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Nonclassical secretion of human catalase on the surface of CHO cells is more efficient than classical secretion

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Abstract

There is a great demand for improved production of therapeutic proteins using mammalian cell expression systems and transgenic animals. There have been intensive endeavors to optimize production at the transcriptional and translational levels, but comparatively little attention has been paid to the secretory level, especially to nonclassical secretion. To compare the efficiencies of classical and nonclassical secretion, we expressed GFP-tagged human catalase conjugated with a classical signal peptide and with several short peptides derived from mouse Engrailed 2 (mEN2) homeoprotein for nonclassical secretion and internalization in CHO cells. Surprisingly, the results showed that the secretory efficiency was significantly greater (up to 2.3 fold) than classical secretion when the fusion protein was driven by the secretory sequence (SS) of mEN2, and up to 1.9 fold when the classical secretion process was modified by incorporating the internalization sequence (IS) of mEN2. The effect of these short peptides on nonclassical secretion and internalization may indicate potential applications in the improved production of complex therapeutic proteins in mammalian cell expression systems and transgenic animals.

Keywords: Signal peptide; Nonclassical secretion; Homeodomain; Human catalase

1. Introduction

Recombinant DNA technology has been used for producing therapeutic proteins for decades. Currently, most therapeutic proteins are produced from bacteria and mammalian cell expression systems. Although bacteria grow quickly, are easier to handle for genetic modification and cost less in industrial production than mammalian cell fermentation, they have unavoidable disadvantages in producing mammalian proteins. A major problem is that they cannot carry out complex post-translational modifications of recombinant proteins. Bacteria also lack appropriate chaperones, and that may put the recombinant protein at risk of misfolding. These disadvantages can be overcome by using a mammalian cell expression system (Wurm, 2004). However, although intense efforts have been made to optimize recombinant protein production in mammalian cells, such expression systems are still economically uncompetitive compared to their prokaryotic counterparts. An alternative way to improve recombinant protein production from mammalian sources is to utilize the animal mammary gland as bioreactor. Using this approach, the protein of interest is targeted for expression in the animal mammary gland, and then purified from the milk. Compared with large-scale cultivation of cells, the lower production cost and the higher production capacity of safer, pathogen-free product makes the mammary gland bioreactor very attractive. In addition, the purification process follows the same basic principles that are used to purify recombinant products from mammalian cell cultures (Clark, 1998; Dunn et al., 2005; Hennighausen, 1990; Morcol et al., 1994). ATryn, the first recombinant

Abbreviations: mEN2, mouse Engrailed 2; ER, endoplasmic reticulum; SS, secretory sequence; NES, nuclear export sequence; IS, internalization sequence; PTS, peroxisomal targeting sequence.

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protein derived from a transgenic animal, was approved for use in Europe by the European Medicines Agency in 2006. This heralds the commercial exploitation of animal transgene expression systems.

Much work is currently being undertaken to optimize the production of recombinant protein from mammalian cells and transgenic animals, focusing mainly on improving protein production through modification at the transcriptional and translational levels, e.g. the selection of strong promoters, incorporation of enhancer and insulator, and codon modification (Fiering et al., 2000; Lee and Young, 2000; Verrijzer et al., 1995; Zhang et al., 2005). However, less attention has been paid to improving the efficiency of secretion, especially in transgenic animals.

Soluble proteins destined for secretion are usually characterized by an N-terminal signal peptide, which mediates co- or post-translational import to the lumen of the endoplasmic reticulum (ER). The translocated proteins are then delivered to the cell surface by ER/Golgi-dependent vesicular transport (Rapoport et al., 1996, 1999; Walter et al., 1984). However, several groups of proteins such as the transcriptional factors Engrailed, Hoxa5 and Pax6 and the viral proteins HIV Tat and Herpes virus VP22 have been identified as secretory proteins, but lack signal peptides. The secretion of the these proteins has been termed "non-classical" (Arnoys and Wang, 2007; Brooks et al., 2005; Cleves, 1997; Prochiantz, 1999, 2000; Prochiantz and Joliot, 2003; Sagan et al., 2006). Although several nonclassical secretion pathways may exist, the molecular mechanisms remain to be revealed (Derossi et al., 1996; Joliot et al., 1997; Nickel, 2003).

