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Trappin ovine molecule (TOM), the ovine ortholog of elafin, is an acute phase reactant in the lung

Thomas I Brown[*#], Rohit Mistry[+], David D Collie[#], Steven Tate[#] and Jean-Michel Sallenave[*].

[*]Rayne Laboratory, Respiratory Medicine Unit, MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK.

[#]Wellcome Trust Centre for Research in Comparative Respiratory Medicine, The University of Edinburgh, Easter Bush Veterinary Centre, Roslin, Midlothian, UK.

[+]Molecular Genetics, Anderson College, Institute of Biomedical Sciences, University of Glasgow, Glasgow, UK.

Correspondence to Dr. J-M. Sallenave, Rayne Laboratory, Respiratory Medicine Unit, MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh, EH8 9AG, Scotland, UK.

e-mail jsallena@staffmail.ed.ac.uk; phone 00-44-1-31-650-6949; fax 00-44-1-31-650-4384

Running title: The ovine ortholog of elafin

Abstract

As large animal models continue to play an important role in translating lung-directed therapeutic strategies from laboratory animals to man, there is an increasing interest in the analysis of endogenous regulators of inflammation at both a genomic and a therapeutic level. To this end we have sought to characterise the ovine ortholog of elafin, an important regulator of inflammation in man. We have isolated both the elafin cDNA and gene which have a similar structure to other species orthologs. Interestingly, we have isolated two alleles for ovine elafin, which contain a very high number of transglutamination repeats, thought to be important in binding elafin to the interstitium. The mainly mucosal mRNA distribution for ovine elafin suggests that ovine elafin may, like its human ortholog, have functions in innate immunity. This is supported by analysis of elafin and the related protein SLPI in ovine bronchoalveolar fluid in response to locally administered lipopolysaccharide and confirmation of them acting as 'alarm' antiproteases. We have also cloned the ovine elafin cDNA into an adenoviral vector and have demonstrated correct processing of the secreted protein as well as biological activity. Over-expression of ovine elafin in a lung-derived epithelial cell line has a protective effect against the enzymes human neutrophil and porcine pancreatic elastase. The identification of the ovine elafin gene and its translated protein are important in developing practical strategies aimed at regulating inflammation in the large mammalian lung.

Keywords : anti-protease, elastase, sheep, trappin.

Abbreviations

SLPI : secretory leukoprotease inhibitor; BAC : bacterial artificial chromosome; RACE : rapid amplification of cDNA ends; WAP : whey acidic protein; REST : rapidly evolving seminal vesicle transcribed; TOM : trappin ovine molecule; HNE : human neutrophil elastase; PPE : porcine pancreatic elastase; EIC : elastase inhibitory capacity; PAGE : polyacrylamide gel electrophoresis; UTR : untranslated region; LPS : lipopolysaccharide.

Introduction

The trappins (acronym for **TR**ansglutaminase substrate and W**AP** (whey acidic protein) domain containing **P**rote**IN**, (27)) are a group of proteins characterized by the presence of a N-terminal transglutaminase substrate ("cementoin") domain and a C-disulphide core domain, also called WAP domain, which contains, in some members, an antiproteinase active site. Current knowledge suggests that the two domains may have evolved from exon shuffling and gene duplication between rapidly evolving seminal vesicle transcribed (REST) genes and the WAP domains of ancestral genes (3). Indeed, in humans, co-localisation of the human trappin-2 gene (also known as elafin/elastase-specific-inhibitor/SKALP) and the secretory leukocyte protease inhibitor (SLPI) gene (which contains two WAP domains) in the same region of chromosome 20 is in agreement with this hypothesis (8, 12). Our group is particularly interested in the study of elafin which was identified as an anti-elastase inhibitor in the early 90s (19, 21, 26, 35). The closely related protein SLPI also has important functions as an inhibitor of neutrophil elastase (20 and references therein) and both molecules have been found to be present in man at mucosal sites and also have been shown to be synthesized by epithelial cells and alveolar macrophages. In addition, it has recently been demonstrated that both elafin and SLPI have antimicrobial functions and may be able to prime the innate immune system (20).

More than fifteen trappins and many more WAP-containing proteins have been identified to date, throughout the animal kingdom. Although the sequence in the WAP domain predicts that a number of trappin species orthologs are anti-elastase molecules, this has only formally been demonstrated for human and porcine elafin, (19, 21, 26, 32, 35).

Because of our interest in the modulation of lung inflammation in ovine models (2), and the known modulatory activity of antiproteases (20) we set out to isolate the ovine elafin cDNA and gene ortholog and to study its properties.

We identified in this study two allelic forms of the ovine elafin gene (named TOM-1 and TOM-2 for "Trappin-Ovine Molecule" 1 & 2) and analysed their mRNA distribution in a variety of tissues. Furthermore, we have demonstrated that secreted ovine elafin can protect a lung derived epithelial cell line from proteolytic damage due to both human neutrophil elastase and porcine pancreatic elastase. The distribution, anti-elastase activities and kinetics of expression *in-vivo* of ovine elafin predict that it may also have mucosal protective functions in inflammation. This

should prove a valuable tool in developing therapeutic strategies in a large animal model of lung inflammation, currently being developed in our laboratories.

Experimental Procedures

Molecular biology reagents

Primers were synthesised and supplied by MWG-Biotech AG (Milton Keynes, UK). Primer design for 5'RACE (GSP1) was based on preliminary ovine elafin sequence (R. Mistry, unpublished data). Subsequent primers were based on the newly determined ovine elafin sequence. Elafin gene-specific reverse primer (GSP1) 5'- CAT GGC GCA CCG GAT CA - 3' ; Elafin gene-specific forward primer (GSP2) 5'- ATG AAG ACC AGA AGC TTC TTG GTC C - 3'; Full length reverse primer (r-el) 5'- CTG GGG ATC CAT ACA GGT CTT – 3'; Bgl II forward elafin primer (Bgl-f-el) 5'- GCC GGA GAT CT**G ACA AC**A TGA AGA CCA GAA GCT TCT TGG TC – 3' (Bgl II site underlined; Kozak sequence in bold); Sac I elafin reverse primer (Sac-r-el) 5'- TAC GTG AGC TCT CAT CAC TGG GGA TCC ATA CAG GTC TT – 3' (Sac I site underlined); Forward BAC primer (fBAC) 5'- TGA AAA GGG TCC AAT CAA CG – 3'; Reverse BAC primer (rBAC) 5'- GAG CCA CGC TTA GTG AGG AG – 3'.

Taq DNA polymerase, magnesium chloride 25mM, DNA polymerase buffer, dNTPs, oligo (dT) primer, EcoR1, Buffer H for EcoR1, BglII, Buffer D for BglII, Sac I, Buffer J for SacI, M-MLV reverse transcriptase, 5x M-MLV RT buffer were supplied by Promega UK (Southampton, UK).

