension was centrifuged at 500 rpm for 2 min. and the cell pellet was resuspended in DMEM / 10 % FCS at a concentration of $2x10^6$ cells/ml using coulter counter. The cells were then plated onto tissue culture dishes (5 ml / 60 mm dish) that had been precoated for 30 min. at room temperature with a solution of 50 µg/ml of poly (D,L)-lysine (sigma). 24 hr later cells were detached from the substrate by gentle washing and resuspended at a concentration of $1x\ 10^5$ cells /ml in DMEM/10 % FCS. Two ml were plated in 35 mm poly (D,L)-lysine coated tissue culture dishes. After 24 hr the medium was changed to DMEM containing 1 mg/ml BSA and either bFGF or OSF-1 was added at concentrations ranging from 10 ng to 10 µg/ml. After 24 hr incubation, cells were examined visually for neurite outgrowth and pictures of various fields were taken using a Nikon phase contrast microscope. For the PC-12 neurite outgrowth assay the procedure described by Neufeld et al. was followed (17).

RESULTS

Expression of human OSF-1 in *E. coli*. We wished to express the mature form of OSF-1. In order to introduce a start codon in front of the mature coding sequence, a PCR fragment was cloned into expression vector pHSG741. The resulting plasmid pOSF201 is able to express at high level (approximately 26 % of total cellular protein) the unfused, mature 17 kDa rhOSF-1 in the cytoplasm under the control of the *trp* promoter. Fig. 1A shows the accumulation of rhOSF-1 after tryptophan starvation of the cells and Fig. 1B shows that rhOSF-1 is present in the insoluble fraction after lysis of the bacteria.

Purification and renaturation. The insoluble fraction contained several distinct *E. coli* proteins in addition to rhOSF-1, which amounted to 70 % of the total insoluble fraction (Fig. 1B, lane 3). rhOSF-1 inclusion body is very rigid and high concentration of SH-reagent (> 10 mM DTT) and denaturants (> 8 M urea or > 6 M guanidine-HCl) are both necessary for complete solubilization. Solubilized, denatured rhOSF-1 from inclusion bodies was subjected to gel permeation chromatography and the fractions containing rhOSF-1 were pooled (Fig. 2). The protein concentration was diluted to 0.1 mg/ml and renaturation was done as described in Materials and Methods. After ultracentrifugation, soluble protein was subjected to heparin sepharose chromatography (Fig. 3). Almost 60 % of the soluble rhOSF-1 bound to the heparin sepharose column and was subsequently eluted with 2M NaCl. From a 100 ml shaker flask bacterial culture, typically 3 mg of heparin bound rhOSF-1 was recovered. Purity of the eluted rhOSF-1 was 87% (as measured by scanning under reducing conditions; see Fig. 3 inset). rhOSF-1 preparations of this purity was used for amino acid composition analysis and for the biological assays.

Amino acid sequence analyses. Table 1 shows the result of the amino acid composition analysis of rhOSF-1. The experimental data is in good agreement with the theoretical values. Next, 19 cycles of Edman degradation were performed using rhOSF-1 from inclusion bodies. The resulting

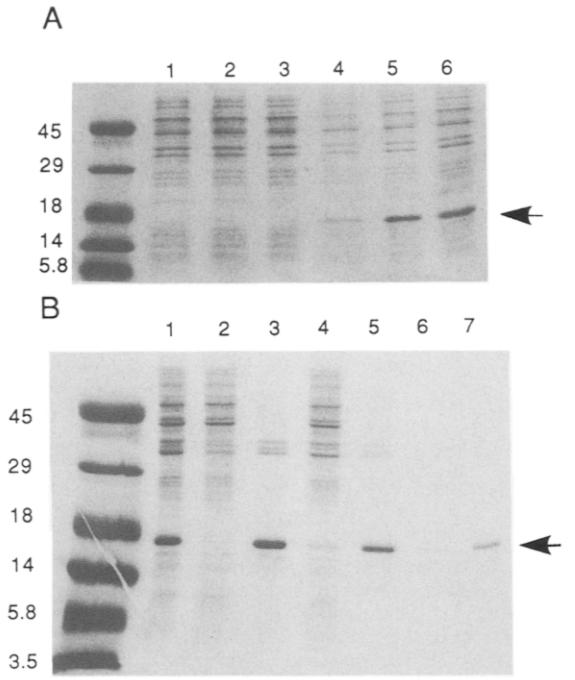

Fig. 1: SDS-PAGE analysis of rhOSF-1 expressed in *E. coli*. A: Total cellular proteins from *E. coli* harboring pHSG741 (lanes 1-3) and pOSF201 (lanes 4-6) were analyzed on a standard 15 % polyacrylamide gel (25) under reducing conditions and stained with Coomassie blue. Cells of an overnight culture were 100 fold diluted into M9-GC medium and aliquots were lysed at 5.5 hours (lanes 1, 4), 11 hours (lanes 2, 5), and 22 hours (lanes 3, 6) after inoculation. **B:** Characterization of inclusion bodies. Total cellular proteins (lane 1) were separated into soluble (lanes 2, 4) and insoluble (lanes 3, 5) fractions using different g forces (lanes 2, 3 : 15,000 g, lanes 4, 5 : 5,000 g). Insoluble material analyzed in lane 3 was resuspended and incubated for 30 min with 8 M urea in PBS. The suspension was centrifuged at 15,000 g for 10 min. Aliquots of supernatant (lane 6) and sediment fractions (lane 7) are shown. Molecular mass of the standard proteins are given in kDa. The position of rhOSF-1 is indicated by arrows.