We found that recombinant human catalase was actively synthesized in the mammary glands of transgenic mice, but the protein was less effectively secreted to the milk by the classical secretion pathway (manuscript in preparation). Human catalase is a homotetrameric enzyme with heme and NADPH as cofactors (Safo et al., 2001). Catalase is mainly present in the matrix of peroxisomes and is responsible for the decomposition of hydrogen peroxide into water and oxygen, decreasing the levels of reactive oxygen species (ROS), which are closely related to the aging process (Schriner et al., 2005; Zhou and Kang, 2000). We suppose that the complex three-dimensional structure of human catalase, normally a non-secreted protein, may inhibit its smooth transport through the ER and Golgi by the classical secretory pathway. However, it may not be retarded in the ER or Golgi compartments by the nonclassical secretion pathway. In this study, we combined short peptides derived from the mouse Engrailed 2 (mEN2) homeodomain, and the bovine β-casein signal peptide, with human catalase and assessed the efficiency of secretion of these recombinant proteins in CHO cells via the nonclassical and classical pathways. The ultimate aim is to design an ideal short peptide for the efficient secretion of complex therapeutic proteins to the milk of transgenic animals.

2. Materials and methods

2.1. Expression vector constructs

The CDS of the human catalase gene was amplified from a commercial EST clone (Imgage 4515735, Invitrogen) with a pair of primers: forward, 5′-TTGCTAGCAGATGAAGGTC CTCATCC-3′ and reverse, 5′-TTCTGCAGCAGATTTGCCTT CTCCCT-3′. PCR amplification was performed under the following conditions: denaturation at 95 °C for 5 min, then 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min for a total of 30–35 cycles. The 1.7 kb long PCR product with NheI and PstI restriction sites on each end was then subcloned into a mammalian cell expression vector, pEGFP-N1 (Clontech), fusing GFP to the C-terminus of human catalase. Synthetic oligonucleotides corresponding to the sequences of the bovine β-casein signal peptide (Sig), NES and SS of mEN2, and SigIS–SigSS combinations were cloned upstream of the CDS to obtain the ultimate expression vectors (Fig. 1).

2.2. Cell culture and transfection

CHO cells (Invitrogen) were cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg /ml streptomycin. The cells were incubated in a humidified atmosphere of 5% CO_2 in air at 37 °C, and transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Optimal transfection efficiency was achieved using a mixture of 4 μg DNA and 10 μl Lipofectamine 2000 reagent in 2 ml culture medium containing 6-8 × 10^5 cells at 90% confluence.

2.3. RT–PCR and Western blot analysis

Total RNA was extracted from the transfected CHO cells (grown on six-well cell culture plates for 24 h at 37 °C) using TRIzol (Invitrogen) according to the manufacturer's instructions. The purified RNA was further incubated with DNase I (Sigma) to digest the contaminating DNA. Oligo dT-adaptor

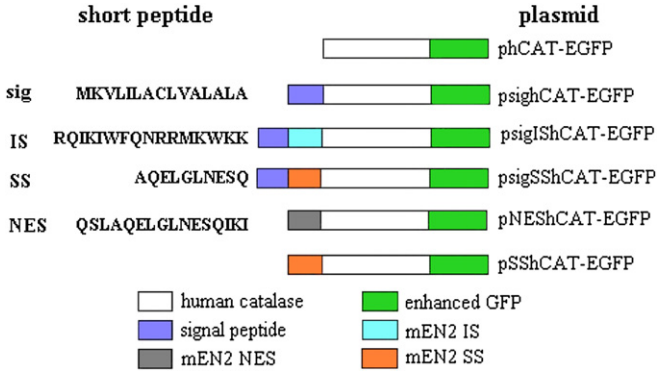

Fig. 1. Scheme of expression vector construction. The plasmids encoding the proteins to be expressed in the CHO cells are depicted. Each protein is fused with enhanced GFP on its carboxylic terminus. The plasmid phCAT-EGFP encodes GFP-fused human catalase - without conjugation of the short peptide on the N-terminus; the plasmids psighCAT-EGFP, pNEShCAT-EGFP and pSShCAT-EGFP encode fusion proteins correspondingly combined with the bovine signal peptide MKVLILACLVALALA, mouse Engrailed 2 (mEN2) nuclear export sequence (NES) QSLAQELGLNESQIKI, and mEN2 secretion sequence (SS) AQELGLNESQ; the plasmids psigIShCAT-EGFP and psigSShCAT-EGFP encode fusion proteins with combinations of the signal peptide and the mEN2 secretion sequence, mEN2 internalization sequence (IS) RQIKIWFQNRRMKWKK, respectively.