Shuttle vector pDC516 was obtained from Microbix Biosystems Inc. (Toronto, Canada). Shuttle vector pIRES-GFP#3 was kindly donated by Dr. Mary Hitt (Dept. of Pathology and Molecular Medicine, McMaster University, Hamilton, Ontario, Canada).

RNA preparation and Rapid amplification of cDNA ends (RACE)

Ovine tissues were obtained locally from a selection of 5 month old crossbred lambs (Moredun Institute, Edinburgh, UK). The tissue samples obtained from sheep organs were treated as follows. Samples of each tissue were removed at autopsy and frozen at –80^{o}C. Approximately one gram of each of these was then homogenised and the RNA extracted using Trizol (Invitrogen, Paisley, UK). RNA quality was assessed by running 10µl on a 1% agarose gel. Quantification of RNA in samples was by dual wavelength spectrophotometry.

RACE-PCR for ovine elafin was performed using ovine tracheal mucosal RNA. Firstly in order to obtain 5' information, we used a primer specific for ovine elafin, GSP1 (derived from partial sequence already obtained from

preliminary experiments, not shown), and the 5' forward Generace Primer. This was followed by a nested PCR using GSP1 and nested 5' forward Generace Primer. Cycling parameters were as follows: initial incubation of 92°C for 5 minutes, followed by 92°C 1 minute, 55°C 1 minute, and 72°C for 1 minute (30 cycles followed by a final amplification step of 72°C for 5 minutes). Products were run out on a 1% agarose gel. These conditions were used for both the original and nested PCRs.

To perform the 3'RACE a slightly different strategy was used. Reverse transcription of the original RNA sample was performed using the Generace oligo-dT supplied in the Generacer™ kit and M-MLV RT (Promega). PCR was then performed using the forward primer GSP2 and the reverse Generace primer in order to obtain the full-length cDNA sequence. GSP2 was designed from the sequence derived from the 5'RACE procedure. PCR conditions were similar to those above except for a magnesium chloride concentration of 2.2mM. This was followed by a nested PCR using GSP2 and nested reverse Generace primer.

DNA Preparation and Sequence Analysis

Genomic DNA was extracted from lung and liver samples of the above animals by proteinase K digestion in NTES (50mM Tris, 50mM EDTA, 100mM NaCl, 1% SDS, pH 8), followed by phenol/chloroform extraction and washing with 100% isopropanol followed by 70% ethanol. The DNA was re-suspended in water and kept at -20 °C.

DNA Sequence analysis was done both in house and by MWG-Biotech AG, and the sequence data analysed with the on-line computer software packages NCBI Open Reading frame finder (NCBI), NCBI Blast (NCBI) and BCM Multiple Sequence Alignment tools (BCM).

Extraction of PCR products and cloning reactions

All PCR products were run on 1% agarose gels and bands of interest were cut out and purified using the QIAQuick Gel Extraction Kit (Qiagen, Crawley, W. Sussex).

Cloning of the extracted RACE cDNA fragments was achieved by using the TOPO TA Cloning® Kit for Sequencing included with the GeneRacer™ kit. The cDNAs were ligated into pCR®4-TOPO® plasmid followed by transformation into TOP10 *E.coli* cells (Invitrogen). Plasmid DNA was purified with the Promega SV Miniprep system.

Bacterial Artificial Chromosome (BAC) library screening and subcloning

An ovine BAC library was screened using primers specific for elafin by Dr. F. Piumi at Institut National de la Recherche Agronomique (INRA), Laboratoire mixte INRA-CEA de Radiobiologie et d'Etude du Genome (LREG), Jouy-en-Josas, France. One positive clone was isolated and DNA prepared with Qiagen Plasmid Maxi Kit (Qiagen, Crawley, UK). 1µg of the BAC positive clone was digested with EcoR1, run out on a 1% agarose gel, and blotted onto GeneScreen^{TM} membranes (PerkinElmer Life Sciences (UK) Ltd, Cambridge, UK). This was probed for one hour with [α-^{32}P]dCTP labelled full length cDNA coding for ovine elafin, using Express-Hyb^{TM} (Clontech, Palo Alto, USA) following the manufacturers protocol. Positive bands were identified with a Storm Phosphoimager system (Amersham Biosciences UK Ltd., Little Chalfont, UK) and further subcloned into plasmid PDC516 for sequencing using various internal primers.

Tissue screening of ovine elafin by Northern Blot and RT-PCR analysis

Twenty µg of each tissue RNA sample were denatured in 50% formamide, 16% formaldehyde, 20mM MOPS, pH 7.0, 5mM sodium acetate, and 1mM EDTA, for 15 minutes at 65°C, electrophoresed in a 1% agarose gel containing 2.2M formaldehyde and transferred to GeneScreen^{TM} membrane. The membrane was hybridised with [α-^{32}P]dCTP labelled full length cDNA coding for ovine elafin in ExpressHyb^{TM} following the manufacturer's recommendations. The membrane was then visualised on a Storm^{TM} Phosphoimager system.

One µg of each tissue RNA sample was used in a reverse transcriptase reaction using M-MLV RT (Promega). Product from this reaction was split into two PCRs, using either forward and reverse ß-actin primers or the full length elafin primers GSP-2 and r-el. Conditions were as for the RACE elafin PCR except for there being 35 cycles for elafin and 28 cycles for the ß-actin.

Comparison of ovine elafin products synthesised by PCR from positive BAC clone, genomic DNA and RT-PCR synthesised cDNA

In order to compare the elafin cDNA, identified by the RACE procedure, with the gene sequences, PCR was performed using as template purified elafin BAC DNA and ovine liver DNA alongside cDNA synthesised from ovine skin and pIRES-GFP-ovine elafin, using the non intron-spanning primers fBAC and rBAC. PCR parameters

were as for the RACE elafin PCR. Products from the genomic DNA PCR were ligated into pCR^{R}4-TOPO^R plasmid which was transformed into TOP10 *E.coli* cells. Plasmid was extracted with Promega SV Miniprep system and elafin sequences were sequenced.

An additional PCR was performed on ovine genomic DNA using the primers GSP2 and r-el. Products from this PCR were also cloned into pCR^{R}4-TOPO^R as above and sequenced.

Phylogenetic Tree Analysis of WAP domains of the elafin and SLPI family members

To investigate the phylogenetic relationships of the ovine forms of elafin we constructed a phylogenetic tree by the neighbour-joining method (18) using data from multiple alignment of the amino acid sequences of the whey acidic protein (WAP) domains of members of the trappin family, the known SLPI sequences (human, mouse, rat, pig and sheep), the murine elafin-like proteins 1 and 2 (5), and the human vasopressin molecule which contains a rudimentary WAP motif and can be assumed to be evolutionarily related to the elafin and SLPI families of proteins.