sequence GKKEKPEKKVKKSDXGEWQ is identical to the known one (1,26) at the N-terminus of the mature protein. In the first cycle, methionine is only 10% against glycine, indicating that the N-terminal methionine is almost quantitatively removed by the *E. coli* MAP.

Neurite outgrowth activity. rhOSF-1 stimulated the neurite outgrowth of primary cortical neurons at concentrations ranging from 500 ng to 10 µg/ml (Fig. 4). bFGF (used as a positive control) was active at concentrations ranging from 10 ng to 250 ng/ml and the extent of neurite outgrowth was larger with rbFGF than with OSF-1 (Fig. 4). When tested on PC12 cells, rhOSF-1 did not stimulate neurite outgrowth even at concentrations as high as 10 µg/ml (both bFGF and NGF stimulated neurite outgrowth at concentrations as low as 1 ng/ml for bFGF and 10 ng/ml for NGF). In the ACE proliferation assay (18) rhOSF-1 did not have noticeable activity at concentrations as high as 10 µg/ml, whereas bFGF stimulated cell proliferation at concentrations as low as 10 pg/ml.

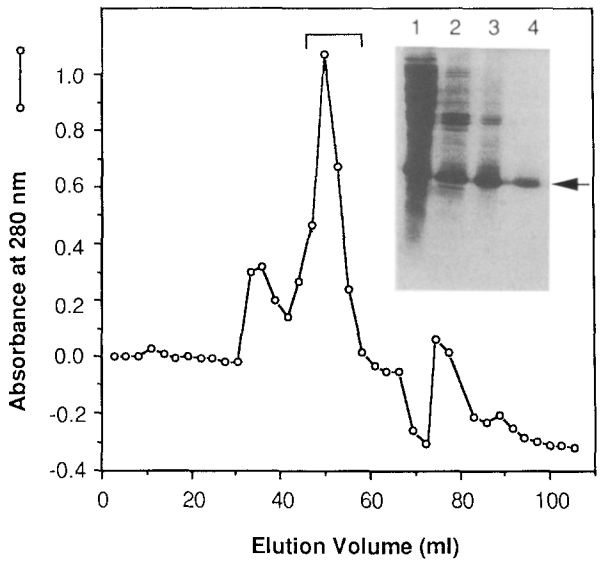

Fig. 2: Gel permeation chromatography of denatured rhOSF-1. 19 mg of unfolded protein from the insoluble fraction was subjected to chromatography using a Sephacryl S-300 column. The absorbance was measured against fraction 1 as the reference (negative absorbance values might be the result of oxidation of DTT during chromatography). The bracket indicates the pooled fractions used for subsequent renaturation. Inset: SDS-PAGE of rhOSF-1 (position indicated by an arrow) after purification steps Lane 1, total cellular protein, lane 2, insoluble fraction, lane 3 after washing inclusion bodies with n-octylglucopyranoside, lane 4, pooled fractions after chromatography.

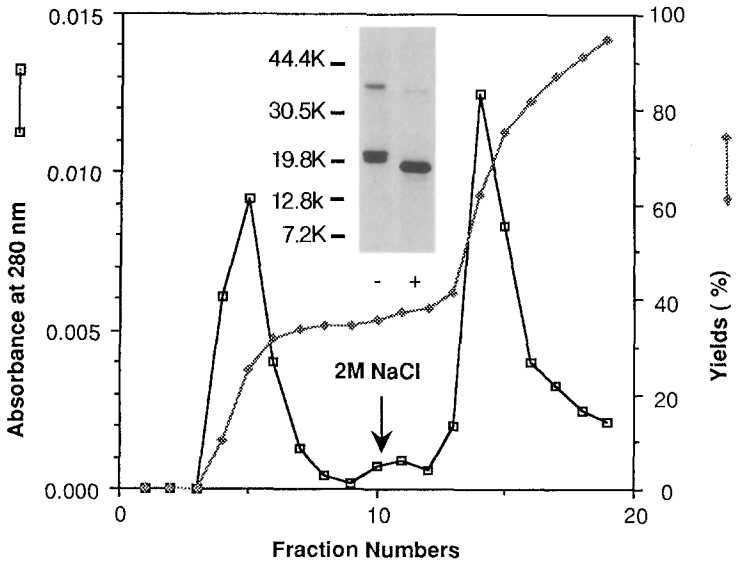

Fig. 3: Heparin sepharose chromatography of renatured rhOSF-1. Renatured soluble rhOSF-1 in refolding buffer was subjected to a heparin sepharose CL-6B column. The column was washed with 6 ml of refolding buffer and the bound rhOSF-1 was eluted with the same buffer containing 2 M NaCl. Inset: SDS-PAGE of rhOSF-1. The peak fraction after heparin sepharose chromatography was dialysed against PBS, and 5 µg of protein each was subjected to SDS-PAGE in the absence (-) and presence (+) of reducing reagent. (Inset; the molecular mass of the standard proteins are given in Da).

