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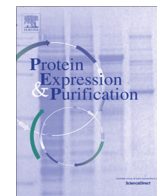
- VC1 domain of human RAGE, secreted from *P. pastoris*, is functionally active.
  - Intracellular retention of V indicates that V and C1 form a single folding unit.
  - Yeast-secreted VC1 is monomeric and more stable than its *E. coli* counterpart.
  - Glycosylation/deamidation of recombinant VC1 mimics the natural pattern.
  - High stability/solubility of glycosylated VC1 will allow future *in vitro* studies.
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# Protein Expression and Purification

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## An improved expression system for the VC1 ligand binding domain of the receptor for advanced glycation end products in *Pichia pastoris*

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### ABSTRACT

The receptor for the advanced glycation end products (RAGE) is a type I transmembrane glycoprotein belonging to the immunoglobulin superfamily and binds a variety of unrelated ligands sharing a negative charge. Most ligands bind to the extracellular V or VC1 domains of the receptor. In this work, V and VC1 of human RAGE were produced in the methylotrophic yeast *Pichia pastoris* and directed to the secretory pathway. Fusions to a removable C-terminal His-tag evidenced proteolytic processing of the tag by extracellular proteases and also intracellular degradation of the N-terminal portion of V-His. Expression of untagged forms was attempted. While the V domain was retained intracellularly, VC1 was secreted into the medium and was functionally active in binding AGEs. The glycosylation state of VC1 was analyzed by mass spectrometry and peptide-N-glycosidase F digestion. Like RAGE isolated from mammalian sources, the degree of occupancy of the N-glycosylation sites was full at Asn25 and partial at Asn81 which was also subjected to non-enzymatic deamidation. A simple procedure for the purification to homogeneity of VC1 from the medium was developed. The folded state of the purified protein was assessed by thermal shift assays. Recombinant VC1 from *P. pastoris* showed a remarkably high thermal stability as compared to the protein expressed in bacteria. Our *in vivo* approach indicates that the V and C1 domains constitute a single folding unit. The stability and solubility of the yeast-secreted VC1 may be beneficial for future *in vitro* studies aimed to identify new ligands or inhibitors of RAGE.

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### 1. Introduction

The receptor for the advanced glycation end products (RAGE) is a protein unique to mammals. In humans, it is expressed at low levels in many cell types, in particular leukocytes, dendritic cells, endothelial cells, smooth muscle cells, neurons and microglia and is abundant in the lung [1]. This receptor is important for human biology. It promotes neurite outgrowth, differentiation and migration in the nervous system, and is involved in bone metabolism and lung homeostasis [1]. It contributes to the inflammatory response [2] and participates to innate and adaptive immunity, where the activation of human RAGE (hRAGE) leads to the production of reactive oxygen species (ROS). Furthermore, prolonged and strong activation of hRAGE can result in the pathogenesis of several diseases. Thus, hRAGE plays two opposing roles, one beneficial for

human biology and one that promotes the disease establishment and progression.

High levels of hRAGE and its ligands lead to several dysfunctions through the sustained activation of different signal transduction pathways depending on cell type, context and ligand. The signaling pathways include (i) the p21ras/mitogen-activated cascade of protein kinases (MAPKs) such as ERK1/2 and the stress-activated kinases (JNK and p38) and (ii) the JAK/STAT pathway, resulting in the nuclear translocation of NF-κB and activation of transcription of inflammatory genes, of RAGE gene itself and of the interferon-stimulated response, (iii) the cdc42/rac pathway that is involved in cell growth and migration, and finally (iv) the NADPH oxidase pathway that leads to ROS formation [1,2]. Sustained activation of RAGE signaling results in tissue damage as observed in complications of diabetes such as cardiovascular disorders, atherosclerosis, nephropathies and chronic inflammation. For these reasons, various studies indicate that antagonizing RAGE activation, by use of inhibitors or through binding to soluble ectodomain (sRAGE), has potential therapeutic effects in several

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inflammatory-based diseases including Alzheimer disease [3,4]. Small molecules acting as RAGE antagonists have been recently reported and a compound developed by Pfizer (PF-04494700) has reached phase II clinical trials [3–5].

hRAGE is a type I transmembrane glycoprotein of 404 amino acids and a member of the immunoglobulin (Ig) superfamily that resembles cell adhesion molecules in some structural and functional aspects [6,7]. The human RAGE contains a 22 residue signal peptide and an extracellular portion that comprises a variable (V) and two constant (C1 and C2) Ig-like domains. A single transmembrane domain connects the V<sub>2</sub>-C1-C2 segment to a short C-terminal tail that is responsible for intracellular signaling. RAGE binds a wide spectrum of ligands [8], the majority of which are: AGEs, i.e., products generated by the non-enzymatic glycation and subsequent oxidation of proteins, amyloid- $\beta$ -peptides,  $\beta$ -sheet fibrils and members of the S100/calgranulin protein family [9]. Additional ligands include: extracellular matrix proteins, such as collagens I and IV, Mac-1 (member of the  $\beta$ 2-integrin family), the High Mobility Group protein Box-1 (HMGB-1 or amphoterin), which is a prototypic Damage-Associated Molecular Pattern (DAMP) molecule [6], the lipopolysaccharide of bacterial walls (LPS), the complement component C1q [10], chondroitin sulfate and heparan sulfate [11,12] and nucleic acids [13]. hRAGE ligands do not share sequence or structural similarity but all display a negatively charged surface at neutral pH and many ligands show the tendency to oligomerize [14]. The 3D-structure of the RAGE extracellular portion (V-C1-C2), of VC1 and V, has been determined, providing an insight into the putative molecular mechanism of RAGE activation [14]. Most hRAGE ligands bind to the highly basic V domain through electrostatic and hydrophobic interactions. Due to its peculiar binding properties, RAGE is also regarded as a pattern-recognition receptor. A model of RAGE activation proposes that RAGE molecules preassemble in the plane of the membrane and this potentiates signaling after the binding of the ligand to the V domain [2]. Moreover, two RAGE molecules are also able to associate in head-to-head fashion possibly mediating the interaction between cells similarly to adhesion proteins (homophilic adhesion).

We are interested in studying the binding of hRAGE to AGEs, since the RAGE-AGE axis is crucial in the etiology of many diseases. Indeed, the formation of AGEs is accelerated in the presence of hyperglycemia and oxidative stress (for a review, see [15]). In this work, we probed the expression of V and VC1 in the lower eukaryote *Pichia pastoris*. This microorganism has an efficient apparatus of protein glycosylation, folding and secretion that may be beneficial for the expression of naturally secreted glycoproteins. In addition, glycosylation, together with the formation of disulfide bonds, confers stability to proteins and also improves their solubility. To our knowledge, the expression of the isolated V and VC1 domains in a eukaryotic system has not been attempted to date, although *P. pastoris* proved to be an appropriate host for the expression of the full ectodomain [16]. Here, we report the expression/secretion of VC1 and V domains of hRAGE in *P. pastoris*. A protocol for VC1 purification to homogeneity from the culture medium, the analysis of its glycosylation profile, the improved quality of the protein in comparison to the form produced in bacteria and the proof of its functionality are presented.