LPS-induced up-regulation of ovine elafin and SLPI in bronchoalveolar lavage fluid

Bronchoalveolar lavage fluid was collected to assess the regulation of ovine elafin and SLPI following local lung challenge with LPS. Briefly, twelve cross-bred sheep (10 female and 2 male) were anaesthetised with 20mg/kg intravenous thipoentone (Intraval sodium, Merial Animal Health Ltd., Harlow, Essex, UK), intubated and maintained on 2-3% halothane in oxygen and nitrous oxide. 1mg lipopolysaccharide (LPS) from E. coli O26:B6 diluted in 5ml distilled water was instilled via bronchoscope into random segments. Bronchoalveolar lavages from these sheep were collected a week prior to the experiment and 6, 24 and 168 hours after the administration of LPS. The lavages were spun at 400g for 7 minutes and 200µl of the resultant supernatant was used in a dot blot onto GeneScreen^{TM} membrane and blocked with 5% milk powder in TPBS. The membrane was then probed with either polyclonal anti-human SLPI antibody (24) or monoclonal Trab-2O anti-elafin antibody (Hycult, Nl). Resultant films after exposure to X-omat radiograph-quality film were scanned and densitometry measurements taken using a UVP white light Transilluminator (UVP, Inc. Upland, CA USA). Control membranes probed with secondary antibody alone showed no detectable protein (not shown).

Construction of Adenoviral vector expressing ovine elafin

Full length ovine elafin cDNA in pCR^{R}4-TOPO^R (see above) was used as a template for two consecutive PCRs with firstly Bgl-f-el and FLAG-r-el, followed by Bgl-f-el and Sac-r-FLAG-el (to yield BglII-elafin-FLAG-SacI). PCR conditions were as for the RACE PCR for elafin. This product was ligated into shuttle vector pDC516 (which contains the murine cytomegalovirus (MCMV) promoter) using standard techniques.

This plasmid was used to create a replication-deficient adenovirus (Ad) by cotransfection of 293 cells with pBHGfrt(del)E1,3FLP (a kind gift from Dr. M. Hitt, McMaster University). Ad-ovine elafin was obtained through homologous recombination between the two plasmids using the FLP recombinase/frt mechanism (15).

The presence of the correctly inserted ovine elafin cDNA into the viral genome was confirmed by Hind III digestion followed by Southern blot analysis (not shown). Infection of A549 cells with this virus confirmed correct processing of the ovine elafin protein (not shown).

Adenovirus-mediated protection of A549 cells against proteolytic enzyme damage

A549 cells (11) were plated out in 48 wells plates at 50,000 per well and kept in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% foetal calf serum (FCS), penicillin G (final concentration 100U/ml), streptomycin sulphate (final concentration 100µg/ml) and L-glutamine (final concentration 2µM) overnight at 37°. The following day the cells were washed with fresh medium and incubated for 45 minutes with Ad-ovine elafin, Ad-human elafin (25), Ad-murine SLPI (6), or Ad-murine Eotaxin (6) in fresh medium. The cells were then washed with serum free DMEM and medium replaced with DMEM containing 2% USG serum substitute (Gibco, BRL). After 72hrs the medium was removed, the cells washed twice with PBS and medium replaced with fresh DMEM with no serum or USG and containing 1µg/ml human neutrophil elastase (HNE) (Elastin Products Company, Inc., Missouri, USA) or porcine pancreatic elastase (PPE) (Sigma-Aldrich Co. Ltd., Dorset, UK). Plates were incubated overnight and photomicrographs taken.

To quantify elastase-mediated injury, medium containing detached cells was spun at 13,000 rpm for 5 minutes. The cell pellet was then washed in sterile PBS twice and re-suspended in distilled water, followed by two successive freeze/thaw rounds. The suspension was further centrifuged (13,000rpm, 5 minutes) and the supernatant was assessed for protein content (indirect read-out of cell detachment) using the bicinchoninic acid method (Pierce) using purified albumin (Pierce) as standards.

Residual HNE and PPE activity in the supernatants was assayed in 96 well microtitre plates. 10µl of supernatant diluted with 40µl assay buffer (50mM Tris, 0.1% Triton, 0.5M NaCl, pH 8.0) was incubated at 37°C with the chromogenic substrates N-methoxysuccinyl-ala-ala-pro-val-p-nitroanilide (for HNE activity) or N-succinyl-ala-ala-ala-p-nitroanilide (for PPE activity) diluted to 1µg/ml in assay buffer. Both chromogenic substrates were supplied by Sigma-Aldrich Co. Ltd, Dorset, UK. Enzyme activity was shown by a change in absorbance measured at 405nm over 90 minutes.

Results

Isolation and sequence analysis of ovine elafin cDNA

Using ovine tracheal mucosal RNA, and PCR primers annealing to a portion of the ovine elafin sequence already available (Mistry et al, unpublished) 5' RACE PCR produced a product of 612bps which was cloned into pCR^{R}4-TOPO^R plasmid for sequencing. This PCR product contained the sequence corresponding to the Generacer^R oligo ligated to the 5' terminus of the mRNA followed by 18bps of 5'UTR and the ovine elafin cDNA sequence through to the priming site for the gene-specific primer. This information allowed 3' RACE to proceed. This yielded a product of 887bps which was also cloned into pCR^{R}4-TOPO^R plasmid for sequencing and was shown to contain sequence 3' of the stop codon corresponding to 3' UTR through to the polyadenylation signal. This product terminated with the sequence corresponding to the Generacer^R oligo dT sequence used in the reverse transcription step of the RACE procedure. Altogether, the 5' and 3' RACE procedure allowed us to identify an open reading frame for a protein of 220 amino acids which showed homology with human, simian, porcine and bovine elafin (see fig. 1) and will be referred to as ovine elafin. The protein consists of a putative hydrophobic signal sequence of 23 amino acids at the N terminus followed by a distinct region of hexapeptide repeats of consensus sequence PVKGQD which corresponds to a potential transglutaminase substrate domain (27), and a C terminal portion containing a presumed disulphide core region with eight cysteine residues in positions corresponding to those in the human elafin protein WAP domain.

Sequence analysis of the ovine elafin gene

Screening of an ovine BAC library was performed at the Institut National de la Recherche Agronomique (INRA) using probes generated by PCR (see materials and methods). This screening identified one clone for elafin. Southern blot analysis using 1μg of BAC clone DNA allowed identification of a positive EcoR1 band of approximately 10kb from the elafin clone. This band was purified and subcloned into PDC516 for sequencing with sequential internal primers through to the polyadenylation signal at the 3' end and proximally through towards the promoter region. This approach only enabled us to obtain full 3' sequence information (up to the polyA signal) and no 5' information. Indeed, because the BAC library was generated by partial Hind III digestion, and since the elafin 5' promoter region contains a Hind III site, we were unable to obtain 5' information using this methodology and only obtained sequence

from this restriction site forwards. The elafin promoter region sequence was obtained by using PCR (with ovine genomic DNA as template obtained locally – see Materials and Methods) with a primer designed to anneal to the 5' end of the human elafin promoter along with a reverse primer annealing to a portion of the first intron of the ovine elafin gene. This PCR product was cloned into pCR^{R}4-TOPO^R and sequenced. This allowed us to show that the elafin gene spans approximately 2.5kb and is split into 3 exons and 2 introns (Fig. 2, Genbank accession number AY344541).