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primers were used to synthesize cDNA by AMV Reverse Transcriptase at 42 °C for 1 h, then a 184 bp length fragment of the human catalase gene was amplified by the following primers: forward, 5′-TGCTGAATGAGGAACAGAGG-3′ and reverse, 5′-GTGTGAATCGCATTCTTAGG-3′. PCR amplification was performed under the following conditions: denaturation at 95 °C for 5 min followed by 94 °C for 30 s, 56.5 °C for 30 s and 72 °C for 1.5 min for a total of 30–35 cycles.

Cells transfected with appropriate constructs and control vector were seeded in six-well plates, each well containing 2 ml of fresh medium, and harvested after incubation at 37 °C for 24 h. Samples of the medium were collected from the same wells. To avoid interference from detached cells, all medium samples were centrifuged at 1000 rpm for 10 min at 4 °C and the supernatants were transferred to fresh tubes. A cell lysis buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1% Triton X-100 and several protein inhibitors such as sodium pyrophosphate, β-glycerophosphate, EDTA, Na_3VO_4 and leupeptin (Beyotime Biotechnology, China) was added to the wells. After incubation for 5 min at room temperature, cell debris was removed by centrifugation at 10000 rpm for 10 min at 4 °C. Supernatants (cell extracts), together with the medium samples, were assayed for recombinant protein. The protein concentrations in the samples were estimated using a Bradford protein assay kit (Beyotime Biotechnology). Proteins were separated by electrophoresis on 10% SDS–polyacrylamide gels, then transferred to nitrocellulose membranes (Amersham) and blotted with sheep polyclonal antibody to catalase (1:1000) (Abcam) and HRP-conjugated rabbit anti-sheep IgG antibody (1:2500) (KPL) as recommended by the manufacturer.

2.4. Catalase activity

Transfected CHO cells were grown on T-25 flasks for 24 h at 37 °C. The culture medium and cell extract were collected for measurements of human catalase activity by a catalase analysis kit (Beyotime Biotechnology) as per the manufacturer's instructions. Briefly, samples were treated with excess hydrogen peroxide for decomposition by catalase for an exact time, and the remaining hydrogen peroxide coupled with a substrate was treated with peroxidase to generate a red product, *N*-4-antipyryl-3-chloro-5-sulfonate-*p*-benzoquinonemonoimine, which absorbs maximally at 520 nm. Catalase activity was thus determined by measuring the decomposition of hydrogen peroxide spectrophotometrically. The protein concentration was measured with a Bradford protein assay kit (Beyotime Biotechnology).

2.5. Confocal microscopy

Transfected CHO cells were grown on glass coverslips for 24 h at 37 °C, and fixed with paraformaldehyde (4% w/v) for 10 min at 4 °C without permeabilization. Sheep polyclonal antibody to catalase (1:1000) (Abcam) and phycoerythrin (PE)-conjugated donkey anti-sheep IgG antibody (1:2000) (Abcam) were used for cell surface immunodetection. The specimens were counterstained with DAPI, mounted in an anti-bleach medium (2.5% w/v DABICO, 90% v/v glycerol, 50, ChinamM Tris) and viewed with a Nikon C1 Si confocal microscope.

2.6. Quantification of secretion efficiency

Confocal sections (low magnification) were processed by NIH Image J. Briefly, PE-derived red fluorescence and GFP-derived green fluorescence were quantified (Area × Intensity) in the successfully transfected CHO cells. The red/green ratio was a measure of the secretory efficiency of human catalase driven by the different short peptides.

