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High yield expression of biologically active recombinant full length human tuftelin protein in baculovirus-infected insect cells

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ABSTRACT

Tuftelin is an acidic protein expressed at very early stages of mouse odontogenesis. It was suggested to play a role during epithelial–mesenchymal interactions, and later, when enamel formation commences, to be involved in enamel mineralization. Tuftelin was also detected in several normal soft tissues of different origins and some of their corresponding cancerous tissues. Tuftelin is expressed in low quantities, and undergoes degradation in the enamel extracellular matrix. To investigate the structure and function of tuftelin, the full length recombinant human tuftelin protein was produced. The full length human tuftelin cDNA was cloned using Gateway™ recombination into the Bac-to-Bac™ system compatible transfer vector pDest10. This vector adds a hexahistidine tag to the N-terminus of the expressed protein, enabling one-step affinity purification on nickel column. The recombinant human tuftelin protein was transposed into the bacmid and expressed in *Spodoptera frugiperda* (*Sf*9) insect cells. The yield of the purified, his-tagged recombinant full length human Tuftelin (rHTuft$^+$) was 5–8 mg/L culture. rHTuft$^+$ was characterized by SDS–PAGE, Western blot, ESI-TOF spectrometry, restriction mapping and MS/MS sequencing. The availability of the purified, full length recombinant human tuftelin protein opened up the possibility to investigate novel functions of tuftelin. Application of rHTuft$^+$ agarose beads onto embryonic mouse mandibular explants caused changes in the surrounding epithelial cells, including morphology, orientation and spatial organization. Further studies using DiI labeling, revealed that rHTuft$^+$, placed on the tooth germ region, brought about recruitment of adjacent embryonic mesenchymal cells. These findings support the hypothesis that tuftelin plays an important role during embryogenesis.

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Introduction

Tuftelin was initially identified, cloned and sequenced from a bovine ameloblast (enamel forming cells) enriched cDNA expression library by Deutsch et al. [1]. Later on, tuftelin expression was identified in numerous types of tissues, mineralizing and non-mineralizing, of several embryonic origins (mesenchymal, epithelial and neuronal). The entire human tuftelin gene, cDNA, and derived protein were deciphered and characterized in details [2,3]. The human tuftelin is a single copy gene that spans 43,279 bp on chromosome 1q21.3 ([2–4], GenBank Accession Nos. NP064512.1, NM020127, NC000001.9 region: 149779405... 149822683). It is composed of 13 coding exons, containing an open reading frame of 1170 bp, coding for the full length 390 amino acid protein ([2], GenBank Accession No. NP064512.1). Two alternatively spliced tuftelin isoforms, coding for 365 and 331 amino acid proteins, were also identified in the human tooth bud ([2], UniProt - UniProtKB No. Q9NNX1).

Computer analysis of the cDNA derived full length tuftelin proteins from human, bovine and mouse, revealed deduced hydrophilic acidic proteins of 390 amino acids. High similarities of amino acid sequences were found between human and bovine tuftelin (89%) and human and mouse tuftelin (88%). The human tuftelin has a predicted molecular weight of 44,264 Da, and an isoelectric point of 5.7. The bovine and mouse tuftelin proteins have predicted molecular weights and isoelectric points of 44.3 kDa and pI 5.7, and 44.6 kDa and pI 5.8, respectively. These similarities indicate high conservation within vertebrate species [2]).

Since tuftelin was first found and cloned from odontogenic tissue, most of the accumulating data on tuftelin is related to odontogenesis (tooth development). The vertebrate tooth is a typical example of an epithelium-mesenchyme composed organ, with evolutionarily conserved elements regulating different aspects of its development [5]. It involves many complex biological processes, including epithelial-mesenchymal reciprocal interactions, differentiation, morphogenesis and mineralization [6–8].

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Tuftelin was first identified in the extracellular enamel matrix [1]. During the process of development and mineralization, the extracellular enamel matrix, which is originally rich in proteins and water but relatively poor in mineral, looses most of its proteins (and water), which are degraded by specific proteases and replaced by mineral ions, calcium and phosphorus, the enamel finally becoming highly mineralized and mature tissue [1,9,10]. However, the initial expression of tuftelin mRNA during mouse tooth development (which has homologues stages in human and other species), was found at embryonic day E13, while the protein was detected at E17 [11]. Since tuftelin mRNA is detected already at E13, when no enamel or dentin have as yet been formed, it is possible that low amounts of tuftelin protein are also expressed, suggesting that tuftelin might have multiple roles, being also involved in the mesenchymal-ectodermal reciprocal interactions at this stage [9,11].

Tuftelin was also identified as one of three novel hypoxia-inducible genes in cancerous cell lines [12]. We showed that under physiological conditions, tuftelin mRNA levels were significantly higher in kidney and testis, which are normally exposed to low oxygen tension/hypoxic conditions, compared to brain, liver and lung [13]. Furthermore, we recently found, that tuftelin is induced during hypoxic conditions both *in-vivo* in the mouse brain and *in-vitro* in PC12 cell lines (model for neural cell differentiation) (unpublished data).

Since the tuftelin protein is present in relatively low amounts in the different tissues, and probably undergo degradation during enamel development, isolation and purification of sufficiently large quantities of tuftelin is extremely challenging.

Here we describe, for the first time, the expression of a recombinant full length human tuftelin protein (rHTuft^{+}) using the eukaryotic baculovirus system. This has made possible the commencement of investigations into novel function of tuftelin by providing relatively high amounts of biologically active tuftelin protein.

Materials and methods

Construction of the recombinant human tuftelin baculovirus

All procedures were performed according to the manufacturer's protocols, unless otherwise stated (Gateway™, Bac-to-Bac™, Invitrogen).

A full length human tuftelin cDNA was cloned into a TA plasmid; human tooth bud total RNA (0.29 μg) was reverse transcribed using a random hexamer primer and Superscript II reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Single stranded cDNA (1 μl) was PCR-amplified with the human tuftelin gene specific primers: sense—5′-ATGAACGGGACGCGGAACTGGTG-3′ and anti sense—5′-TCTCCAGGCAGCTCAGGTTTCCACC-3′ (underlined start and stop codons, respectively). RT-PCR product was cloned into the pCR 2.1 vector (TA Cloning Kit, Invitrogen), and transformed for propagation into One Shot® INVαF′competent cells (Invitrogen). The yielded TA_HTuft clone was purified and sequenced for verification using an automated DNA sequencer (ABI prizm 377, Perkin-Elmer, USA).