## 2. Materials and methods

### 2.1. Strains and growth conditions

The *Escherichia coli* DH5 $\alpha$  strain was used for DNA propagation and manipulation. *P. pastoris* GS115 strain (*his4*) (Invitrogen) was used for the heterologous expression of hRAGE domains. The

GS115-*sub2* strain, inactivated in the gene encoding the major secreted subtilisin-like protease, was kindly provided by Dr. Michel Monod (Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland). *P. pastoris* cells were routinely propagated on plates of YPDA (1% yeast extract, 2% peptone, 2% glucose and 2% agar) at 30 °C. To induce the expression of recombinant proteins, His<sup>+</sup> Mut<sup>s</sup> cells were shifted from Buffered Glycerol-complex Medium (BMGY) (1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB, 4  $\times$  10<sup>-5</sup>% biotin, 0.1 M potassium phosphate, pH 6.0) to Buffered Methanol-complex Medium (BMMY) (1% yeast extract, 2% peptone, 0.5% methanol, 1.34% YNB, 4  $\times$  10<sup>-5</sup>% biotin, 0.1 M potassium phosphate, pH 6.0) according to the manufacturer's instructions. Alternatively, MGY and MMY media, having the same composition as above but without potassium phosphate buffer, were used. Cells were routinely grown in flasks at 28 °C under strong agitation and growth was monitored through the increase in optical density at 600 nm (OD<sub>600</sub>).

### 2.2. Construction of the expression plasmids

Recombinant plasmids for integrative recombination in *P. pastoris* were obtained by cloning *Xho*I-digested PCR fragments into the *Xho*I site of pHIL-S1 (Invitrogen), to generate in-frame fusions with the secretion signal of *P. pastoris* PHO1, encoding an extracellular acid phosphatase. PCR was carried out using as a template pET-15b-VC1<sup>243</sup> [17], harboring the cDNA encoding a portion of hRAGE (residues 23–243), high-fidelity fusion DNA polymerase (NEB) and appropriate primers (Table 1). The DNA fragments encoding VC1<sup>233</sup> and VC1<sup>233</sup>-His were obtained using VC-For/VC233-Rev and VC-For/VC233His-Rev oligonucleotide pairs, respectively. Similarly, the DNA encoding V<sup>121</sup> and V<sup>121</sup>-His were obtained using the primer pairs VC-For/V121-Rev and VC-For/V121His-Rev. Both PCR products and the digested vector were gel-purified before ligation. Screening of the transformants was performed by colony PCR using 5'-AOX1/VC233-Rev and VC-For/3'AOX1 for VC1<sup>233</sup>, 5'-AOX1/VC233His-Rev and VC-For/3'AOX1 for VC1<sup>233</sup>-His, 5'-AOX1/V121-Rev and VC-For/3'AOX1 for V<sup>121</sup>, 5'-AOX1/V121His-Rev and VC-For/3'AOX1 for V<sup>121</sup>-His (Table 1). Recombinant plasmid DNA, named pHIL-S1-VC1<sup>233</sup>, pHIL-S1-VC1<sup>233</sup>-His, pHIL-S1-V<sup>121</sup> and pHIL-S1-V<sup>121</sup>-His, were purified from positive clones. The correct orientation, the in-frame fusion, and the absence of random mutations in the inserts were verified by DNA sequencing (BMR Genomics, Padova, Italy).

### 2.3. Transformation of *P. pastoris* and expression of recombinant forms

Plasmids, linearized with *Bgl*II, were transformed into *P. pastoris* cells using the “EasyComp” chemical transformation method (Invitrogen). Selection of His<sup>+</sup> transformants and screening for Mut<sup>s</sup> phenotype were performed as previously described [18]. To induce the expression of the recombinant proteins, the positive clones were grown overnight at 28 °C in 10 mL of glycerol-containing medium (BMGY or MGY) under strong agitation and at a ratio between volume of culture and capacity of the flask of 1:10. Then, appropriate amount of cells were collected and transferred in methanol-containing medium (BMMY or MMY) in order to obtain an initial OD<sub>600</sub> of 1. Growth was monitored by increase of OD<sub>600</sub>. Fresh methanol was added daily to 0.5% (v/v) final concentration. To monitor protein expression, supernatants from 1 mL-aliquots of culture, withdrawn at intervals after induction, were obtained by centrifugation, flash-frozen and stored at -20 °C until analysis by SDS-PAGE. Total protein extracts from flash-frozen cell pellets were prepared as previously described [19]. At least three clones were analyzed per construct.

**Table 1**  
Oligonucleotides used in this work.<sup>a</sup>

Primer	Sequence 5'–3'
VC-For	AGCATATTGACTGACT <b>CTCGAGCTCAAACATCACAGCCCG</b>
VC233-Rev	ATCGTCGGGCTCA <b>CTCGAGCTACACAGGCTCCACAGACACG</b>
VC233His-Rev	ATCGTCGGGCTCA <b>CTCGAGCTAGTGATGGTGATGGTGATG</b> GCTGCTGCCGCTGCCGCGGGCACCAGATGGCTGCTGCCG CTGCCGCGGGCACCAGATGC <b>CACAGGCTCCACAGACACG</b>
V121-Rev	ATCGTCGGGCTCA <b>CTCGAGCTAAGGAATCTGGTAGACACGGA</b>
V121His-Rev	ATCGTCGGGCTCA <b>CTCGAGCTAGTGATGGTGATGGTGATGGC</b> TGCTGCCGCTGCCGCGGGCACCAGATGGCTGCTGCCGCTGCC GCGCGCACACAGAT <b>AGGAATCTGGTAGACACGGA</b> GACTGGTTCCAATTGACAAGC GCAAAATGGCATTCTGACATCC
5'-AOX1	
3'-AOX1	

<sup>a</sup> XhoI site is underlined and CDS of VC1 or V is in bold. Stop codon is in italic.

#### 2.4. Purification of recombinant VC1

For protein small-scale purification, 80 mL culture of induced *P. pastoris* cells was centrifuged at 4000×g for 10 min and the supernatant was filtered through cellulose nitrate filters (1.2 μm pore size, Sartorius) to remove residual cells and debris. After dialysis (Spectra/Por membranes, cut-off 6–8000) at 4 °C for 16 h against 10 mM Na-acetate pH 5.0, the sample was applied to a cation-exchange RESOURCE S column (1.6 × 15 cm; GE Healthcare) connected to an ÄKTA-FPLC system (GE Healthcare) and equilibrated with 10 mM Na-acetate pH 5.0. The column was washed with the same buffer until the A<sub>280</sub> reached the baseline. The protein was then eluted with 10 mM Na-acetate pH 5.0 containing 1 M NaCl. The fractions containing the recombinant protein, as judged by SDS–PAGE, were combined and applied to a Superdex 75 (10/30) gel filtration column (GE Healthcare) equilibrated with 10 mM Na-acetate, pH 5.0, 1 M NaCl. Protein in the fractions was precipitated by trichloroacetic acid and analyzed by SDS–PAGE. To estimate the native molecular mass of VC1, the column was calibrated with standard proteins under the same conditions (BSA dimer, 132 kDa; BSA monomer, 66 kDa; carbonic anhydrase, 29 kDa; cytochrome c, 12.3 kDa).