Tissue distribution of ovine elafin

Total RNA was extracted from multiple ovine tissues and analysed by Northern blot. A 750bp ovine elafin RNA species was detected in tongue, trachea, small intestine, large intestine (strongest signal) and skin (see fig. 3A).

RT-PCR reaction using primers GSP2 and r-el for ovine elafin was performed using the same RNA samples (see fig. 3B). Consistent with the Northern blot analysis, we found RNA to be present in tongue, trachea, small intestine, large intestine and skin. Interestingly, products of two different lengths were obtained, approximately 660bp (the expected size as predicted from the gene sequence) and 800 bp.

Genomic PCR for ovine elafin using a combination of non intron-spanning and intron-spanning primers

On account of isolating two different ovine elafin mRNA products by RT-PCR (see fig. 3B), although only one gene was isolated from the BAC library screening, we performed additional PCR reactions using non-intron spanning primers and a variety of templates to determine whether these RNA products are splice variants from a single gene or products of different/allelic genes. Fig.4A. shows the PCR products when fBAC and rBAC primers are used with various templates : genomic ovine DNA (obtained locally), cDNA synthesised by RT-PCR from ovine skin RNA (shown to be positive for elafin expression – see fig. 3), DNA extracted from the elafin positive BAC clone, and finally DNA extracted from the expression plasmid pIRES-GFP#3 containing the cDNA for ovine elafin isolated by RACE-PCR (see above). Two bands were seen with the first two templates (approximately 400bps and 525bps). The low molecular weight band corresponded to the ovine elafin cDNA product isolated by RACE-PCR (coding for a 220 amino acid protein) and present in the pIRES-GFP#3 plasmid containing the ovine elafin cDNA. The higher molecular weight band was of unknown origin. The two products obtained with the genomic DNA template were isolated and cloned into pCR^{R}4-TOPO^R and sequenced. As predicted, the low molecular weight product sequence

matched exactly the sequence from the ovine elafin original sequence while the high molecular weight product sequence showed an increase in number in PVKGQD repeats (26 instead of 19). The existence of two different genomic forms was further confirmed using a different set of PCR primers (the intron-spanning primers GSP2 and r-el), yielding 2 distinct products of approximately 1500 and 1600bps (not shown). These products were cloned into pCR^{R}4-TOPO^R and sequenced and confirmed the presence of a second, heavier form of the ovine elafin otherwise identical to the original gene isolated by BAC library screening. The original form of the gene described will here after be referred to as Trappin Ovine Molecule-1 (TOM-1) and the higher molecular weight form, coding for the longer form of ovine elafin of 262 amino acids, will be referred to as TOM-2. Alignment of TOM-1 and the known region of TOM-2 are shown in a diagrammatic representation in fig. 4B.

Southern Blot analysis of ovine genomic DNA (from the same animal as the RNA used in the RT-PCR fig. 3B) is shown in fig. 5 and indicates the presence of only one ovine elafin gene showing that indeed TOM-1 and TOM-2 are two allelic forms of the same gene.

In vivo up-regulation of ovine SLPI and elafin in response to bacterial lipopolysaccharide

Bronchoalveolar lavage samples were collected one week prior to and 6, 24 and 168 hours after LPS administration. Dot blots were performed using these lavages and probed with either a polyclonal antibody against human SLPI or the monoclonal antibody Trab-2O to detect ovine elafin (fig. 6).Although both SLPI and elafin concentrations in the BALF from LPS treated sheep increase with time compared to pre-experiment values, the kinetics and degree of elafin and SLPI up-regulation are different. SLPI increases significantly (1.5 fold) at 6 hours ($P<0.0005$) and at 24 hours ($P<0.005$) while the increase in elafin levels is much more dramatic (8 fold) ($P<0.005$ at 24 hours).

Protection of A549 cells against proteolytic damage

In order to show potential anti-elastase activity attributable to the protein encoded for by the ovine elafin cDNA, A549 cells were infected with Ad-ovine elafin. In order to compare its activity with other known anti-proteases cells were also infected with Ad-human elafin and Ad-murine SLPI (fig. 7). Ad-murine eotaxin was used as a negative control. Ad-ovine elafin and Ad-human elafin conferred protection of the A549 cells, irrespective of the enzyme used. Ad-murine SLPI protected the cells against HNE only, whereas, as anticipated, the negative control Ad-murine eotaxin was ineffective against either enzyme.

This array of results was obtained using qualitative (fig. 7 panel A) and quantitative (fig. 7 panel B) methods. In agreement with the above, the protection by Ad constructs against HNE and PPE activity was paralleled by measuring residual enzymatic activity in the supernatants (fig. 7 panel C).

Discussion

Neutrophil elastase inhibitors have been shown to be important endogenous molecules involved in limiting the extent of neutrophil-induced inflammation in humans, as well as in a variety of animal models (4, 10, 17, 30). Among these inhibitors, our group is particularly interested in the study of elafin, a member of the trappin multigene family (27). The 'trappin' name has been coined by Schalkwijk and collaborators (27) as an acronym for **TR**ansglutaminase substrate and w**AP** (whey acidic protein) domain containing **P**rote**IN**, to illustrate its property of being 'trapped' in a tissue (at least in the skin - 13, 29) and therefore potentially acting as an anchored protein. Trappins consist of an N-terminal transglutaminase substrate domain with hexapeptide repeats (PVKGQD), and a C-terminal four disulphide core, containing, in some members, the antielastase activity (27). Members of this family share the WAP domain with another elastase inhibitor, the secretory leukocyte proteinase inhibitor (SLPI). Trappin members have been isolated from a number of animal species, including man, but, until now, no studies had identified the ovine ortholog of elafin.

We have in the present study isolated both the genes and cDNA coding for this protein and have analysed the anti-elastase activity of the translated product.

We have isolated from a trachea sample a cDNA encoding for ovine elafin (o-elafin) which is composed of 220 amino acids, inclusive of a 23 amino acid signal peptide indicating that the protein is, like other species orthologs, a secreted protein (fig. 1 and 2). Following this signal peptide, we identified a region containing nineteen hexapeptide repeats of the consensus sequence PVKGQD. A very similar region of repeats has been termed 'cementoin' (14) in the trappin family of proteins (27) which the elafin members belong to (see above). This glutamine and lysine rich region is thought to be involved in a transglutaminase reaction resulting in ε-(γ-glutamyl)lysine covalent bonds between the repeat region and matrix proteins such as loricrin and keratin-1, to promote the anchorage of the molecule to the interstitium, at least in the skin (13,29). The second domain of o-elafin is composed of a WAP domain (27), which has strong homology with o-SLPI which we sequenced (Accession number AY346135) and based on the sequence contains the anti-elastase site (see below).