The TA_HTuft recombinant plasmid was PCR amplified, using SDS–PAGE purified specific primers (Fig. 1) containing attB sequences (Invitrogen), for site specific recombination with pDONR201 vector (Gateway Technology, Invitrogen), to generate entry clone containing the full length human tuftelin (pDONR_HTuft). pDONR_HTuft was recombined with the pDEST10 vector (Gateway Technology, Invitrogen), resulting in the production of a baculovirus transfer vector containing the recombinant human full length tuftelin cDNA (pDEST_HTuft), encoding for the 390 amino acid protein, described by Mao et al. [2], (Accession Nos. NM020127.2, NP064512.1). This recombinant construct encodes additional 39 amino acids at the N-terminus, including a hexahistidine tag, a spacer region and rTEV and Enterokinase protease cleavage sites (Fig. 2) followed by the human tuftelin cDNA. Identification of successful recombination was verified by DNA sequencing.

pDEST_HTuft was electro-transformed [14] into DH10BAC™ bacterial cells (Invitrogen), for obtaining the recombinant human tuftelin in the baculovirus genome (bacmid). The transformed DH10Bac bacteria were then isolated on selective plates according to the manufacturers' instructions. Single white colonies were restruck on consecutive plate, to ensure the verification of the bacmid clone. Single colonies were grown in 5 ml LB selective media, and small scale DNA preparations were made from these growths according to the Bac-to-Bac manual. The extracted DNA was analyzed by PCR using pUC/M13 universal primers, to confirm the homogeneity of the clone.

PCR-verified recombinant tuftelin bacmids were used to transfect *Sf*9 insect cells for viral particle formation using Cellfectin™ (Invitrogen). Recombinant baculovirus particles were harvested three days post transfection, by collecting condition medium. One round of plaque purification was performed to eliminate any non-recombinant virus that may be present. The purified plaques underwent three rounds of amplification in *Sf*9 monolayer cells;

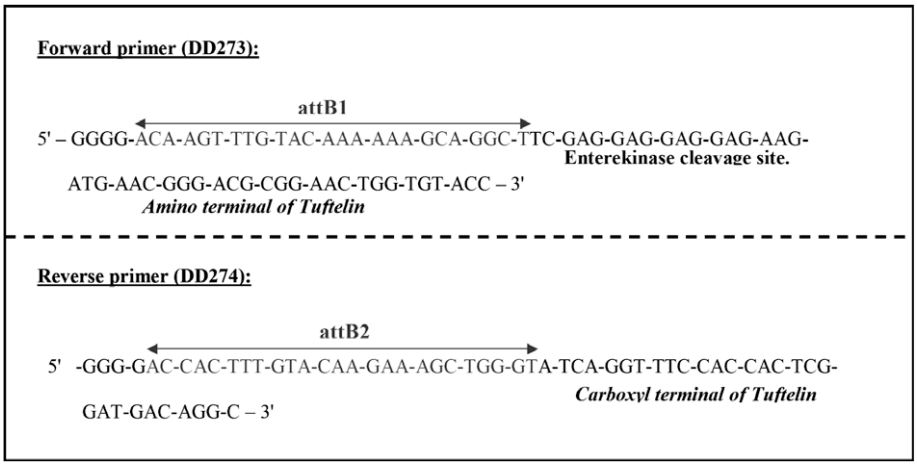

Fig. 1. attB-flanked human tuftelin primers. Sequence and illustration of the GATEWAY® attB-flanked human tuftelin primers.

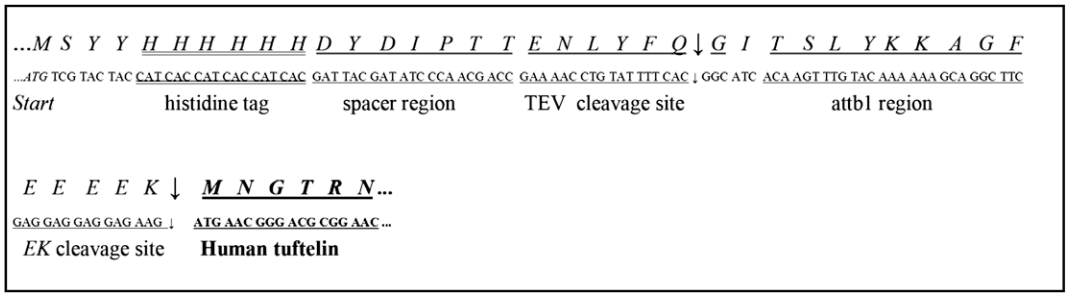

Fig. 2. Structure of the recombinant vector coding for the recombinant human tuftelin protein. The construct contains a hexa-histidine tag, spacer region, TEV protease cleavage site and Enterokinase protease cleavage site followed by the human tuftelin sequence (bold). The arrows indicate the cleavage sites.

First round of amplification included a 25 cm^2 plate and 2nd and 3rd round of amplifications were conducted on 75 cm^2 T-flasks to generate high titer rHTuft$^+$ viral stock. The viral titer was determined by plaque assay to be 2×10^8 Plaque forming unit (pfu)/ml.

Production of full length recombinant human tuftelin protein (rHTuft$^+$) in Spodoptera frugiperda (Sf9) cell culture

Spodoptera frugiperda (*Sf*9) cells adapted to serum-free Bioinsect-1 medium containing 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.25 μg/ml amphotericin, were incubated at 27 °C (±1 °C) in either monolayer or suspension cultures. All media components were obtained from Biological Industries (Biological Industries, Beit Haemek, Israel). Suspension cultures were grown at a constant orbital agitation speed of 97 rpm. Stock cells grown in suspension were seeded at 0.8–1.5 $\times 10^6$ cells/ml and the cells were diluted every 2–3 days, while still in log phase (4–6 $\times 10^6$ cells/ml). Viable cell counts were determined by Trypan blue staining (Sigma–Aldrich) and cultures with viabilities below 97% were discarded.