3. Results

3.1. Expression of biologically active human catalase in CHO cells

CHO cells transfected with plasmids pEGFP-N1, phCAT-EGFP, psighCAT-EGFP, psigIShCAT-EGFP, psigSShCAT-EGF, pNEShCAT-EGFP and pSShCAT-EGFP were respectively named CHO_{EGFP}, CHO_{hCAT}, $CHO_{sighCAT}$, $CHO_{sigIShCAT}$, $CHO_{sigSShCAT}$, $CHO_{NEShCAT}$ and CHO_{SShCAT}. The transfection efficiency was 70% for CHO_{EGFP}, but this decreased to around 40% for the larger constructs. Twenty-four hours after transfection, human catalase mRNA was actively transcribed and the GFP fusion proteins were expressed in all transfected cells except CHO_{EGFP} (Fig. 2). Newly-secreted human catalase mostly accumulated on the cell surface and only traces, not detectable by Western blotting, diffused into the culture medium (data not shown).

In order to examine the effect of the appended short peptides and GFP on enzyme activity, catalase activity was

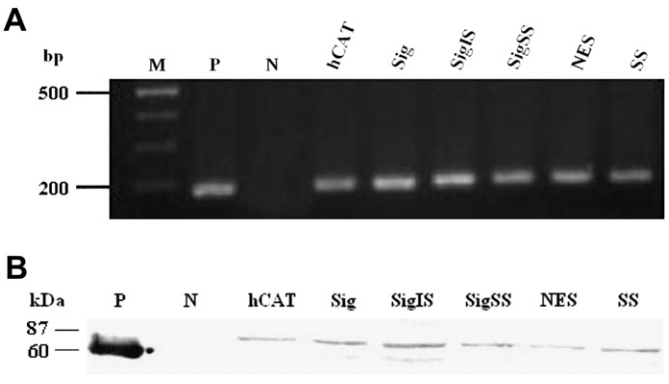

Fig. 2. Transcriptional and translational analysis of recombinant human catalase. RT–PCR was performed using RNA extracted from transiently transfected CHO cells; the RNA was subjected to DNase to eliminate DNA contamination before the reaction. P: positive control, PCR using plasmid as template; N: negative control, CHO_{EGFP}; hCAT, Sig, SigIS, SigSS, NES and SS represent CHO_{hCAT}, $CHO_{sighCAT}$, $CHO_{sigIShCAT}$, $CHO_{sigSShCAT}$, $CHO_{NEShCAT}$ and CHO_{SShCAT}, respectively (A). Sheep anti-human catalase IgG was used to examine the transient expression of recombinant human catalase tagged with the 27 kDa GFP. P: the 60 kDa human catalase; N: lysates of CHO_{EGFP}; hCAT, Sig, SigIS, SigSS, NES and SS correspond to lysates of CHO_{hCAT}, $CHO_{sighCAT}$, $CHO_{sigIShCAT}$, $CHO_{sigSShCAT}$, $CHO_{NEShCAT}$ and CHO_{SShCAT} (B).

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measured in the cell culture supernatants and transfected cell lysates. The trace recombinant human catalase in the culture medium was outside the detection limit of the kit. It did not contribute to the catalase activity in the medium. The catalase retained within the cell, combined with different short peptides and tagged with GFP, retained most or at least part of its native activity, which contributed significantly to the catalase activity in the transfected cells (Fig. 3).

3.2. Nonclassical secretion is more effective than classical secretion

Catalase is a non-secretory protein; the monomer is originally synthesized on cytosolic polyribosomes and then targeted to the peroxisome by the C-terminal peroxisomal targeting sequence (PTS). Heme is incorporated to produce mature active catalase after import (Purdue and Lazarow, 1996). We expected that the attachment of the bovine β-casein signal peptide to the N-terminus would drive the synthesized catalase to the ER and from there to the Golgi compartment, and it would ultimately be exported to the extracellular surface by exocytosis. In contrast, the addition of mouse transcript factor EN2-derived SS and NES short peptides was expected to deliver the human catalase to the cell surface by a pathway different from the ER/Golgi-dependent classical secretion pathway.