For a large-scale purification, the supernatant from 400 mL culture was obtained as described above. After concentration to ~30 mL by ultrafiltration using a 30,000 cut-off membrane and dialysis against 10 mM Na-acetate, pH 5.0, for 16 h at 4 °C, the solution was applied to a cation-exchange Mono S HR5/5 column (GE Healthcare) equilibrated with 10 mM Na-acetate, pH 5.0 and connected to an ÄKTA-FPLC system (GE Healthcare). After column washing with the same buffer, elution was performed with a 0–1 M NaCl gradient in the same buffer. Fractions were analyzed by SDS–PAGE.

#### 2.5. Electrophoresis and immunoblotting procedures

*P. pastoris* culture supernatants and chromatographic fractions were denatured by boiling for 3 min in SDS sample buffer (0.0625 M Tris–HCl, pH 6.8, 2.3% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol and 0.01% Bromophenol blue). SDS–PAGE was performed on 13% or 16% polyacrylamide gels (14 × 16 cm or 8 × 10 cm). Proteins bands were stained by Coomassie Blue Biosafe (Bio-Rad, USA). After Western blotting, V or VC1 were detected by using a 1:1000 dilution of an affinity-purified goat polyclonal antibody raised against an N-terminal peptide of hRAGE (N-16, Santa Cruz Biotechnology) or a 1:10,000 dilution of a monoclonal anti-poly(His) antibody (mAb) (Novagen) in combination with the appropriate peroxidase-conjugated anti-goat (Sigma) or anti-mouse secondary antibodies (Jackson Laboratories) and LiteAblot<sup>®</sup>PLUS Western blotting detection reagent (Euroclone, Italy). Quantification of the

intensity of VC1 bands on immunoblot was performed using a ChemiDoc MP system (Bio-Rad). The level of VC1 accumulated in the medium was determined by comparison to a calibration curve obtained using known amounts of VC1 expressed in *E. coli* (60–240 ng/lane). VC1 was purified from *E. coli* as described [20].

#### 2.6. Treatment with peptide N-glycosidase F

N-peptide glycosidase F (PNGase F) treatment was performed on either concentrated culture supernatant or purified proteins. In order to remove the N-linked oligosaccharide chains, 0.5–1 μg of protein (5 μL) were denatured at 95 °C for 3 min in the presence of 0.2% SDS. Then, samples were diluted to 50 μL and their composition was adjusted to 0.02% SDS, 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethanesulfonyl fluoride (PMSF), 50 mM Na-acetate, pH 5.5. Each sample was halved and one aliquot was used as a control while the other was added with 0.3 U of PNGase F (Roche). The samples were incubated at 37 °C under gentle agitation for 16 h before SDS–PAGE analysis.

#### 2.7. VC1 digestion

Bands excised from gels were digested according to an established procedure [21]. Briefly, after destaining with 50% acetonitrile, 25 mM ammonium bicarbonate, gel pieces were incubated with 10 mM dithiothreitol at 56 °C for 60 min and then with 55 mM iodoacetamide at room temperature for 45 min. About 1 μg sequencing-grade trypsin (Roche Diagnostics S.p.A, Monza, Italy) dissolved in 50 mM ammonium bicarbonate was added to each sample. After overnight digestion at 37 °C, the liquid phase was withdrawn. Gel pieces were washed first with 30% acetonitrile, 3% trifluoroacetic acid for 10 min, and then with 100% acetonitrile for 10 min. The three extracts were combined and dried on a vacuum concentrator. Tryptic peptide mixtures were then dissolved in 15 μL 0.1% formic acid for mass spectrometry analysis.

#### 2.8. Mass spectrometry analyses

Peptides (5 μL) were injected on a reverse phase C18 HALO PicoFrit column (75 μm × 10 cm, 2.7 μm particle size, 100 Å pore size, New Objective, USA) using an UltiMate 3000 RSLCnano liquid chromatographic system online with an LTQ-Orbitrap mass spectrometer (Thermo Scientific, USA). Peptide separation was performed by a 35% acetonitrile linear gradient in 0.1% formic acid over 40 min, at flow rate of 300 nL/min. Eluting peptides were electrosprayed using a nanoESI source (Thermo Scientific, USA). The instrument operated in data-dependent mode to acquire both full MS and MS/MS spectra. Full MS spectra were acquired in profile mode by the FT analyzer in a scan range 300–1500 m/z, using capillary temperature of 220 °C, AGC 5 × 10<sup>5</sup> and resolution 100,000 FWHM at m/z 400. Tandem mass spectra were acquired by the linear ion trap (LTQ) for the 3 most intense ions. MS/MS spectra acquisition was set as follows: centroid mode, resolution 15,000, precursor ions isolation width 2.5 m/z, AGC target 1 × 10<sup>4</sup> and normalized collision energy 30 eV. Dynamic exclusion was enabled to reduce redundant spectra acquisition: two repeat counts, 30 s repeat duration, 45 s exclusion duration. Monoisotopic precursor selection was enabled; singly and unassigned charged ions were not fragmented. Instrument control and spectra analysis were operated by the software Xcalibur 2.0.7 and Chromeleon Xpress 6.80.

#### 2.9. Peptide identification

The software Proteome Discoverer (v 1.3, Thermo Scientific) was used to extract peaks from spectra and to match them to a

dedicate protein database containing: (i) the sequences of VC1 and VC1-His preproteins obtained from the translation of the cDNA cloned in *P. pastoris* (Supplementary File 1) and (ii) *P. pastoris* proteome (8073 entries downloaded from UniProt database on 27.06.2014). Trypsin was selected as the cleaving protease, admitting a maximum of two missed cleavage sites. Peptide and fragment ions tolerance were set to 5 ppm and 0.3 Da, respectively. Cysteine carbamidomethylation was set as fixed modification and methionine oxidation was allowed as variable modifications. In PNGase F-treated samples, Asn-Asp conversion was also allowed as additional variable modification (monoisotopic delta mass equal to 0.984 Da). Low-confidence identifications were filtered out by selecting peptide confidence <0.05 and at least 2 peptides/protein. Regarding the analysis of glycosylated peptides, the extracted ion chromatograms (XICs) of selected *m/z* values, corresponding to peptides of interest, were manually obtained from Quan Browser (Xcalibur software version 2.0.7) using tolerance 5 ppm and mass precision 5 decimals.

### 2.10. Filter binding assay

AGE-bovine serum albumin (AGE-BSA) was produced as described previously [22]. Serial dilutions of solutions of BSA, and AGE-BSA (BSA modified with ribose, also named BSA-R) were spotted on a nitrocellulose membrane. After saturation with 5% (w/v) dried milk powder in TBS-T (10 mM Tris-HCl, pH 7.4, 166 mM NaCl, 0.05% Tween-20), the membrane was incubated over night at 4 °C with purified VC1 (5 µg/mL) in 20 mM HEPES, pH 7.1, 100 mM NaCl. The membrane was then washed twice with TBS-T for 10 min. To identify bound VC1, the membrane was incubated with a 1:1000 dilution of anti-RAGE antibody in TBS-T-BSA (5% BSA in TBS-T). After washing the membrane four times with TBS-T for 10 min and incubation with a 1:80,000 dilution of secondary peroxidase-conjugated anti-goat IgG in TBS-T, immune complexes were detected as described above.