Calibrating parameters, for best yield, included harvest time post infection (24, 48 and 72 h), and various MOIs (1–10). Using Coomassie blue staining and Western blot analyses for screening, the best infection conditions for rHTuft$^+$ production were determined at MOI 7, when cells were harvested 72 h post infection.

These conditions were used for high expression of rHTuft$^+$ in *Sf*9 cells suspension cultures of log phase (viability above 98%) at final concentration of 2.5 $\times 10^6$ cells/ml with culture medium containing 0.5% (v/v) Fetal Calf Serum (Biological Industries, Beit Haemek, Israel). Harvested cells were separated from the supernatant by centrifugation at 1000 rpm for 5 min, at 4 °C, pellets were washed twice by re-suspension in 1× PBS containing 1mM PMSF subjected to centrifugation at 1000 rpm for 5 min at 4 °C. The isolated cell pellet was stored at −80 °C, until protein purification.

Batch scale production

Crude extracts were prepared by re-suspending cell pellets in lysis buffer (50 mM Tris pH 7.5, 1% (v/v) Triton X100 and 1 mM PMSF), sonication on ice (3× 10 s for 1 ml lysate, at 40% of maximum energy output (Vibra-Cell; Sonics, USA)) and centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatants were analyzed for rHTuft$^+$ expression by SDS–PAGE and Western blot. All harvesting procedures were performed in the presence of protease inhibitor cocktail (P8849, Sigma–Aldrich) in order to minimize degradation of the recombinant protein.

Purification of large scale recombinant human tuftelin protein (rHTuft$^+$)

Cell pellets were resuspended in 1:10 volume of lysis buffer (6 M GuHCl; 0.1 M NaH_2PO_4 pH 7.5; 10 mM imidazole and 0.5% (v/v) protease inhibitor cocktail (P8849; Sigma–Aldrich)). Sonication was performed on ice for 5× 10 s per 10 ml lysate at 40% of maximum energy output (Vibra-Cell; Sonics, USA). The lysate was cleared by centrifugation at 11,000 rpm for 30 min at 4 °C. The supernatant (crude extract) was incubated with pre-equilibrated NTA–Ni^{2+} resin (Qiagen) at a ratio of 1 ml beads/8 ml lysate, rotating constantly (∼15 rpm) at 4 °C for 90 min. The resin was centrifuged for 5 min at 4000 rpm, to remove unbound proteins, and then was packed into a column. Chromatography was performed on an ÄKTA Explorer FPLC system (Amersham Pharmacia) and the absorption (280 nm) and conductivity of the eluent was monitored. The column was washed at a flow rate of 2 ml/min with 5–10 column volumes (CV) of lysis buffer, 20 CV wash buffer 1 (6 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris–HCl; 10 mM imidazole pH 7.0); 10CV wash buffer 2 (wash buffer 1 at pH 6.3); 5CV wash buffer 2 adding 5% (v/v) elution buffer (6 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris–HCl; 500 mM imidazole; pH 4.8) and eluted on a 35–500 mM imidazole gradient using the elution buffer at a flow rate of 0.5 ml/min.

Fractions containing the recombinant human tuftelin protein were combined and extensively dialyzed (SnakeSkin™, MWCO 3500; Pierce), in order to remove the urea, against 50 mM acetic acid at 4 °C and the dialysis buffer was exchanged every 4 h for 2–3 days. rHTuft$^+$ was then aliquoted, lyophilized, and stored at −20 °C. Protein concentration was determined by the Bradford protein assay (Bradford, 1976) using Biorad Protein Assay Reagent (Biorad).

Recombinant protein analyses

SDS–PAGE

All samples were mixed with running buffer (125 mM Tris–HCl, pH 6.8; 4% (w/v) SDS; 0.005% (w/v) bromophenol blue; 20% (v/v) glycerol; 5% (v/v) β-mercaptoethanol).

SDS–PAGE was performed with precast 4–20% Tris–glycine gels in Tris running buffer (25 mM Tris pH 8.8, 1% (v/v) SDS, 0.2 M glycine) using a minicell apparatus (Novex) at 150 V for 50 min. Proteins were stained with Coomassie brilliant blue R-250 (Sigma–Aldrich).

Western blot analysis

SDS–PAGE gels were blotted onto Protran® nitrocellulose membranes (Schleicher&Schuell BioScience), using electrophoretic blotting (Biorad apparatus, Biorad) at 200 mA for 60 min. The membranes were incubated in a blocking solution containing 10% (v/v) PBS; 1 M glucose; 3% (w/v) BSA; 1% (w/v) milk powder; 10% (v/v) glycerol; 0.5% (v/v) Tween 20, agitating at 37 °C for 1 h. The membranes were then incubated agitating overnight at room temperature with rabbit polyclonal antibodies against synthetic bovine tuftelin peptides (LF74 and LF75 mix—[1]) diluted 1:750, or mouse monoclonal antibody against hexahistidine diluted 1:1000 (MMS-

156P, Babco-Convance), in 1% (w/v) gelatine–Tris buffered saline, followed by incubation with Alkaline phosphatase-conjugated secondary antibodies (Promega) for polyclonal and monoclonal antibodies were anti-rabbit IgG (diluted 1:10000) and anti-mouse IgG (diluted 1:7500), respectively.

Visualization was performed with BCIP-NBT reagents (Promega).

Enzymatic peptide mapping and MS/MS sequencing

Purified rHTuft$^+$ (major SDS–PAGE band) was cleaved by Trypsin at pH 8.0 in 25 mM ammonium bicarbonate at 37 °C overnight. The peptide mixture was solid phase extracted with C_{18} resin filled tip (ZipTip Milipore, Billerica, USA) and nanosprayed into the Orbitrap MS system in 50% CH_3CN 1% CHOOH solution.

Mass spectrometry was carried out with Orbi-trap (Thermo Finnigen) using nanospray attachment [15]. Data analysis was performed using bioworks 3.3 package and database searches were performed against the NCBInr database with the Sequest package and with Mascot package (Matrix Science, England). The amino acid sequence tag data was compared and mapped to the full length human tuftelin protein sequence (GenBank Accession No. NP064512.1).