Using both the ovine genomic BAC library described above and genomic DNA obtained locally, we isolated the o-elafin gene TOM-1 (Fig. 1, Accession number AY344541), the sequence of which predicted exactly the cDNA

sequence obtained from tracheal RNA. The gene structure is similar to that for human elafin (23) in that the gene contains 3 exons and 2 introns with similar intron/exon boundaries.

The promoter region shown contains a 'TATA' box at bp 463 but no sequence identifiable as a 'CAAT' box. An ATG initiation codon was detected, directly 3' of a consensus Kozak's sequence (9). The o-elafin leader sequence coding for the signal peptide was found to be very similar to other species' orthologs.

RT-PCR and Northern blot analysis using RNA from various ovine tissues showed o-elafin to be present in tongue, trachea, small intestine, large intestine and skin. This is similar to porcine elafin's distribution where elafin has been shown by in-situ hybridisation to be secreted by goblet cells in the tracheal mucosa and large intestinal crypts (31) and to human elafin which has been detected in bronchial mucus (19) and psoriatic skin (26, 35) at the protein level, and in the epiglottis, pharynx, vocal fold and psoriatic skin at the mRNA level (16).

To summarise tissue distribution of mRNA, o-elafin is transcribed in the upper respiratory tract, oral cavity, skin and gastrointestinal tract. The mainly mucosal tissue distribution of o-elafin is consistent with it being, like its human orthologs, an antimicrobial 'defensin-like' molecule (20). Indeed, evidence is forthcoming that, in addition to their antiprotease activity, SLPI and elafin have important functions in innate immune responses (20).

Interestingly, we obtained either one or two o-elafin cDNA products, depending on the tissues (fig. 3). The origin of the two bands has been shown to be two allelic forms of the same gene (TOM-1 and TOM-2) (fig. 4). The differential expression of TOM-1 and TOM-2 in the different tissues is un-explained at present. The reason why we only obtained TOM-1 from the BAC library analysis while both TOM-1 and TOM-2 were obtained from genomic DNA probably lies with the origin of the samples: indeed, the BAC library used (INRA, France) was derived from a different ovine breed (Romanov (34)) from the one used for the RNA and subsequent RACE analysis (Suffolk crosses from the UK). Our results suggest that the Romanov breed animal used to construct the BAC library only has TOM-1 while the Suffolk breed has both TOM-1 and TOM-2. It is therefore possible that the two allelic forms of o-elafin may be caused by the interbreeding of the commercial Suffolk lowland flock.

The large transglutaminase substrate domain present within the o-elafin molecule is of interest with regards to its potential interaction with other tissue components. As mentioned above this region is thought to be involved in cross-linking to extracellular matrix proteins at least in the skin. Human elafin has been shown to be cross-linked in this way to the cornified envelope of keratinocytes (13, 29). Human elafin contains 5 repeats loosely based on the

consensus sequence PVKGQD. The o-elafin orthologs discussed here (TOM-1 and TOM-2) contain 19 or 26 repeats with far higher conservation of the consensus sequence, suggesting, potentially, higher levels of transglutaminase-mediated cross-linking. Interestingly, recent work has implicated transglutaminase repeats to be of importance in competitively inhibiting tissue transglutaminase and in turn phospholipase-A2 hence leading to a down-regulation of pro-inflammatory mediators in a hypersensitivity model (28). This link between members of the trappin family and down-regulation of phospholipase-A2 leads to the fascinating possibility that the extensive transglutaminase substrate region of the o-elafin proteins may provide a particularly high anti-inflammatory activity to the secreted protein compared to those already studied. The transglutaminase substrate domain has also been identified as one of the *cis* acting elements implicated in the gene multiplication seen in the members of the trappin family (Furutani et al identify a correlation between number of repeats and number of trappin family members (3) i.e. the fewest repeats are seen in human and peccary in each of which only one trappin family member has been described. Surprisingly, given the high number of repeats identified here, we only isolated one trappin, ovine elafin, which was shown to exist in two allelic forms. This is the first time a trappin family member gene has been shown to be present in more than one allelic form.

The phylogenetic tree analysis (fig. 8) shows the close relationship between the WAP domain of TOM-1 and TOM-2, and the other trappin family members, with the second WAP domain of the SLPI orthologs. This suggests a divergence in evolution of the trappin WAP motifs and the second SLPI WAP motif from a common ancestral protein subsequent to the divergence of the first and second WAP motifs of the SLPI family from each other. Interestingly, the hominid trappins (human elafin and simian elafin) have diverged from the rest of the known trappins (bovidae, hippopotamidae, tayassuidae and suidae). The two o-elafin proteins (TOM-1 and TOM-2) are most closely linked with bovine elafin and bovine trappins 4 and 5.

In order to ascertain whether the ovine elafin protein is behaving like a local acute phase reactant we assessed the levels of ovine elafin in a model of lung inflammation triggered by local bronchoscopic administration of LPS. The dramatically increased level of elafin in inflammatory conditions such as described here finds an echo in some of our previous *in vitro* and *in vivo* human work. We showed (33) that elafin levels were sharply increased in BALF from human patients with farmer's lung disease. By contrast, in the same patients, and in the present study, SLPI levels increased less dramatically. This may be due to the increased sensitivity of elafin to pro-inflammatory cytokines such as IL-1 and TNF (22). The time courses of elafin and SLPI expression presented here add further to

that concept, in agreement with murine SLPI *in vivo* studies (1, 7). Western blot analysis of broncho-alveolar lavage fluid (BALF) for ovine elafin and SLPI using the antibodies utilised in the dot blot analysis above was unsuccessful at detecting protein due presumably to the low concentration of these anti-proteases in the BALF.

In summary, the identification and cloning of the gene encoding for the ovine orthologs of human elafin have allowed us to postulate important and exciting functions of this protein in addition to the anti-protease functions. Furthermore, the *in vivo* demonstration that ovine elafin is an acute inflammatory reactant in the lung indicates that it can be considered as an 'alarm inhibitor' in that organ. Its efficiency as a neutrophil elastase inhibitor makes it an attractive candidate for use in adenovirus-mediated gene transfer in ovine models of neutrophils-driven lung injury, currently being explored in our laboratories. Additionally, specific and potentially very important effects of the transglutaminase substrate domain in this protein warrant further investigation.

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Figure Legends

Fig. 1. Sequence for the gene coding for ovine elafin.

The 'tata' box, 5' Kozak sequence and 3' polyadenylation signal sequence are boxed. Exons are underlined. Predicted signal cleavage site is between amino acids 23 and 23 (between A and A). Annealing sites for the primers GSP1, GSP2, r-el (used in RACE-PCR), fBAC, rBAC (used in PCR screening of BAC library) and r-inv-el (used to amplify the promoter region) are indicated.