### 2.11. Thermal stability analysis

0.5 mg/mL purified proteins were added with the fluorescent dye SYPRO Orange (Sigma-Aldrich), according to the manufacturer's instructions in 10 mM Na-acetate, pH 5.0 containing no salt, 150 or 300 mM NaCl. 25 µL-aliquots were loaded in a multi-well plate (MJ white, Bio-Rad) and subjected to a 15–99 °C temperature ramp at a rate of 2 °C/min using the real time PCR apparatus MiniOpticon MJ Mini (Bio-Rad). The intensity of fluorescence emission in the range 540–700 nm was recorded after each 0.2 °C increment. Each protein under each ionic strength condition was

analyzed in triplicate. The melting temperature of each protein (*T<sub>m</sub>*) was identified as the inflection point of the plot of the fluorescence emission as a function of temperature and was determined as the maximum of the first derivative of such a function.

### 2.12. Generation of a 3D model for glycosylated VC1

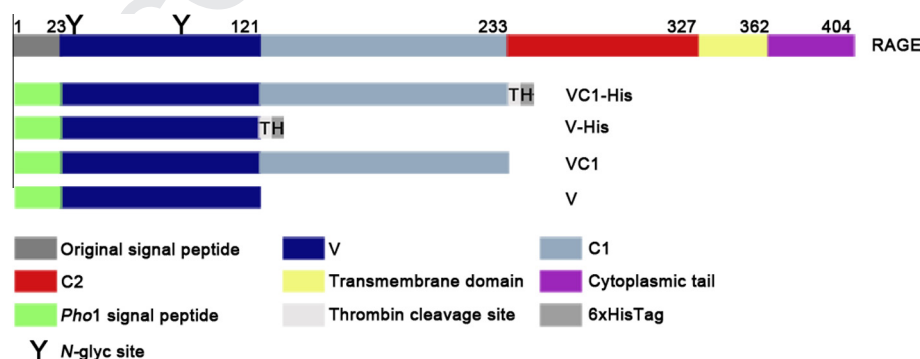
A model of the glycosylated VC1 tandem domain of RAGE was generated using the coordinates of VC1 with PDB accession code 3CJJ [14] and the program SWEET [34]. The typical “core” type oligosaccharide moiety of *P. pastoris*, comprising 8 mannose residues (Man) and 2 *N*-acetyl glucosamine residues (GlcNac), was used in the model [35].

## 3. Results

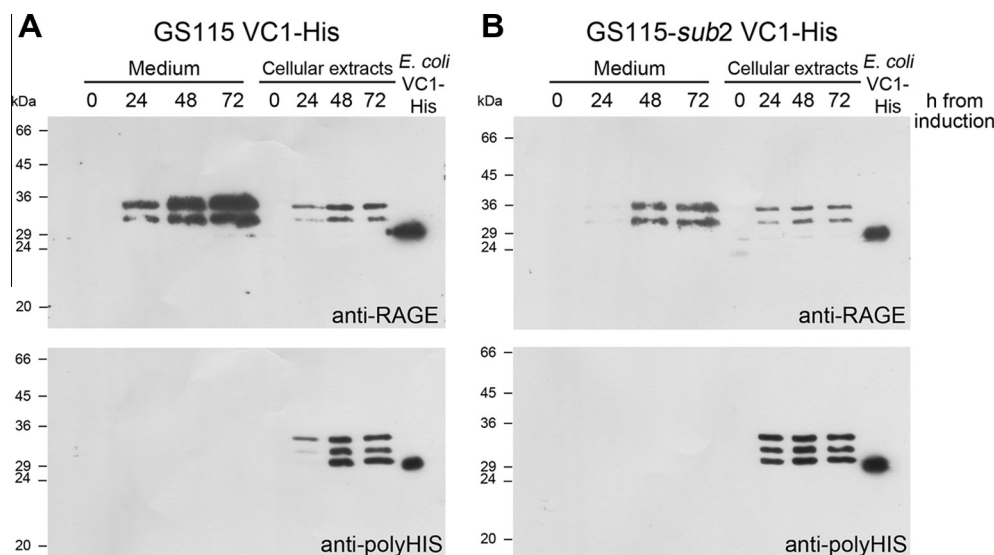
### 3.1. Expression of His-tagged VC1 fragment of hRAGE reveals proteolytic activities in the medium and inside *P. pastoris* cells

To produce and secrete the VC1 and V domains in *P. pastoris*, the human N-terminal signal peptide was replaced by the signal sequence of *P. pastoris* PHO1 encoding the extracellular acid phosphatase Pho1p (see scheme in Fig. 1). Expression was driven by the strong methanol-inducible promoter AOX1. VC1 and V domains were tagged with a thrombin cleavable hexa-His sequence at the C-terminus to facilitate purification and allow the removal of the tag to avoid interference in binding studies (Fig. 1). Two bands of about 34 and 36 kDa were detected by immunoblot with anti-RAGE antibody in the medium of *P. pastoris* cells expressing VC1-His, but these polypeptides did not react with anti-poly(His) mAb, indicating that the tag was removed (Fig. 2A). To ascertain whether the tag was cleaved inside the cell or after secretion, whole cell extracts were analyzed. Both anti-RAGE antibody and anti-poly(His) mAb recognized the two bands, indicating that the tag was removed outside the cell (Fig. 2A). Interestingly, in the cell extracts an additional band of about 30 kDa was detected exclusively by the anti-poly(His) antibody suggesting that this protein is devoid of the N-terminal epitope recognized by the anti-RAGE antibody (Fig. 2A). This N-terminal truncated form likely represents an intermediate of VC1 degradation that probably occurs at the level of the ER.

MS analysis of the secreted VC1 protein did not detect the C-terminal His-tag (data not shown). These results are consistent with the proteolytic cleavage of the His-tag by an extracellular protease. To verify whether the tagged VC1 forms were substrates of Sub2p, the major secreted subtilisin-like protease of *P. pastoris* [23], we analyzed the production of the tagged VC1 in a



**Fig. 1.** Schematic representation of the constructs used in this work. The upper scheme shows the domain structure and the N-glycosylation sites of the full-length hRAGE. After the N-terminal signal peptide, three immunoglobulin-like domains (V, C1 and C2) are present, followed by the trans-membrane domain and a short cytoplasmic tail. The numbers indicate the amino acid sequence positions at the domain borders. The sequence of the thrombin recognition site (T), present in the constructs carrying a C-terminal 6x His Tag (H), is LVPRGS (with cleavage between R and G).



**Fig. 2.** Immunoblot analysis of culture supernatant and extracts of cells expressing VC1-His. Culture supernatants and cell extracts of GS115 (A) and GS115-*sub2* (B) expressing VC1<sup>233</sup>-His were analyzed by immunoblotting using anti-poly(His) mAb or anti-RAGE antibody as indicated. VC1-His expressed in *E. coli* was used as positive control. Culture supernatants (110  $\mu$ L) of the indicated time points were denatured and loaded on SDS-slab gels. Cell extracts were obtained as described in Section 2. Cell extracts immunostained with anti-Poly(His) mAb contain an extra band of ~33 kDa ascribable to a VC1 proteolytic product lacking the N-terminal epitope.