Ex-Vivo mandibular explant culture

All experiments were approved by the Animal Care Ethical Committee, Hebrew University-Hadassah, Jerusalem.

Mice mating

ICR:Hsd (CD-1) females were mated with CD1 males. The following day females were assumed to be pregnant if a vaginal plug was present upon examination. The morning of this day was considered as embryonic day (E) 0.5. The exact developmental stage of embryos was calculated by developmental signs. All mating were performed and monitored by Harlan Biotech laboratories, Kiryat Weizmann, Israel.

Embryo collection

Female mice at E14.5 pregnancy stage were sacrificed by cervical dislocation. The uterus, containing the embryos, was removed and placed in Dulbecco's Modified Eagle Medium (DMEM) (with L-glutamine) containing 20 μg/ml penicillin/streptomycin (Biological Industries, Beit Haemek, Israel) at 4 °C. The embryos were separated from extra-embryonic membranes in tissue culture grade PBS under a stereomicroscope (Zeiss Stemi 2000-C) and the embryos heads were further dissected to separate the mandible from the head.

Preparation of culture dishes

Metal grids were prepared from stainless steel mesh of 0.25 mm diameter wire (Goodfellows) by cutting, approximately, 35 mm triangles and bending the edges. The metal grids were autoclaved and placed in Falcon 60 mm Center-well Organ Culture dishes (Becton Dickinson). Paper filters (Whatman) were cut to fit the outer surface diameter of the Center-well Organ Culture dish. The paper filters were autoclaved, inserted into the dish, and soaked with sterile H_2O to assist in maintaining humidity of the culture. The main well was filled with Dulbecco's Modified Eagle Medium (DMEM) (with L-glutamatmine) containing 20 u/ml penicillin/streptomycin (Biological Industries, Beit Haemek, Israel) up to the mesh surface.

White 0.lμ VCWP Millipore nitrocellulose filters (Millipore Corp.) were cut to 6 mm diameter circles using a sterile filter punch. The filters were rinsed in sterile solutions, under gentle agitation, as follows: 3 times in 70% ethanol for 1 h, overnight in 70% ethanol, 3 times in H_2O for 1 h, overnight in H_2O, and were stored at 4 °C in sterile PBS supplemented with 20 u/ml penicillin/streptomycin (Biological Industries, Beit Haemek, Israel), for a maximum of 3 weeks. The dissected mandibles were placed on the circular Millipore filters (1 mandible per filter), positioned on the mesh grid in the organ culture dish, and incubated for 2 h in Nuaire incubator (Nuaire Inc., MN, USA) (37 °C, 5% CO_2, 40% O_2), prior any treatment.

rHTuft$^+$ affi-gel beads preparation

Affi-gel agarose beads (Biorad) (75–100 μm) were separated by size under stereomicroscope, washed thoroughly in PBS, air dried, and suspended in rHTuft$^+$ (0.1–1 μg/μl) dissolved in 0.05 M acetic acid solution, at 37 °C for 30 min. Prior to use, beads were washed in PBS (neutralizing the acid), and the beads were then placed on target regions on the mandibular explants. Control beads were prepared in a similar manner by using BSA (0.5 μg/μl) or PBS.

The mandibular explants were then cultured in the Nuaire incubator for 48 h. The explants were then removed from the dish and fixed in 4% para-formaldehyde (PFA) (in PBS) overnight at 4 °C, dehydrated, paraffin embedded and sectioned (5 μm). All culture dishes contained triplicates of mandible explants.

Preparation of 250 μm thick frontal sections of mouse embryonic mandibles for DiI studies

E14.5 mandible explants were dissected out and sliced into 250 μm frontal slices, using the Mcllwain tissue chopper (Mickle Laboratory Engineer ing Co., Ltd), as described by Matalova et al. [16]. Briefly, the slices were separated and those showing tooth germs were placed on Milipore filters previously coated with Matrigel (BD Biosciences). Beads (soaked in 1 μg/μl rHTuft$^+$) were then placed at the incisor tooth germ region. Small amounts of DiI (Molecular probes, USA) were injected into the mesenchymal cells in the vicinity of the tuftelin beads using a mouth pipette. The slices were then covered with Matrigel (BD Biosciences). The position of the DiI labeled cells was recorded using a Leica dissecting microscope under superimposition of bright field and dark field to monitor the DiI at 0, 1 and 2 days. PBS beads served as control.

Results and discussion

Production of the full length recombinant human tuftelin protein (rHTuft$^+$)

Since tuftelin is expressed in relatively low amounts and is partially degraded during tissue development [17], production of relatively large amounts of purified recombinant protein is essential to study novel functions of tuftelin. Such expression of a recombinant tuftelin protein has not been reported so far.

We chose to produce the recombinant human tuftelin protein, using the eukaryotic Gateway-baculovirus expression system. The major potential advantages of this system includes: high expression level, post translation modification, and production of biologically active protein. The production of the recombinant protein in the eukaryotic baculovirus system also avoids the presence of *Escherichia coli* endotoxin, which could interfere with our *ex-vivo* studies.

The Gateway technology enabled us to clone the human tuftelin cDNA by recombination rather then the conventional restriction and ligation method. The latter proved to be challenging regarding the human tuftelin cDNA.

The full length recombinant human tuftelin protein (rHTuft$^+$) produced in eukaryotic *Sf*9 insect cells corresponded to the full length human tuftelin mRNA transcript coding for the 390 amino acid human tuftelin described by Mao et al. [2] (GenBank Accession

No. NP064512.1), and has an additional 39 amino acids at the N-terminus, comprising a hexahistidine tag, a spacer region and a potential protease cleavage site. Fig. 2 describes the N-terminal region of the recombinant human tuftelin construct and its corresponding amino acid sequence.

Production and purification of rHTuft$^+$

Expression of rHTuft$^+$ was confirmed by SDS–PAGE and Western blot analyses on batch scale production (for details see Materials and methods). Fig. 3 shows the presence of a major band of about 55 kDa in the infected cell extract that was not observed in the control (uninfected) cell extract. This band reacted with the anti-tuftelin (polyclonal) antibodies against synthetic tuftelin peptides (LF74 and LF75 mix), suggesting that this band represents the recombinant human tuftelin protein (Fig. 3). Some additional bands were also detected including a significant band at about 30 kDa. These bands probably represent degradation products, as discussed below.