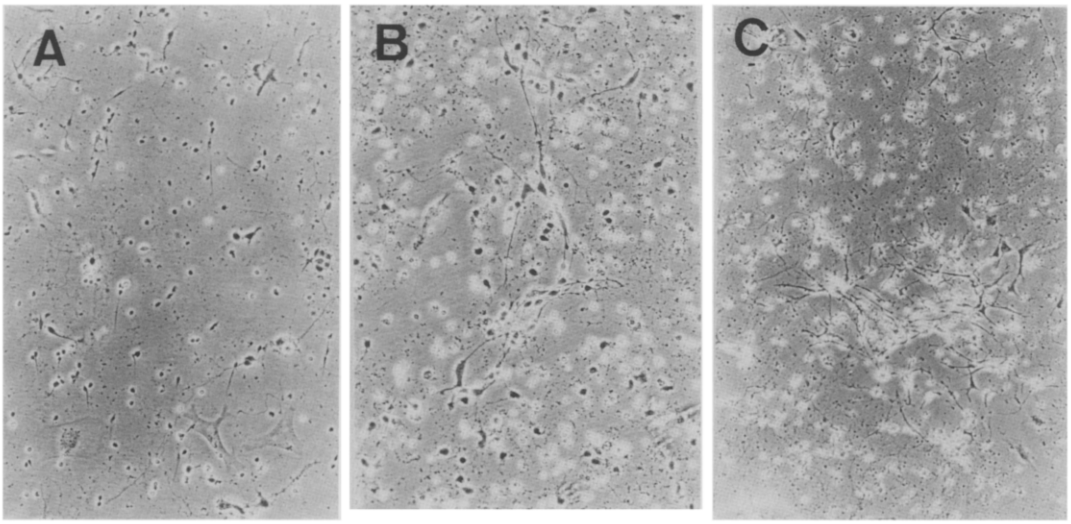

Fig. 4: Biological assay for rhOSF-1. Panel A: culture of cortical neurons without addition of growth factor, panel B: after treatment with 1.0 µg/ml rhOSF-1, panel C: after treatment with 30 ng/ml bFGF.

DISCUSSION

The human mature OSF-1 was expressed in *E.coli* at high levels (26% of total cellular protein). The 136 residue rhOSF-1 forms very rigid inclusion bodies, which were purified under denaturing conditions. The protein was renatured and regained its heparin binding activity, which is a characteristic of the natural protein. Using this property, rhOSF-1 was further purified and subsequently used for biochemical characterization and neurite outgrowth assays. Amino acid composition analysis of rhOSF-1 demonstrated the proteins identity. N-terminal sequencing revealed the correct sequence and showed that in approximately 90% of the molecules the N-terminal methionine was removed. This finding is in agreement with other studies, in which it was shown, that glycine in second codon position favors removal of the N-terminal methionine by the *E. coli* MAP (19, 20, 21). As a result of the employed expression strategy, the rhOSF-1 has the identical amino acid sequence as the processed natural protein (26). Using the model of Wilkinson and Harrison (22) to predict the solubility of recombinant proteins in *E. coli* we calculated a probability of 83 %. Despite this prediction, rhOSF-1 forms very rigid inclusion bodies. Lowering the growth temperature of the bacterial culture to 27 °C, which had been reported to favor the formation of soluble recombinant proteins (23), did not result in the expression of the soluble form of rhOSF-1 (results not shown). It had been reported, that the addition of reduced and oxidized glutathione at optimum ratios to create "oxido shuffling" conditions might improve the overall yield of correctly folded recovered proteins (24). When we employed different concentrations and ratios of reduced/oxidized glutathione in the backfolding experiments with rhOSF-1, the heparin binding activity was not improved (results not shown). The heparin-bound renatured rhOSF-1 tends to form dimers, whose amount diminished under reducing conditions (Fig. 3 Inset). SDS-PAGE

analysis of the renatured rhOSF-1 revealed double bands at the position of the monomer only under non-reducing conditions (Fig. 3 Inset). Although the reason for this behavior of rhOSF-1 is not understood in the moment, the double bands might be explained by the existence of disulfide isomers. The observations that rhOSF-1 stimulates the neurite outgrowth of primary cortical neurons while having no effect on the proliferation of vascular endothelial cells confirm earlier studies which have shown that HBNF, the native form of bovine brain OSF-1, lacked mitogenic activity for endothelial cells (2) while being a neuronotrophic factor. The finding that renatured rhOSF-1 from *E. coli* has neurite outgrowth activity indicates that the protein has regained at least partially its natural conformation. The expression of a biologically active rhOSF-1 shall overcome the limited availability of the natural protein in order to elucidate its biological role, in particular in bone cell biology.

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