*sub2*-deficient strain (Fig. 2B). VC1-His forms were detected in cell extracts but only the untagged forms were present in the medium suggesting that a secreted protease other than Sub2p is responsible of the proteolytic cleavage of the tag.

### 3.2. The histidine tagged V domain of hRAGE does not properly fold when expressed in *P. pastoris*

Fusion of the V domain to the His-tag, resulted in the accumulation in *P. pastoris* cells of polypeptides of about 20, 21 and 16 kDa recognized by the anti-poly(His) mAb (Fig. 3, left panel). Treatment with PNGase F indicated that such recombinant products represent different glycosylated forms of the V domain (data not shown). These proteins reached a maximal level after 48 h induction (Fig. 3, left panel). None of these polypeptides reacted with the anti-RAGE antibody (Fig. 3, right panel), indicating that the N-terminal epitope was removed, as described above for the 30 kDa form of VC1-His. Moreover, no V-His forms were detected in the culture supernatant (data not shown). Despite several attempts to overcome the block of secretion by employing a mixed induction regimen or lowering the growth temperature, no secretion of V-His was observed.

These results strongly suggest that the isolated V-His domain is incorrectly folded in *P. pastoris* and likely undergoes degradation in the ER and/or in the vacuole.

### 3.3. Expression and secretion of the VC1 domain of hRAGE in *P. pastoris*

VC1 and V domains were subsequently expressed in *P. pastoris* without a tag (Fig. 1). No specific bands of the V domain were detected in the culture supernatant, even after 30-fold medium concentration, by SDS-PAGE and Coomassie staining or by immunoblotting (data not shown). No further attempts to secrete this protein were performed and the focus was placed on untagged VC1. The growth kinetics of cells expressing VC1 is shown in Fig. 4A. After the shift to methanol containing medium (time zero), cells grew exponentially for about 24 h and then the growth rate progressively decreased. Two bands of ~34 and ~36 kDa were detected by SDS-PAGE analysis in the culture medium at 24, 48 and 72 h after induction but not in the medium of non-induced

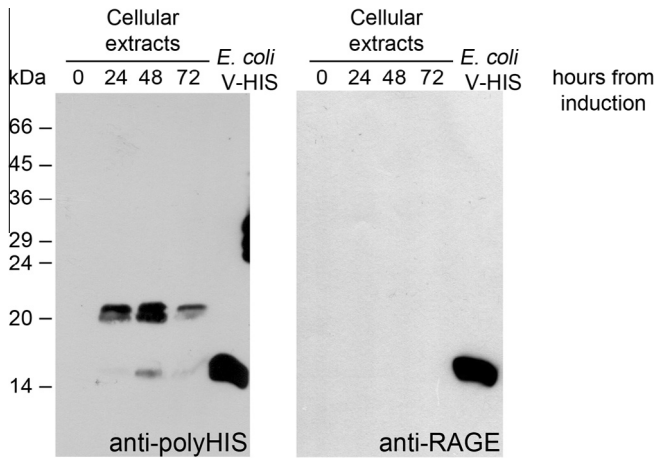
cells (Fig. 4B) and cross-reacted with anti-RAGE antibody (Fig. 4C). The two recombinant VC1 forms were named p34 and p36. Mass spectrometry (MS) analysis confirmed the identity of the two bands as proteins forms with the expected human VC1 sequence with coverage of 77.36% for p36 and 81.60% for p34. Moreover, the identification of the RAQNITAR peptide as the N-terminal end indicated that the cleavage of the Pho1p signal peptide occurs at the expected site.

VC1 levels in the medium were quantified, as described in the Materials and methods. The highest level, about 6 mg of total VC1 per liter culture medium, was reached after 72 h of induction, a time concomitant with the growth arrest (Fig. 4A). Protein production was highly reproducible among different recombinant clones. No substantial improvement in protein production was obtained by lowering the growth temperature to 23 °C or using unbuffered media (MMY instead of BMMY). Therefore, buffered rich medium (BMMY) and 28 °C were chosen as standard growth conditions.

### 3.4. VC1 is N-glycosylated in *P. pastoris* and mimics the human site occupancy

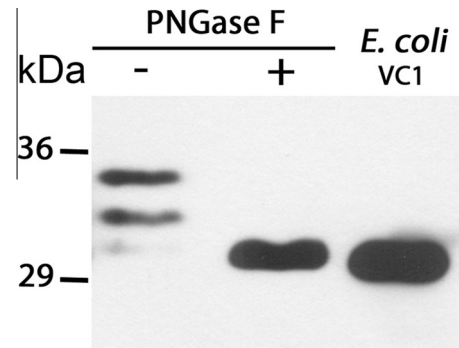
p34 and p36 polypeptides displayed a lower electrophoretic mobility compared to VC1 expressed in *E. coli* (p32<sup>Ec</sup>), a sign of the presence of post-translational modifications. Since two potential N-glycosylation sites are located in the V domain, VC1 was subjected to treatment with PNGase F, an enzyme that removes the N-linked oligosaccharide chains and concomitantly converts the asparagine residue (Asn) into an aspartic acid (Asp). Upon deglycosylation, p36 and p34 were no longer detected and a polypeptide of ~32 kDa appeared. This indicates that VC1 is N-glycosylated in *P. pastoris* and that the observed mobility differences are due to heterogeneity of the glycan moiety (Fig. 5). These results are compatible with the presence of 1 and 2 “core” type N-linked oligosaccharide chains in p34 and p36, respectively.

hRAGE contains two potential N-glycosylation sites, or “sequons” (consensus sequence: NX(S/T) where X  $\neq$  P), namely Asn25 (NIT) and Asn81 (NGS) where the numbering refers to the precursor protein. The two sites correspond to amino acid position 26 and 82 in the recombinant precursor but for the sake of clarity



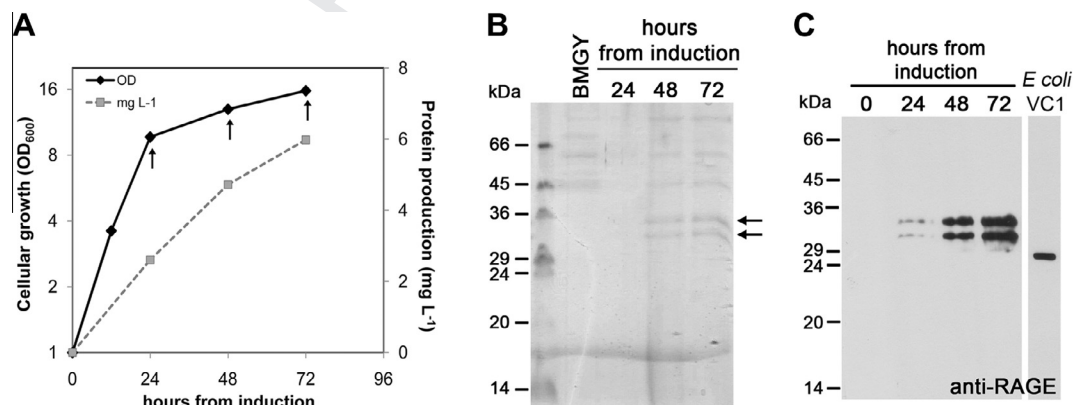
**Fig. 3.** Immunoblot analysis of extracts of cells expressing V-His. Total extracts of GS115 cells expressing V<sup>121</sup>-His harvested after the indicated induction times were analyzed by immunoblotting using both anti-poly(His) mAb and anti-RAGE antibody. V-His expressed in *E. coli* was used as positive control. The cell extracts were obtained as described in Section 2. The lack of reactivity with the anti-RAGE antibody is ascribable to a proteolytic cleavage occurred intracellularly at the N-terminus as shown in Fig. 2 for VC1.