Fig. 2. Comparison of the known elafin protein sequence between species.

The shaded area corresponds to the region of hexapeptide repeats with the consensus sequence PVKGQD. The eight conserved cysteine residues within the whey acidic protein domain (WAP) are indicated by asterisks. Where there is sequence overlap (ie. ignoring the size difference between the proteins) the ovine elafin sequence shows 70% homology at the amino acid level with the simian, 68% with the human, 49% with the porcine and 80% with the bovine elafin sequence.

Fig.3. Tissue distribution of ovine elafin RNA as assessed by Northern Blot and RT-PCR.

A. Northern Blot. 20μg of total RNA was loaded for each tissue. Ribosomal 18S and 28S RNA sub-units are indicated by arrows. The membrane was probed with the full length cDNA (660bps) corresponding to the coding portion of the gene for ovine elafin.
B. RT-PCR for ß-actin and ovine elafin from RNA extracted from the various organs indicated. The position of molecular weight markers is indicated (1kB ladder). Primers used for amplification of ovine elafin were GSP2 and r-el yielding two products of approximately 660bps and 800bps.

Fig. 4. The relationship between the two ovine elafin alleles (TOM-1 and TOM-2) coding for proteins of 220 amino acids and 262 amino acids respectively.

A. PCR was performed using the non intron-spanning primers fBAC and rBAC as detailed in the text. Templates used were ovine genomic DNA, cDNA synthesised by reverse transcription of RNA isolated from ovine skin, DNA isolated from an ovine BAC clone positive by PCR for the elafin gene, and the expression plasmid pIRES-GFP#3 containing the cDNA for ovine elafin (220 amino acid form). The lower molecular weight product of approximately 400bps is due to amplification of exon 2 of the ovine elafin gene or cDNA (and also inserted into pIRES-GFP#3); the longer product of approximately 525 bps corresponds to amplification of a longer form of ovine elafin.

B. The promoter sequence and 3' sequence is known for the TOM-1 gene only. 100% homology exists between the known portion of TOM-2 and the corresponding portion of TOM-1, except for the fact that TOM-2 contains 7 extra repeats with the consensus PVKGQD as shown. Numbers below the representation of TOM-1 denote base-pairs from the start of the gene sequence. TOM-2 has been sequenced after PCR of ovine genomic DNA using the primers GSP 2 and R-el as shown.

Fig. 5. Southern blot of ovine genomic DNA probed for ovine elafin.

20µg of genomic ovine DNA was digested with restriction enzymes (Xbal, SacI, EcoR1 and BamH1), fractionated on a 0.8% agarose gel, then blotted onto a nylon membrane. The membrane was probed with radiolabelled full length cDNA corresponding to the coding sequence of TOM-1. The sizes of the bands seen are indicated.

Fig. 6. Secretion of SLPI and elafin in bronchoalveolar lavage collected from sheep lung segments exposed to lipopolysaccharide.

1mg E. Coli LPS was administered to discrete lung segments of twelve sheep. Bronchoalveolar lavage was collected a week prior to and 6, 24 and 168 hours after administration of the LPS. 200µl of bronchoalveolar lavage from each animal at each time point was used in a dot blot and probed with either (A) human anti-SLPI polyclonal antibody, or (B) Trab 2-O monoclonal antibody detecting ovine elafin. Results are shown as median arbitrary densitometry units against time after dosage of LPS with error bars showing inter-quartile ranges. Also shown are representative dot

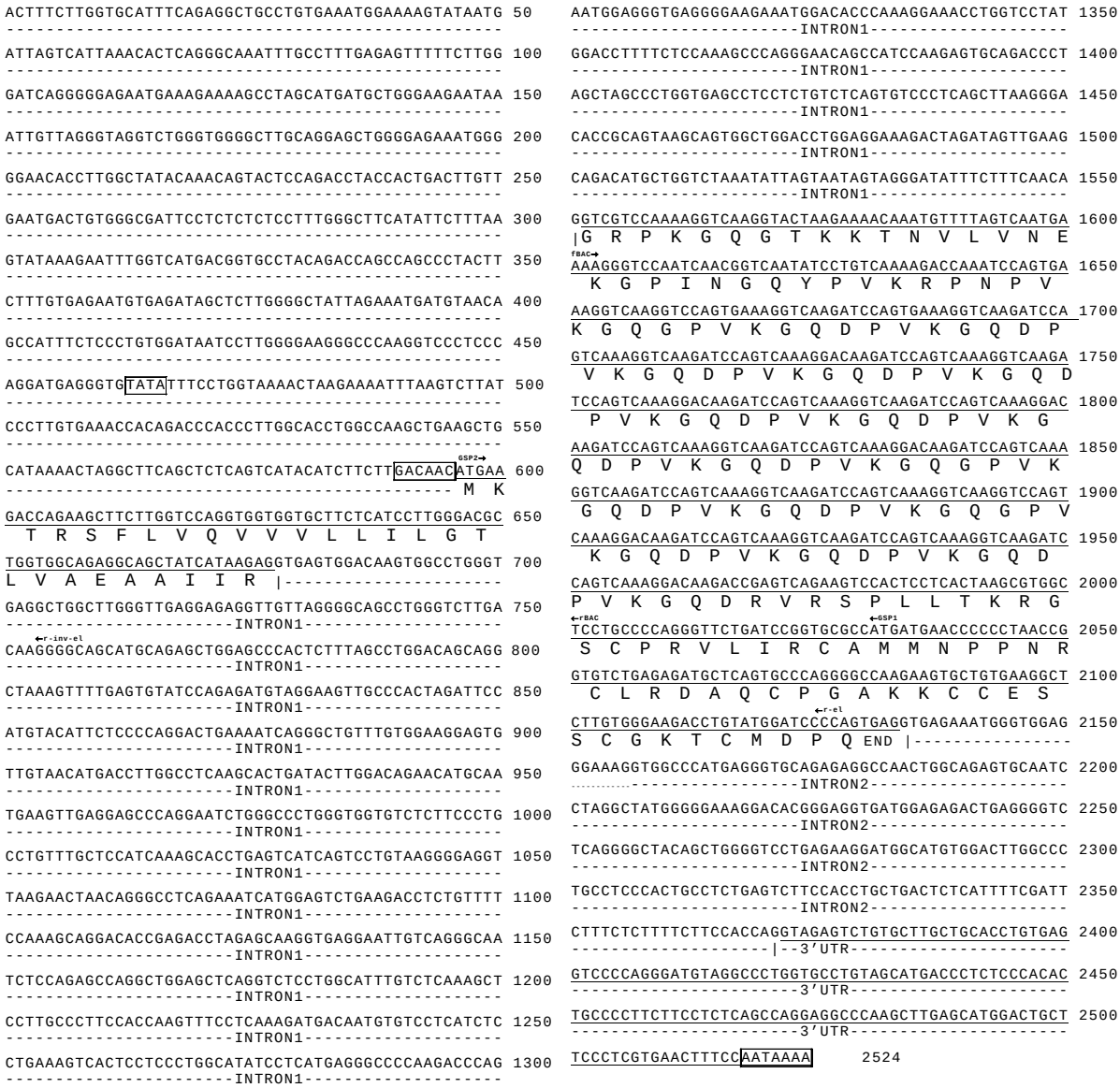
blot results for each protein. ** signifies a P value of <0.005 and *** signifies a P value of <0.0005 compared to pre experimental values when Mann Whitney analysis was performed. All results are based on n=12.