One liter of 2.5×10^6 *Sf*9 cells/ml suspension was infected with the recombinant human tuftelin baculovirus at an MOI of 7, and harvested 72 h post infection. Purified rHTuft$^+$ was obtained by one-step Ni–NTA^{2+} metal affinity chromatography (for details see Material and methods). The expressed rHTuft$^+$ did not bind to the Nickel affinity column under native conditions, (data not shown). Purification was, therefore, performed under denaturating conditions (for details see Material and methods), revealing efficient binding and elution of rHTuft$^+$ using the immobilized metal affinity chromatography.

Elution fractions containing the purified protein, revealed the presence of the major band at around ~55 kDa, and some lower MW bands, suggesting the presence of rHTuft$^+$ and some of its degradation products (Fig. 4). The selected fractions were combined and extensively dialyzed against 50 mM acetic acid, to remove the Urea, the imidazole and possible remnants of the solutions used in the purification procedures. The dialyzed protein was aliquoted, lyophilized and stored at −20 °C. Resuspended lyophilized rHTuft$^+$ reacted both with the anti-tuftelin antibodies and anti-his tag (6× his) antibodies (Fig. 5), revealing the major rHuft$^+$ protein band and its degradation products, including the 30 kDa band. Excluding very few minor bands, all of the degradation products reacted with the anti-tuftelin antibodies. The majority of them, including the 30 kDa band, also reacted with the anti-histidine antibodies, suggesting they contained the N-terminus of rHTuft$^+$ (Fig. 5). The few bands that did not react with the anti-tuftelin or with the anti-histidine antibodies might be degradation products

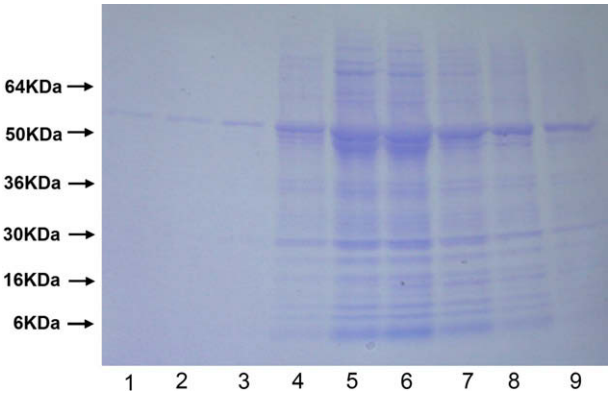

Fig. 4. Immobilized metal (Ni–NTA^{2+}) affinity chromatography of rHTuft$^+$ under denatured conditions. Coomassie blue stain of SDS–PAGE of selected fractions for further studies. Samples taken from elution fractions (lanes 1–9) of the Ni–NTA^{2+} column, using 500 mM imidazole.

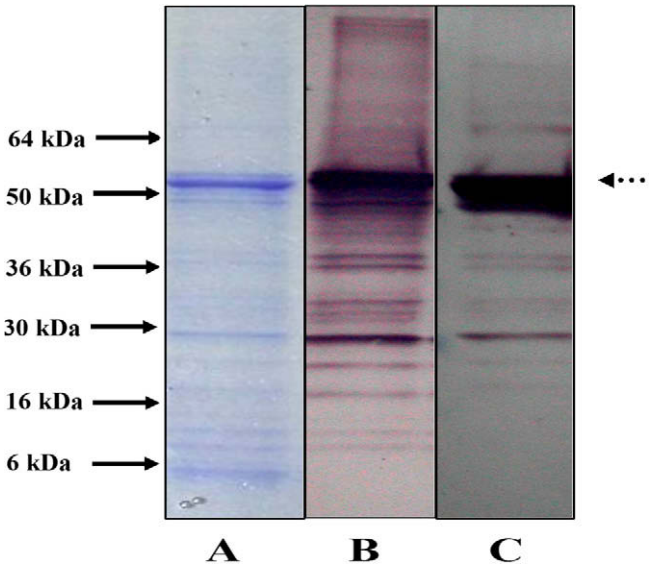

Fig. 5. Lyophilized rHTuft$^+$. (A) Coomassie blue stain of SDS–PAGE of samples from resuspended lyophilized aliquot of rHTuft$^+$, revealing the major band and its lower degradation products. (B) Western blots on the same SDS–PAGE with LF 74, 75 mix anti tuftelin antibodies. (C) Western blots on the same SDS–PAGE with anti-His tag antibodies.

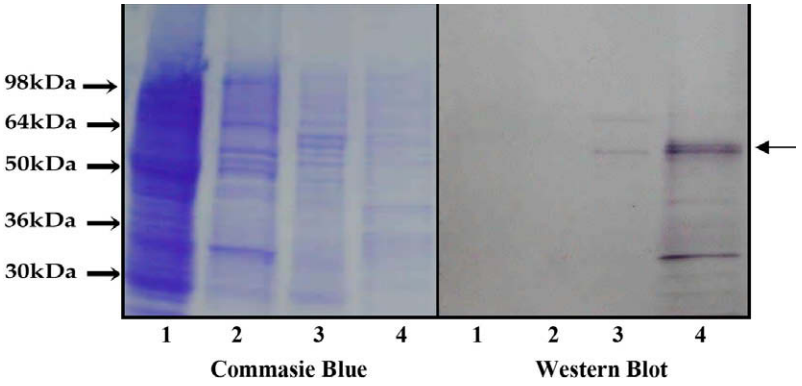

Fig. 3. Batch purification of rHtuft$^+$ under denatured conditions. 1—Crude extract of uninfected *Sf9* cells. 2—Purification of the non infected crude extract on Ni–NTA^{2+} beads. 3—Crude extract of *Sf9* cells infected with the recombinant human tuftelin bacmid. 4—Purification of the infected crude extract on Ni–NTA^{2+} beads. Western blot was carried out using tuftelin antibodies LF 74, 75 mix. A band of approximately 55 kDa was observed in the infected samples: crude extract and after purification, along with some degradation products. No reaction was observed in the control (uninfected) samples.

containing the C-terminal region of rHTuft$^+$, which the LF74, and LF75 antibodies do not recognize since they were produced against synthetic epitops from the middle and N-terminal regions of the tuftelin protein, respectively [1]. The possibility that these bands originate from the *Sf9* cells or the baculovirus cannot be ruled out. However, our previous experience with similar infection of *Sf9* cells with another enamel protein did not yield these bands under similar expression and identical purification conditions [18].