here they will be referred to the numbers of the human pre-protein. To address the question whether such sites are N-glycosylated in VC1 from *P. pastoris*, we mapped the Asp residues generated by the enzymatic removal of N-linked chains by MS analysis. Tryptic digests of gel-purified secreted VC1 were analyzed by nano LC-MS/MS. Peptides were identified by database matching and the intensity of the peaks corresponding to the tryptic peptides containing the deamidated Asn25 and Asn81 were quantified relative to their unmodified form. The extent of p34 and p36 glycosylation was evaluated on the tryptic peptides RAQN<sup>25</sup>ITAR and VLPN<sup>81</sup>GSLFLPAVGIQDEGIFR. Their natural, non-glycosylated forms were expected to appear in MS spectra at theoretical m/z values of 465.26740 and 1121.61512, respectively, for the double charged ions. In the presence of Asn to Asp conversion, RAQD<sup>25</sup>ITAR and VLPD<sup>81</sup>GSLFLPAVGIQDEGIFR were expected to display the increased m/z values of 465.75940 and 1122.10712. The high resolution of LTQ-Orbitrap XL and fragmentation spectra unambiguously discriminated the natural peptides from their deamidated variants.



**Fig. 5.** Analysis of the recombinant VC1 glycosylation. Immunoblot analysis using anti-RAGE antibody of culture supernatant harvested after 72 h of induction and incubated in the absence (-) or presence (+) of PNGase F. Purified recombinant VC1 produced in *E. coli* was used as a control.

Supplementary Fig. 1 reports the extracted ion chromatograms. The peptide RAQN<sup>25</sup>ITAR was not identified in the untreated VC1 sample (p34 and p36 bands), suggesting that this peptide is fully glycosylated in *P. pastoris* (Supplementary Fig. 1A). Consistently with this hypothesis, its variant RAQD<sup>25</sup>ITAR was identified in the deglycosylated p32. This observation was confirmed by the peaks corresponding to the two peptide variants; the peak at m/z 465.26740 was barely visible in p34 and p36, while an intense peak at m/z 465.75940 was indeed visible in the deglycosylated sample (about 3 orders of magnitude). This indicates that chemical deamidation is negligible and thus Asn25 is fully glycosylated in VC1. In contrast with RAQN<sup>25</sup>ITAR, the peptide VLPN<sup>81</sup>GSLFLPAVGIQDEGIFR was detected in the untreated VC1 sample, indicating that Asn81 is present both in glycosylated and unglycosylated state (Supplementary Fig. 1B). Consistently, its deamidated variant was identified upon enzymatic deglycosylation (p32 band). Moreover, the peptide VLPD<sup>81</sup>GSLFLPAVGIQDEGIFR was also detected in the untreated VC1 sample indicating that chemical deamidation occurs at Asn81 as already reported for hRAGE expressed in human cell lines [24]. Following PNGase F treatment, the ratio of peak intensities of deamidated versus unmodified peptide increased, confirming that a subpopulation of Asn81 is glycosylated as reported also by Srikrishna et al. [25]. In order to view the localization of both glycosylation sites, we generated a 3D molecular model of the glycosylated VC1 domain



**Fig. 4.** Kinetics of accumulation of recombinant VC1 in *P. pastoris* culture medium. (A) Growth kinetics at 28 °C of a representative culture of *P. pastoris* cells transformed with pHL-S1-VC1<sup>233</sup> plasmid (solid line). Time zero indicates the moment of the shift from BMGY to the inducing medium, BMMY. The arrows indicate the time points of the withdrawal of culture aliquots for analysis. The recombinant protein level (dashed line) in the culture supernatant was estimated by densitometry as reported in Section 2 using immunoblot with anti-RAGE antibody and recombinant VC1 isolated from *E. coli* as a calibrator. (B) SDS-PAGE of culture supernatant (160 µL) collected at the indicated induction times. Protein bands were stained by Coomassie blue. (C) Immunoblot analysis of the same samples of panel (B). Culture supernatant volumes loaded on the gel were 112 µL for 0 and 24 h, and 100 µL for 48 and 72 h. Recombinant VC1 produced in *E. coli* was used as a control.



(Supplementary File 2). In the model, Asn25 and Asn81 carry a branched Man<sub>8</sub>-GlcNac<sub>2</sub> oligosaccharide that is representative of the short oligosaccharides present on secreted glycoproteins in *P. pastoris* and mainly distributed between Man<sub>8,9</sub>-GlcNac<sub>2</sub> (75%) and Man<sub>10,11</sub>-GlcNac<sub>2</sub> (25%) [35].

Overall, our analyses suggest that Asn25 is fully occupied whereas only a subpopulation of Asn81 is glycosylated as occurs in human cells [24]. Finally, the difference between p34 and p36 electrophoretic mobilities suggests that the *N*-linked chains are of “core” type, consistently with the notion that hypermannosylation of the *N*-linked oligosaccharide chains is rare in *P. pastoris* in contrast to *Saccharomyces cerevisiae*.