The immunofluorescence results from transiently transfected CHO cells showed that in CHO_{EGFP}, GFP was vigorously expressed in the cytosol and diffused into the nucleus (Fig. 4A). In CHO_{hCAT}, in contrast, the GFP visible in the cytoplasm has a distribution resembling that of native catalase (Fig. 4D–F). In $CHO_{sighCAT}$, unlike CHO_{hCAT}, the bovine β-casein signal peptide drove a fraction of the fusion protein to the cell surface where it was successfully secreted, although most of it was retained in the cytosol and nucleus (Fig. 4H,I). NES was found to export the catalase to the outside surface of the nuclear envelope, but not the plasma membrane; the fusion protein was almost wholly retained in the cytosol (Fig. 4P–R). SS showed a marked ability to deliver the fusion protein to a cytoplasmic region immediately adjacent to the plasma membrane, and a large fraction was translocated to the cell surface by an unknown mechanism (Fig. 4S–U), IS, a membrane penetrating peptide, is mainly used to deliver drugs into cells (Lindgren et al., 2000; Morris et al., 2001). We found that, when led by the bovine β-casein signal peptide, IS was also able to transport a cargo from the cytosol to the cell surface (Fig. 4J–L). In addition, the secretion efficiency of the signal peptide alone was enhanced by the addition of IS (Fig. 5); however, a combination of the bovine β-casein signal peptide with the short peptide SS severely decreased the secretion efficiency (Fig. 4M–O), although the signal peptide and SS were each independently able to deliver cargoes to the extracellular space. In conclusion, classical secretion differed from nonclassical secretion in terms of efficiency. Our results indicate that the classical secretion of GFP-tagged human catalase by the bovine β-casein signal peptide can be enhanced up to 2.3 fold by nonclassical secretion using the short peptide SS; the classical secretion efficiency can only be enhanced up to 1.9 fold by modifying the protein with the penetrating peptide IS (Fig. 5). Surprisingly, the combination of bovine β-casein signal peptide and SS short peptide suppressed both classical and nonclassical secretion; it decreased the efficiency of classical secretion to 37% (Fig. 5). In a word, nonclassical secretion of recombinant human catalase is more efficient than classical secretion, even when classical secretion is modified by the penetrating peptide.

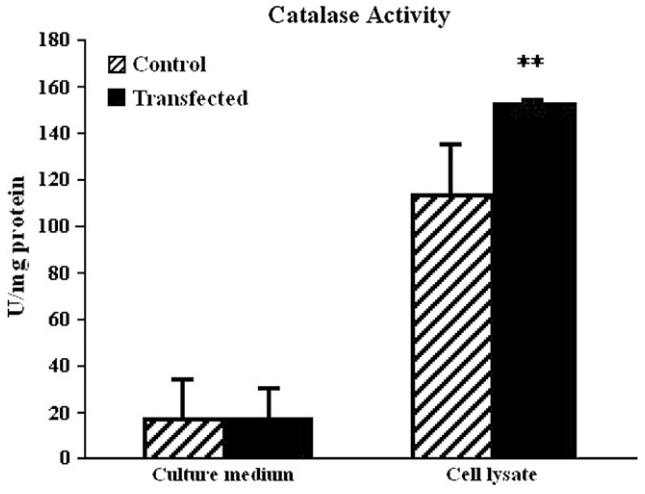

Fig. 3. Human catalase activity. Catalase activity (mean ± SEM) in control CHO_{EGFP} (hatched) and expression vectors transfected (solid); CHO cell culture medium and cell lysate are indicated. The double asterisks indicate $P < 0.01$.

4. Discussion

Secreted proteins occupy about 10% of all the proteins encoded in a genome, and many have valuable medical and industrial applications (Knappskog et al., 2007). Most secreted proteins are exported from cells by signal peptides via the classical secretion pathway (Barash et al., 2002). The signal peptides are 16–26 residue polypeptides at the N-termini of the secreted proteins. They share properties including an initial methionine, a positively charged amino region, a highly hydrophobic central core region rich in leucine, alanine and valine, and a more polar carboxyl terminus with small side-chain residues such as alanine, glycine and serine in the -1 and -3 positions from the cleavage site (Izard et al., 1995). The bovine β-casein signal peptide is responsible for the secretion of the milk protein β-casein in cow mammary glands. It conforms to the above characteristics of a secretory signal peptide. In addition, the leucine to alanine ratio of 4:2 in the central core region is within the range of a functional core region, 7:3 to 2:8 (Izard et al., 1995). The more leucines in the central core region, the greater the probability that a hydrophobic helix may form, resulting in enhanced export efficiency. The high leucine to alanine ratio in the bovine β-casein signal peptide central core region may indicate strong secretion efficiency. Although we observed the translocation of recombinant human catalase to the cell surface (Fig. 4H,I), the