Fig. 7. Ad-ovine elafin protection of A549 cells against human neutrophil elastase and porcine pancreatic elastase – correlation with inhibition of enzymatic activity.

Ad-ovine elafin was shown to protect A549 cells against HNE and PPE (1µg/ml) (panel A). By contrast, the negative control Ad-murine eotaxin provided no protection against either enzyme. Ad-human elafin protected cells against both HNE and PPE whereas Ad-murine SLPI was efficient only against HNE (panel A).These data were confirmed quantitatively, when protein concentration was assessed as a surrogate marker of cell damage (panel B and Materials and Methods). The protection (or lack of) conferred (panels A and B) by the Ad constructs against proteolytic enzymes was paralleled by the residual enzymatic activity recovered in cell culture supernatant (panel C). ** : signifies P<0.005, *** : P<0.0005 compared to Ad-murine eotaxin with Mann-Whitney analysis, n=5 (HNE) or 6 (PPE).

Fig. 8. Whey acid protein (WAP) comparison (SLPI, trappins, mouse elafin-like proteins, vasopressin).

A. Alignment of the 1st and 2nd WAP domains of the currently known SLPI proteins, along with the WAP domains for a variety of trappins including the ovine forms of elafin derived from the alleles TOM-1 and TOM-2, and including the WAP domains of the murine elafin-like proteins 1 and 2 (Hagiwara et al) and human vasopressin. The WAP motifs for all the proteins were aligned by using ClustalW software available online. Conserved cysteine residues are indicated by asterisks. The boxed region indicates the putative active anti-protease active site in the second WAP domains of the SLPI family and in the WAP domain of the trappin family.
B. Phylogenetic tree constructed using a neighbour-joining technique with Poisson corrected data from the above alignment. Bootstrap values above 40 are indicated. Branch length is proportional to evolutionary distance.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GSP2→
M K
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Fig. 1

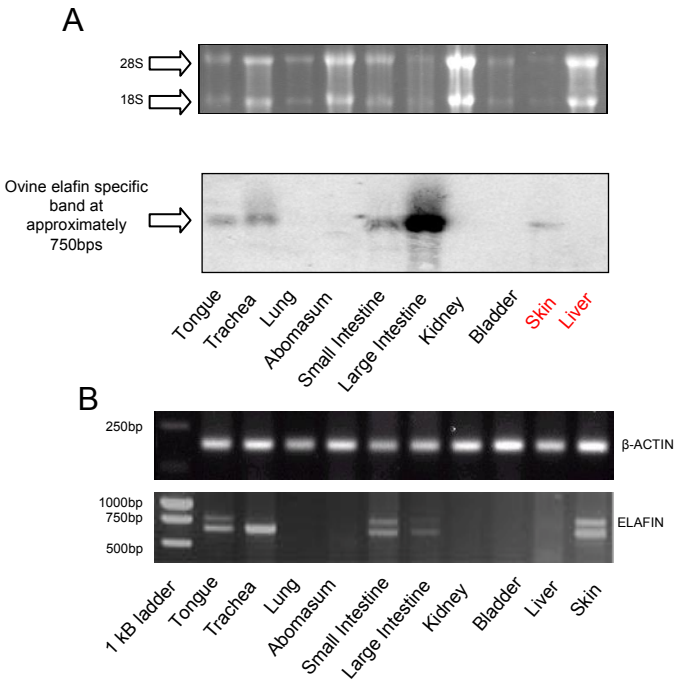
```
        1           15 16          30 31          45 46          60
simian  ---------VVVFLI AGMLVVEAA------ --------------- ---------------
human   MRASSFLI-VVVFLI AGTLVLEAA------ --------------- ---------------
porcine MRSRSFLVLVVVFLI CGTLVAQAAGRIRRP KGKGTKKILALV--- ---------------
bovine  --------------- --------------- --------------- ---------------
ovine   MKTRSFLVQVVVLLI LGTLVAEAAIIRGRP KGQGTKKTNVLVNEK GPINGQYPVKRPNPV

        61          75 76          90 91          105 106         120
simian  -----------VTGV -PVKGQDTVKGRVPF NGQDPVKGQVSVKGQ DRVKGRGPVKG----
human   -----------VTGV -PVKGQDTVKGRVPF NGQDPVKGQVSVKGQ DKVKAQEPVKG----
porcine KGQGPVRGKDQVKGQ GPVKGQDLGKSQDPV KAQLPDKGQDLGKGE DSVKGQDPFKAQ---
bovine  --QEPVKGQDPVKGQ DPVKGQDPVKGQDPV KDQNPVRGQEPVKGQ DPVKGQDPVKGQDPV
ovine   IGQGPVKGQDPVKGQ DPVKGQDPVKGQDPV KGQDPVKGQDPVKGQ DPVKGQDPVKGQDPV

        121         135 136         150 151         165 166         180
simian  --------------- --------------- --------------- -----PVSTKPGSCP
human   --------------- --------------- --------------- -----PVSTKPGSCP
porcine --------------- ------------LPD KLQDPVKAQPAIK-- ---RLILLTKPGSCP
bovine  KGQ------------ ------------EPV KGQDPVKGQDPVKRQ GRIGGPLLTKPGSCP
ovine   KGQGPVKGQDPVKGQ DPVKGQGPVKGQDPV KGQDPVKGQDPVKGQ DRVRSPLLTKRGSCP
                                                                    *

        181         195 196         210 211
simian  NILIRCAMLNPPNRC LKDTD---------- ----------     89
human   IILIRCAMLNPPNRC LKDTDCPGIKKCCEG SCGMACFVPQ    117
porcine RILIRCLMVNPPNRC LSDAQCPGLKKCCEG FCGKACMDPK    167
bovine  RVLIRCAMMNPPNRC LRDAQCPGVKKCCEG SCGKTCMDPQ    134
ovine   RVLIRCAMMNPPNRC LRDAQCPGAKKCCES SCGKTCMDPQ    220
              *       *       *     **    *   *
```