### 3.5. VC1 is a monomer and is functional

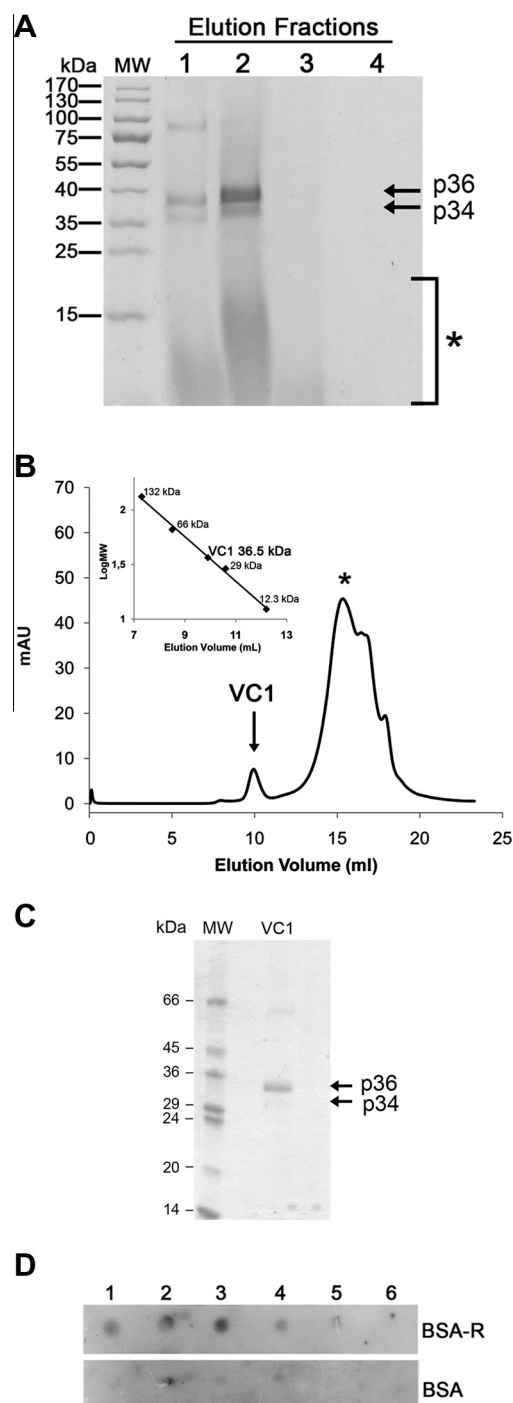
VC1 was purified from the culture supernatant by cation-exchange chromatography as the capture step, exploiting the high charge of the protein (pI = 9.9) and a final gel filtration polishing step. As shown in the SDS-PAGE of Fig. 6A, the one-step elution from the cation-exchange column yielded highly enriched p34 and p36 forms, only contaminated by a small amount of a lower mobility protein and abundant heterogeneous low M<sub>r</sub>-components of the growth medium. Gel filtration studies yielded pure VC1 that eluted in a single sharp peak corresponding to monomeric VC1 forms. The low M<sub>r</sub> contaminants eluted as a broad peak at higher elution volume (Fig. 6B). It was well expected that glycosylated VC1 is monomeric under the chosen acidic and high salt buffer conditions, as previously observed for un-glycosylated VC1 expressed in *E. coli* [14].

The functionality of purified VC1 was tested by nitrocellulose filter-binding assay (Fig. 6D). Different amounts of BSA and AGE-BSA were absorbed onto nitrocellulose in dot-blot format. Following incubation of the membrane with purified VC1, bound protein was detected by anti-RAGE antibody as described under Materials and Methods. VC1 binding to AGE-BSA but not to BSA indicated that the protein is functional (Fig. 6D).

### 3.6. Analysis of the thermal stability of the purified VC1 glycosylated forms

During the optimization and scale-up of the VC1 purification protocol, we developed a procedure based on an ionic-strength linear gradient for the fractionation of the concentrated culture supernatant by high-resolution cation-exchange chromatography. Such an improved procedure yielded the two individual glycosylated species of VC1 in homogeneous form. Indeed, p34 and p36 separated in two partially overlapped peaks, allowing their isolation in homogeneous form at the expenses of a loss in recovery (Fig. 7). Since the pI of the two glycoforms is expected to be the same, the separation likely reflects the different steric hindrance caused by the presence of one or two glycans which affects the affinity of the glycoprotein for the resin. Indeed, p36 with two glycans eluted before p34, which contains just one glycan. As shown in Fig. 7, VC1 glycoforms were well separated from low M<sub>r</sub>-components of the growth medium that eluted during the first part of the salt gradient. The immunoblot shown in Fig. 7 also indicates that no relevant protein degradation occurred during purification. Overall, this procedure of purification provided pure VC1 with a minimum loss of the target protein. Table 2 summarizes the progress of the purification attained from 1 L-culture.

Recently, ThermoFluor emerged as a powerful technique to evaluate the conformational stability of recombinant proteins [26]. Therefore, thermal denaturation analysis of p36, p34 and of the unmodified form produced in bacteria (p32<sup>EC</sup>) was performed at different ionic strengths. Melting temperatures (T<sub>m</sub>) obtained



**Fig. 6.** Purification of VC1 from the culture supernatant and filter binding assay. (A) Result of separation by cation-exchange chromatography and one-step elution of the culture supernatant at 72 h of induction. The Coomassie stained gel is shown. The arrows indicate p36 and p34; \* indicates low M<sub>r</sub>-components of the medium. (B) Profile of gel filtration chromatography on a Superdex 75 column of the partially purified VC1 forms. The peak eluted at 10 mL corresponds to VC1 forms, while those eluted between 13 and 18 mL correspond to the low M<sub>r</sub> components of the medium (predominantly peptones \*). Standard proteins were chromatographed under the same conditions to obtain the calibration curve shown in the inset (inset). (C) SDS-PAGE and Coomassie staining of the fractions corresponding to the VC1 peak. Proteins were precipitated by 10% TCA. (D) Filter binding assay. 2 μL of a concentrated solution of BSA or BSA-R (2 mg/mL) (1) and 1:2 serial dilutions (from 2 to 6) were spotted on nitrocellulose filter that was incubated with purified VC1 as described in Section 2. VC1 specifically recognizes BSA-R indicating that the protein expressed and purified from *P. pastoris* is functional.

for the various proteins are reported in Table 3. The two glycoforms of VC1 produced in *P. pastoris* are equally stable in 150 or 300 mM NaCl whereas p36 appears to be more stable than p34 in the absence of NaCl. In addition, the two glycoforms are more stable than p32<sup>Ec</sup>. In particular, in the absence of NaCl, p32<sup>Ec</sup> is highly unstable ( $T_m = 22.6$  °C). Thus, VC1 forms produced in *P. pastoris* displayed a remarkable improved stability in comparison with the unmodified p32<sup>Ec</sup>. These observations are consistent with the fact that VC1 glycoforms obtained from *P. pastoris* are highly soluble and display no tendency to aggregation that is, in contrast, a typical feature of p32<sup>Ec</sup> (data not shown).

#### 4. Discussion

We here demonstrated that the VC1 tandem domain of hRAGE can be produced in a secretory form in *P. pastoris*. Recombinant VC1 can be purified to homogeneity from the concentrated culture supernatant by cation-exchange chromatography with no relevant protein loss during the purification step. Moreover, no other highly basic protein appears to be secreted by *P. pastoris*. The level of VC1 produced by flask cultivation reached about 6 mg/L after 72 h of induction but by using fermentation-based technologies this level is expected to increase as higher cell density can be reached.

VC1 from *P. pastoris* shows no tendency to aggregate/precipitate. Compared to VC1 produced intracellularly in *E. coli* (p32<sup>Ec</sup>), the secreted protein is remarkably more stable as demonstrated

**Table 2**  
Large-scale purification of VC1 from *P. pastoris* medium.<sup>a</sup>

Purification step	VC1 protein (mg)	Yield (%)
Culture supernatant <sup>b</sup>	6.00 <sup>c</sup>	100
Concentration <sup>b</sup>	5.88	98
Cation exchange chromatography <sup>b</sup>	5.21	86
p36 form	1.45	24
p34 form	1.52	25
Cross-contaminated p34 p36 forms <sup>d</sup>	2.24	37

<sup>a</sup> The starting material was the supernatant from a yeast 1-L culture harvested after 72 h induction at the OD<sub>600</sub> of 13.

<sup>b</sup> Data refer to total VC1, i.e., the sum of the two p36 p34 glycoforms.