Due to low cleavage efficiency of the proteases, experienced in our laboratory [18] and by others, the his-tag was not removed. Several studies showed that the his-tag arm did not affect recombinant protein activity [18–21].

Lyophilized aliquots of rHTuft$^+$ were quantified using the Bradford protein assay, quantifying the total protein content of the lyophilized material. The yield was 5–8 mg protein/l culture (including full length rHTuft$^+$ protein and its degradation products).

Characterization of rHTuft$^+$ using ESI-TOF/MS peptide mapping and MS/MS sequencing

Peptide mapping and MS/MS sequencing of the major rHTuft$^+$ band (Fig. 5, dashed arrow) confirmed the production of the full length recombinant human tuftelin; Seventeen rHTuft$^+$ peptides were identified by MS/MS sequencing (Table 1). The identified peptides cover N-terminal, middle and C-terminal regions of the full length human tuftelin protein (Table 1 and Fig. 6), with an overall 57% coverage. Score rank for the sequenced peptides provides very high statistical confirmation that the sequenced recombinant protein—rHTuft$^+$, is actually the full length human tuftelin, with a certainty of $1{:}10^{76}$.

The rHTuft$^+$ peptide sequences were determined by analysis of their fragmentation masses using ESI-TOF/MS, followed by tandem MS/MS scans. The observed masses of the peptides matched (within <1 Da discrepancies) the calculated masses of expected peptides, resulting from cleavage of rHTuft$^+$ with trypsin, in all but one peptide (peptide 17, Table 1). The observed mass of peptide 17 was ~79.95 Da higher than the expected calculated mass, matching the expected mass of a phosphorylated form of the peptide, corresponding for one phosphorylation site.

Identification of phosphorylation in the recombinant full length human tuftelin protein

As described above, post-translation modifications were examined by searching ESI-TOF/MS data for deviations of the

Table 1
Identified human tuftelin peptide masses and sequences detected by ESI/MS and MS/MS.

Peptide no.	Start–End	Observed mass (MS^{ESI})	Mr (calc)	Delta	Miss	Sequence	Ions score
1	6–27	2494.2011	2494.1911	0.0100	0	R.NWCTLVDVHPEDQAAGSVDILR.L	45
2	28–45	1994.0363	1994.0320	0.0043	0	R.LTLQGELTGDELEHIAQK.A	89
3	50–79	3220.5153	3220.5095	0.0058	0	K.TYAMVSSHSAGHSLASELVESHDGHEEIIK.V	10
4	101–110	1221.6005	1221.5989	0.0016	0	K.SEVQYIQEAR.N	46
5	116–123	946.5093	946.5083	0.0010	1	K.LREDISSK.L	7
6	175–182	886.5143	886.5124	0.0020	0	K.TVQDLLAK.L	16
7	189–203	1775.8340	1775.8261	0.0079	0	R.QHQSDCVAFEVTLSR.Y	15
8	207–217	1243.6063	1243.6156	−0.0094	0	R.EAEQSNVALQR.E	60
9	226–234	1029.5111	1029.5091	0.0021	0	K.EAEVGELQR.R	38
10	236–249	1552.8310	1552.8283	0.0027	0	R.LLGMETEHQALLAK.V	64
11	252–261	1143.5786	1143.5771	0.0015	0	R.EGEVALEELR.S	49
12	288–296	1120.5722	1120.5699	0.0023	0	K.IHHLDDMLK.S	38
13	304–313	1217.6093	1217.6074	0.0019	0	R.QMIEQLQNSK.A	43
14	320–327	916.4876	916.4865	0.0011	0	K.DATIQELK.E	16
15	330–343	1702.8026	1702.7984	0.0042	0	K.IAYLEAENLEMHDR.M	69
16	351–362	1344.6588	1344.6535	0.0053	0	K.QISHGNFSTQAR.A	10
17	373–386	1625.8598	1625.8728	−0.0130	0	R.ISKPPSPKPMPVIR.V Phospho (ST)	58

Trypsin cleaves very specifically at R–X and K–X bonds. If X = P, no cleavage occurs.
Mr(calc)—Peptide masses were calculated using the monoisotopic masses of the amino acid residues.

Fig. 6. ESI-TOF/MS and MS/MS identified peptides of Trypsin cleaved rHTuft$^+$. The human tuftelin sequence is from GeneBank Accession No. NP064512.1. The identified peptides are presented by bold letters on the human tuftelin sequence. The numbers in bold uppercase represent peptide numbers as they appear in Table 1.

measured mass from the calculated peptide masses. One unambiguous indication of phosphorylation was observed in peptide 17, which presented a mass predicted for the existence of one phosphorylation. No unphosphorylated form of this peptide was detected. MS/MS sequencing analysis of peptide 17 revealed a phosphorylated serine at position 378 (Fig. 7). This site was predicted to undergo phosphorylation by PKC (protein kinase C) with the highest score in the full length tuftelin protein (0.998), by several computer analyses programs such as: http://www.expasy.org, http://www. ncbi.nkm.nih.gov and www.cbs.dtu.dk/services/NetPhos. Several additional phosphorylation sites, with lower probability scores, were also predicted, but were not detected. Phosphorylation by mass spectrometry is often challenging because of the low abundance, and low stoichiometry of phosphopeptides (as compared to non-phosphorylated peptides) and other technical issues related to protein purification and mass spectrometry procedures [22].

Functional characterization of the recombinant full length human tuftelin protein (rHTuft^{+}) using the Embryonic Mandibular Explant Culture (ex-vivo system)

To investigate the biological functionality of rHTuft^{+}, the mandibular explant culture system was used. This is a well established model for studying signaling interactions between tooth epithelium and mesenchyme [23,24,7,25]. Tuftelin was first characterized in the developing tooth, and was suggested to have a role in the development and mineralization of the forming tooth bud (odontogenesis), a process which originates in cellular interactions between mesenchymal and epithelial tissues [9,11,26] (for more details, see Introduction). Tuftelin mRNA was identified in the tooth bud already in mouse E13, and tuftelin protein expression was detected in E17 [11]. Thus, on E14.5 and the following 48 h (the explant culture time), tuftelin probably has a biological function in the tooth germ region.