Fig. 2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18S
Ovine elafin specific band at approximately 750bps
Tongue
Trachea
Lung
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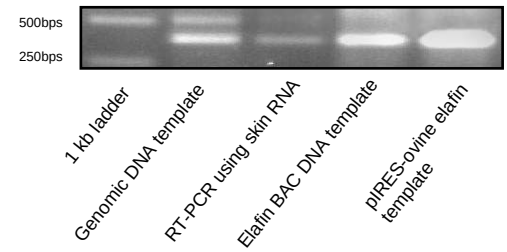
Small Intestine
Large Intestine
Kidney
Bladder
Skin
Liver
B
250bp
β-ACTIN
1000bp
750bp
500bp
ELAFIN
1 kB ladder
Tongue
Trachea
Lung
Abomasum
Small Intestine
Large Intestine
Kidney
Bladder
Liver
Skin

Fig. 3

A


500bps
250bps
1 kb ladder
Genomic DNA template
RT-PCR using skin RNA
Elafin BAC DNA template
pIRES-ovine elafin template

B

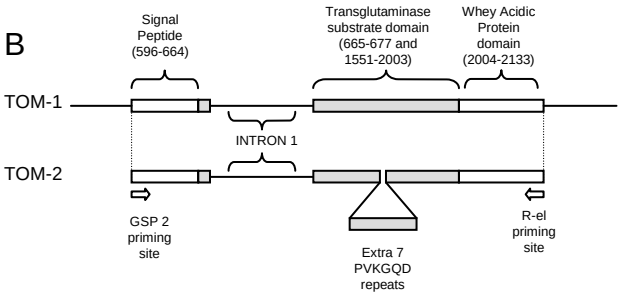

Signal Peptide (596-664)
Transglutaminase substrate domain (665-677 and 1551-2003)
Whey Acidic Protein domain (2004-2133)
TOM-1
INTRON 1
TOM-2
GSP 2 priming site
Extra 7 PVKGQD repeats
R-el priming site

Fig. 4

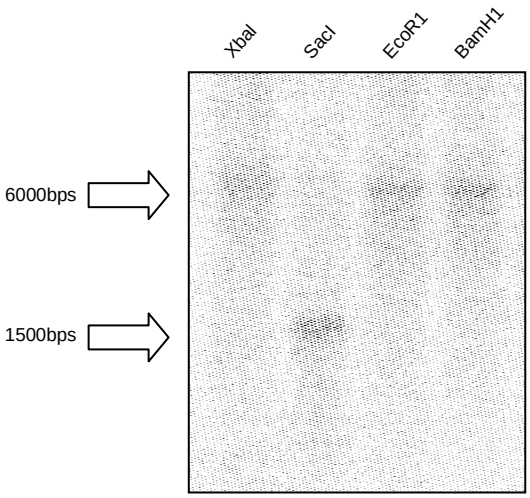


Fig. 5

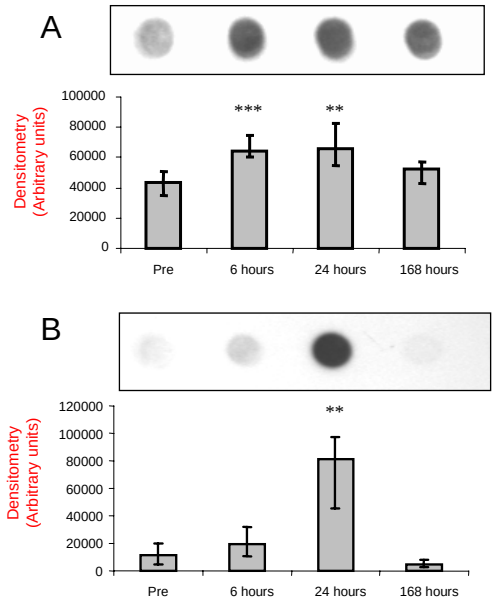
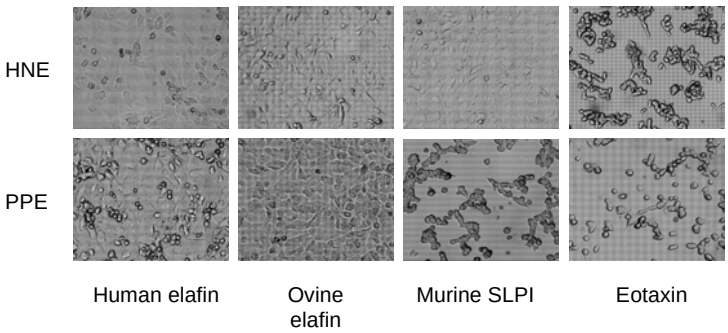
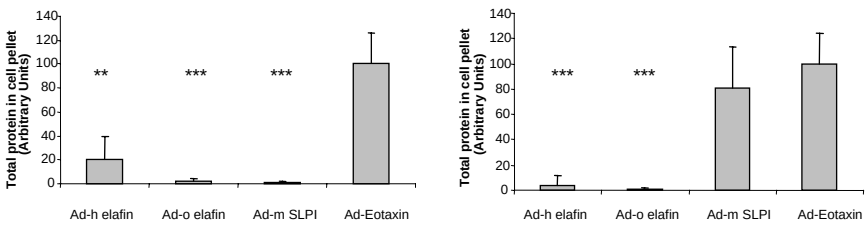

A
Densitometry (Arbitrary units)
100000
80000
60000
40000
20000
0
***
**
Pre
6 hours
24 hours
168 hours
B
Densitometry (Arbitrary units)
120000
100000
80000
60000
40000
20000
0
**
Pre
6 hours
24 hours
168 hours

Fig. 6

A


HNE
PPE
Human elafin
Ovine elafin
Murine SLPI
Eotaxin

B


Total protein in cell pellet (Arbitrary Units)
0
20
40
60
80
100
120
140
**
***
***
Ad-h elafin
Ad-o elafin
Ad-m SLPI
Ad-Eotaxin
Total protein in cell pellet (Arbitrary Units)
***
***
Ad-h elafin
Ad-o elafin
Ad-m SLPI
Ad-Eotaxin

C

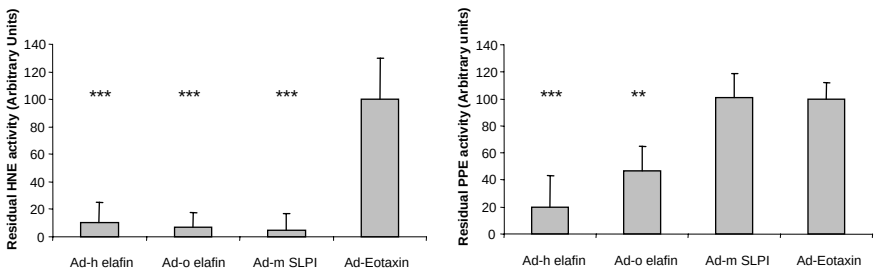

Residual HNE activity (Arbitrary Units)
0
20
40
60
80
100
120
140
***
***
***
Ad-h elafin
Ad-o elafin
Ad-m SLPI
Ad-Eotaxin
Residual PPE activity (Arbitrary units)
***
**
Ad-h elafin
Ad-o elafin
Ad-m SLPI
Ad-Eotaxin

Fig. 7

A

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