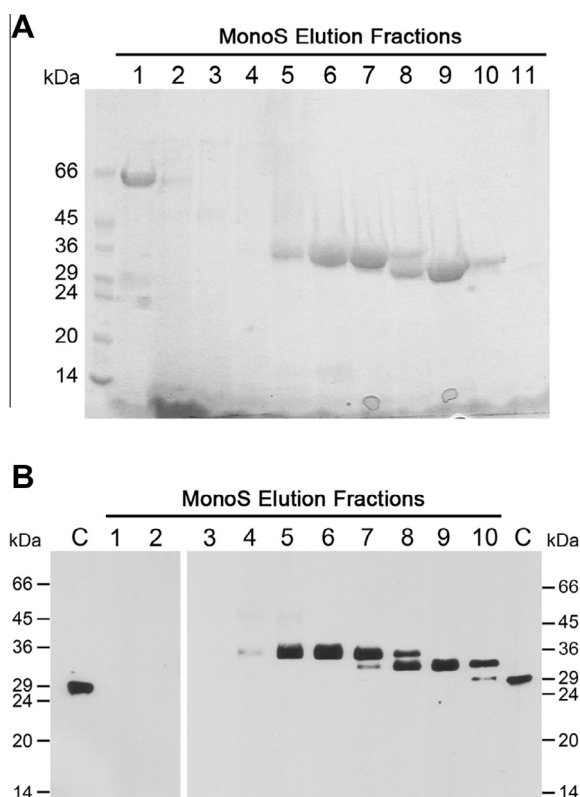
<sup>c</sup> Determined by densitometry, since the high concentration of peptides in the medium precluded the determination of the actual total protein concentration.

<sup>d</sup> Purified VC1 not separated into its individual glycoforms.

**Table 3**  
Melting temperatures of glycosylated and unglycosylated recombinant VC1 forms.

Protein	NaCl (mM)		
	0	150	300
p36	35.0 ± 0.3	43.8 ± 0.1	48.2 ± 0.01
p34	31.6 ± 0.8	43.8 ± 0.2	47.2 ± 0.1
p32 <sup>Ec</sup>	22.6 ± 0.1	37.0 ± 0.2	42.0 ± 0.02

The values indicate the  $T_m$  (°C) ± S.D. (standard deviation) from triplicate samples. VC1 produced in *P. pastoris* (p36 and p34) and in *E. coli* (p32<sup>Ec</sup>) were analyzed in 10 mM Na-acetate, pH 5.0, in the presence of the indicated saline concentration.



**Fig. 7.** Large-scale purification and separation of p34 and p36 glycoforms of VC1 by cation-exchange chromatography. (A) Cation-exchange chromatography on a Mono S column of 400-mL culture supernatant collected after 72 h of induction and 13× concentrated by ultrafiltration (membrane cut-off 30,000). The fractions eluted using a linear ionic-strength gradient were analyzed by SDS-PAGE and proteins were stained by Coomassie blue (B). Immunoblot with anti-RAGE antibody indicates that fractions 5–6 contain only p36, fractions 7 and 8 contain both forms and 9–10 contain only p34. The 66-kDa polypeptide visible in panel (A) (lane 1) is a contaminating endogenous protein. VC1 purified from *E. coli* (p32<sup>Ec</sup>) was used as a control (C).

by thermal shift assays. The increased solubility of glycosylated VC1 most likely arises from masking hydrophobic patches on the surface of VC1 and from hydrophilicity glycans typically confer to proteins. Most importantly, VC1 produced in *P. pastoris* proved to be functional, as verified by binding to AGE-BSA. In contrast with these findings, the V module of hRAGE was retained intracellularly in *P. pastoris*. The ER harbors an ER protein quality control (ERQC) that ensures only properly folded and assembled proteins traffic to the Golgi apparatus. Proteins that do not pass ERQC are diverted by retro-translocation back to the cytosol for degradation by the 26S proteasome in a process termed ER-associated degradation (ERAD). Moreover, a previous work in *P. pastoris* suggested that misfolded proteins can also be transported from the Golgi to the vacuole for degradation [27].

Previous studies indicated that the isolated V, and to a lower extent also VC1 domain, produced in *E. coli* possess a highly dynamic conformation and that V requires C1 association to fold properly [17,28]. Thus, our finding that V does not acquire the proper folding in *P. pastoris* is consistent with, and also extends, previous biophysical *in vitro* studies [17]. Molecular models based on X-ray crystallography and NMR, support the notion that V and C1 domains physically interact through surfaces exposing hydrophobic amino acids [14]. Our results obtained using an *in vivo* approach, support such observations and strongly indicate that V and C1 domains form a single folding unit. Consequently, the V unit should be described as a protein module rather than a domain. Our study also provides evidence of a cellular protease that removes the N-terminal portion of the V module. This protease likely is a component of the ERQC of *P. pastoris*.

This work also showed the full glycosylation occupancy at Asn25 and the partial occupancy at Asn81 in the VC1 forms produced in *P. pastoris*. This glycosylation pattern mimics that observed in human cells [24] indicating that it is driven by intrinsic features of VC1, such as the target sequences, its location in the three-dimensional structure of the protein and the microenvironment. Moreover, we have shown that Asn81 undergoes partial deamidation. Interestingly, as previously reported for Asn81 of hRAGE from mammalian sources and also for different types of

antibodies, specific asparagine residues can undergo non-enzymatic deamidation [16,24]. The susceptibility to deamidation markedly increases if a glycine residue is immediately after the asparagine [29]. In this case, deamidation occurs via cyclization of asparagine by the  $\beta$ -aspartyl shift mechanism in which the main chain peptide nitrogen of the glycine residue acts as a nucleophile on the Asn side chain amide group forming a succinimide intermediate that breaks down in  $\alpha$ - or  $\beta$ -isomeric aspartate products [29]. Interestingly, Asn81 is located in the sequon N-Gly-S providing an explanation of the marked propensity for deamidation of this site in hRAGE.

A polymorphism occurs at the Asn81 sequon in humans and results in the Gly82Ser replacement that leads to G/S or S/S genotypes, often associated with diseases. G/S (heterozygous) individuals are more prone to undergo diabetic retinopathy with respect to G/G (homozygous) patients in the Chinese population [30,31]. An association of the S allele with microvascular complications in diabetic patients has been reported and the S/S genotype has been correlated to low levels of circulating sRAGE [30]. Notably, the G82S replacement increases the occupancy of Asn81 glycosylation site and this could affect AGE-RAGE binding [24].

Finally, glycosylation was proposed to affect RAGE ligand binding specificity. For example, the lack of glycans in bacterial recombinant proteins is accompanied by increased affinity for some ligands, while others have been shown to bind more tightly to glycosylated RAGE [25,32]. Thus, the availability of glycosylated VC1 forms from a eukaryotic microorganism can prove useful in future studies. It is important to note that *P. pastoris* was also engineered to produce humanized N-glycan chains, an important accomplishment in the perspective to produce biotherapeutic products in this yeast [33]. However, irrespective of the role of specific glycan requirements for ligand binding, the VC1 forms produced in *P. pastoris* proved to be more stable and soluble than the non-glycosylated VC1 produced in bacteria, suggesting that the oligosaccharide chains promote the proper folding and are important for protein stabilization and solubility. These features are expected to be beneficial for the *in vitro* studies of the properties of RAGE ligand binding domain and for the set-up of high throughput screening methods for the identification of RAGE antagonists.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.pep.2015.06.012>.

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