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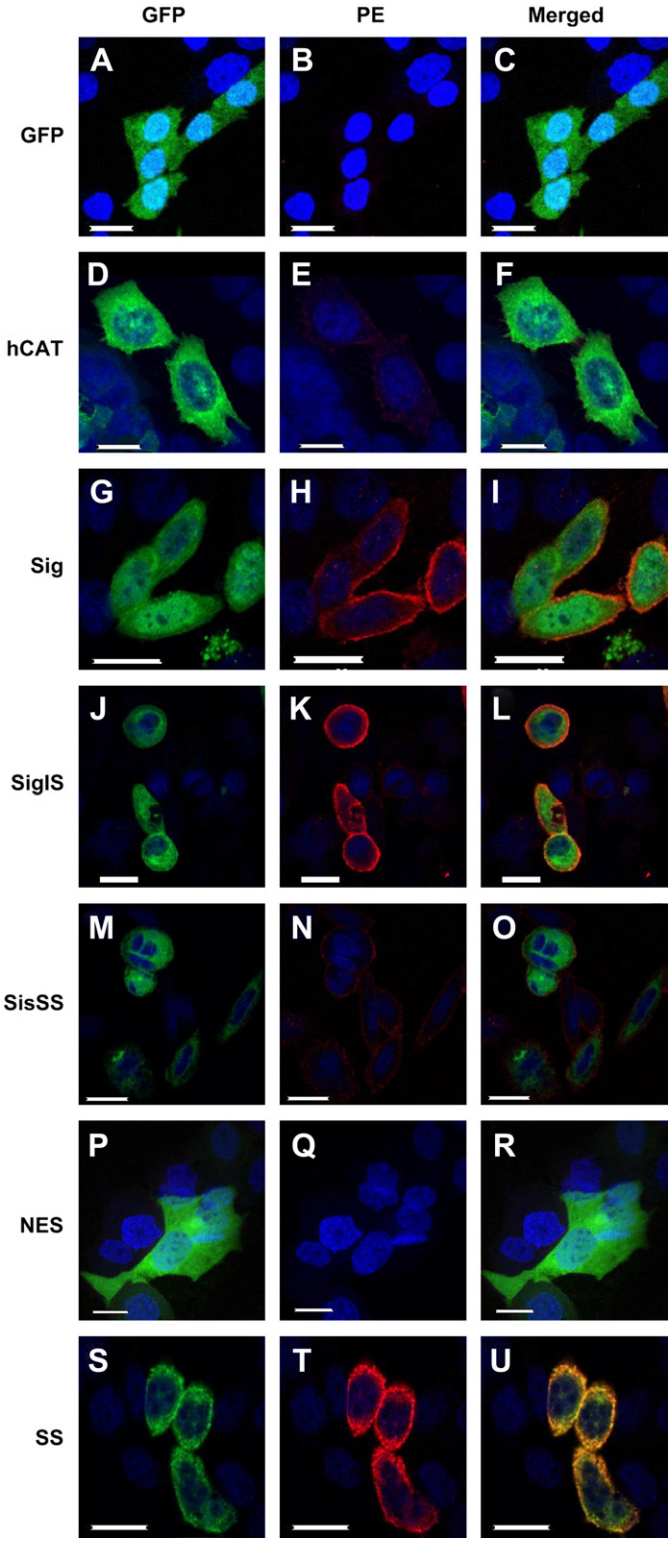

Fig. 4. Translocation of fusion proteins to the extracellular surface of the plasma membrane. Paraformaldehyde-fixed transfected cells were processed with anti-human catalase antibody and second antibody with phycoerythrin (PE) fluorophore, then examined by confocal microscopy. GFP shows the intracellular location of the fusion proteins. The PE-derived red fluorescence shows the proteins secreted on the cell surface. The cell nucleus was indicated by DAPI (blue). The expression of GFP in CHO_{EGFP} is shown in (A), cell surface staining in (B); (C) is the merged image of (A) and (B); (D–F) corresponds to CHO_{hCAT}, (G–I) to $CHO_{sighCAT}$, (J–L) to $CHO_{sigIShCAT}$, (M–O) to $CHO_{sigSShCAT}$, (P–R) to $CHO_{NEShCAT}$ and (S–U) to CHO_{SShCAT}. The results shown are representative of three independent experiments.