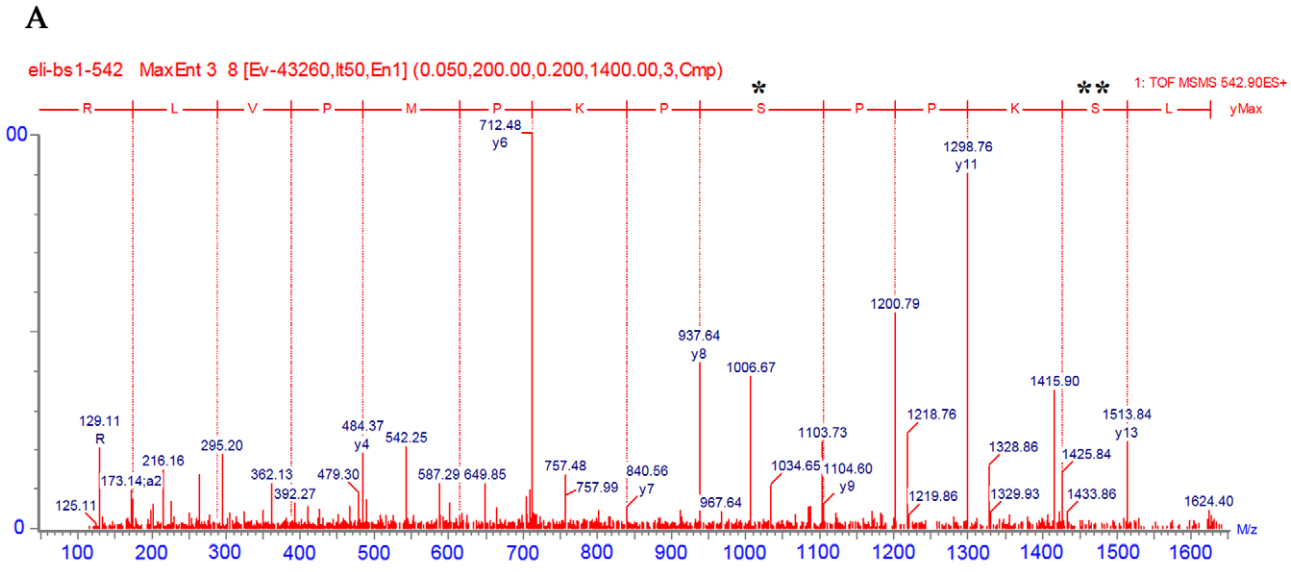

Fig. 7. Detection of phosphorylation using MS/MS sequencing. (A) MS/MS sequencing of peptide 17, which had an observed mass of ~80 Da over the calculated mass using ESI-TOF/MS (Table 1), revealed that one serine residue, at position 378 (*), had an extra ~80 Da over the serine expected mass. Further demonstration of the phosphorylation was achieved by comparing the Serine 378 mass (*) to that of serine 374 (**) (mass is indicated by the major interpeak width—dotted lines). (B) Position of the phosphorylated serine 378 (bold) in the human tuftelin protein sequence.

In the mouse embryonic stage of E14.5, the mandible incisor buds can be visualized, enabling placement of affi-gel beads containing rHTuft$^+$ as close as possible to the buds (using stereoscopic high magnification), inducing a local high concentration of tuftelin protein.

Application of affi-gel rHTuft$^+$ beads

The rHTuft$^+$ beads, introduced to the epithelium surrounding the incisor tooth bud, induced a major morphological effect on the surrounding tissue; the epithelial cells seemed to be recruited from the outer layer of the oral epithelium to surround the rHTuft$^+$ bead. These cells also underwent dramatic morphological changes including; specific orientation, enlarged cytoplasm with eosinophilic appearance or perhaps even extracellular matrix secretion (Fig. 8A and B). When control (BSA) beads were introduced at a similar region no morphological effect was demonstrated except for some spatial interference both in the epithelium and in the

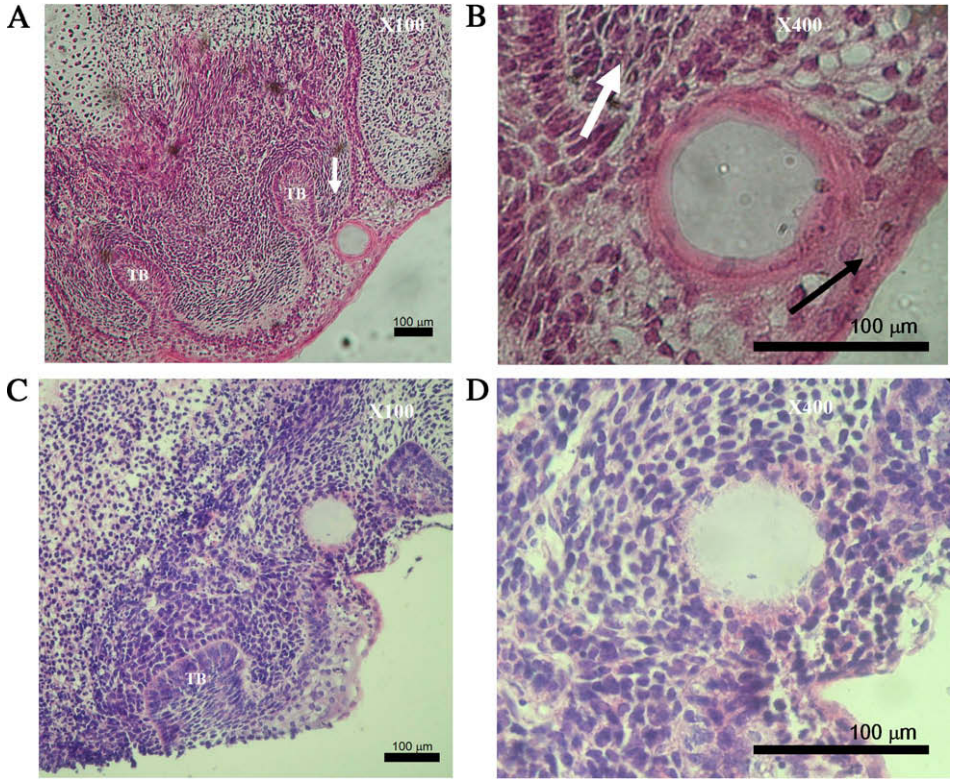

Fig. 8. Effect of rHTuft$^+$ on cells at the tooth bud region. (A and B) The rHTuft$^+$ bead (0.1 µg/µl) was introduced adjacent to the incisor tooth bud. Epithelial cells, apparently recruited from the oral epithelium outer layer, surrounding the bead can be viewed. Black arrow in (B) indicates the recruitment of oral epithelial cells. White arrows indicate mesenchymal cells. (C and D) BSA beads introduced at similar region partially embedded in the epithelium (lower part) and partially in the mesenchyme (upper part of bead). In the control (C and D), no reaction, except for spatial interference, can be noticed neither at the epithelium nor at the mesenchyme. TB—tooth bud.

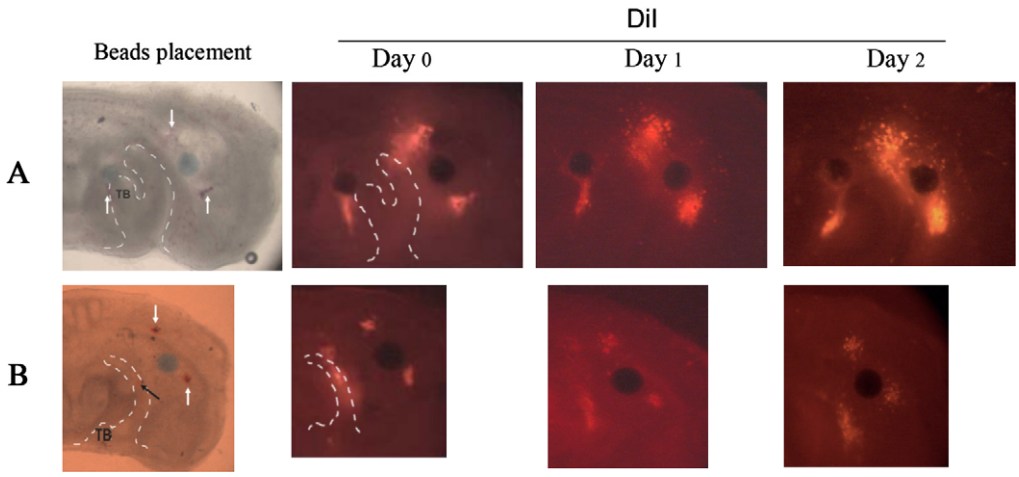

Fig. 9. Recruitment of DiI labeled cells towards the rHTuft$^+$ beads. (A) rHTuft$^+$ beads (experimental), and (B) PBS beads (control), were placed on 250 µm frontal slices of E14.5 mouse mandibles, at the incisor region. Mesenchymal cells (white arrows) and some epithelial cells (black arrow only in the control) in the vicinity of the beads were labeled with DiI. The mandibular slices were photographed 0, 1 and 2 days after DiI application. In the experimental culture (A) progressive mesenchymal cell migration towards the beads can be seen from day 0 to day 2. No such cell migration towards the control PBS bead was observed (B). White dotted line—outline of the vestibular lamina and the tooth bud. TB—tooth bud.

mesenchyme (Fig. 8C and D). These results were repeated in four independent experiments, each performed in triplicates.

Application of affi-gel rHTuft$^+$ beads accompanied by DiI labeling

To evaluate possible recruitment of mesenchymal cells by rHTuft$^+$, affi-gel beads containing rHTuftt$^+$ were placed on a 250 μm slice of mouse E14.5 mandible, in the incisor tooth bud region, and the surrounding mesenchymal cells were labeled with DiI. The rHTuftt$^+$ beads induced migration (recruitment) of DiI labeled mesenchymal cells towards the beads. This was observed already after day 1 (24 h), and further progressed after day 2 (48 h), partially covering the beads (Fig. 9A), suggesting that tuftelin is involved in recruiting mesenchymal cells in mouse embryonic mandibular explants. No cell migration towards the bead was observed using control (PBS) beads (Fig. 9B).

These results indicate that the recombinant human tuftelin protein—rHTuft$^+$, is biologically functional.

Conclusion

This article describes the production, purification and characterization of a biologically active recombinant full length human tuftelin protein (rHTuft$^+$), expressed in the eukaryotic baculovirus expression system. The employed histidine-tagged system enabled high purification through single-step isolation. The yield of purified protein was up to 8 mg per liter culture of full length human tuftelin protein with some degradation products. The substantial, reproducible yield of recombinant human tuftelin was obtained by optimization of infection and purification conditions. Using ESI mass spectrophotometry the recombinant protein was identified as the full length human tuftelin. Further characterization of post translation modifications, identified phosphorylation of Serine 378, as was expected from computerized prediction analyses of possible post translation modifications.

Application of the rHTuft$^+$ protein, using affi-gel beads, onto mouse mandibular explants proved that the rHTuft$^+$ is biologically active, inducing major changes in the surrounding epithelial cells, including morphology, orientation, spatial organization, and possible recruitment. DiI labeling of adjacent mesenchymal cells demonstrated cell migration (recruitment) toward the rHTuft$^+$ beads. The ability of tuftelin to bring about cell recruitment might be of great importance in organogenesis during embryonic mandible and tooth development.

This is the first report of the production of a biologically active full length recombinant tuftelin protein—rHTuft$^+$. The availability of substantial amounts of purified, recombinant, full length human tuftelin, expressed in eukaryotic cells, provides a valuable and reproducible source of biologically active protein. However, it is important to note that some degradation products were always present (Figs. 3–5), and might also contribute to the biological effects observed in this study.

rHTuft$^+$ is currently being used to study the underlying mechanisms associated with the function(s) of the human tuftelin during mouse cranio-facial development.

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