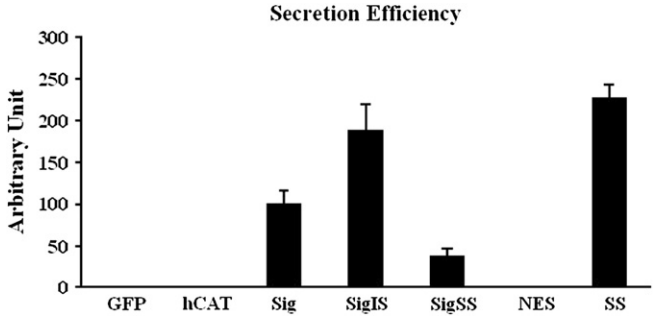

Fig. 5. Quantification of secretion efficiency. Fluorescent signals derived from GFP and PE were calculated by Image J; the red/green ratio of $CHO_{sighCAT}$ was set to 100. The results shown are representative of three independent experiments.

bovine β-casein signal peptide was insufficient to direct most the fusion protein to the extracellular culture medium, so its secretion efficiency may be cargo-dependent.

Human catalase is an antioxidant enzyme and is considered a longevity enzyme. It has potential therapeutic use in delaying the development of cataract and aging-related diseases (Cutler, 2005). Catalase is originally synthesized on cytosolic polyribosomes and imported to peroxisomes post-translationally by the pexisome targeting sequence (PTS). The last four amino acids in this sequence, KANL, have been identified as an effective PTS (Purdue and Lazarow, 1996). In the classical secretory pathway, the bovine β-casein signal peptide is believed to interact with the signal recognition particle (SRP)-ribosome complex and interrupt translation. The complex then binds to the SRP receptor docking protein (DP) on the ER surface and translation resumes. The synthesized polypeptide is translocated into the lumen of the ER, and the signal peptide is cleaved off by signal peptidase (Barash et al., 2002). The newly-synthesized polypeptide undergoes further folding and post-translational modification in the lumen, then translocates to the Golgi organelle to form secretory granules, and is eventually exported from the cell.

The post-translational modification systems in the cytosol and the lumen of ER are different. Generally, various proteins are synthesized simultaneously in the space-limited lumen of the ER. Proteins may interact electrostatically and hydrophobically with each other or with the interior face of the ER membrane. In addition, proteins are prone to form disulfide bonds under the non-reductive conditions in the lumen. These factors might promote incorrect folding of the catalase polypeptide. Misfolded proteins will not be transported to

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Golgi for vesicular secretion, but to the cytosol by the Sec61p complex for degradation. Human catalase is a large protein comprising four identical subunits of 59.7 kDa; with the addition of the GFP protein tag (27 kDa), it has a complex three-dimensional structure. We suggest that the fusion protein may be misfolded in the ER lumen because of its complex stereo configuration, lowering the secretion efficiency. In addition, the peroxisome membrane with its integral catalase PTS receptors is derived from the ER, so the recombinant catalase may interact with newly synthesized receptors in the lumen, blocking transport via the secretory pathway. Another factor that may influence the efficiency of secretion is the structural similarity between the PTS (KANL) and the ER retrieval signal (KDEL). Most ER-residing proteins possess this retrieval signal on the C-terminus, and it is critical for retaining them in the lumen. The PTS may be misrecognized as a retrieval signal, and thus a fraction of the fusion protein is blocked from export.

Nonclassical secretion is independent of the classical ER-Golgi vesicular secretion pathway. Recombinant human catalase was efficiently directed to the cell surface by mEN2 SS-mediated nonclassical secretion (Fig. 4T,U). The significantly improved secretion efficiency may indicate that the recombinant protein is blocked in the ER lumen or Golgi owing to its complex stereo configuration, but there is no interaction with lumen-residing proteins in the nonclassical secretory pathway. The secretion sequence SS is part of the nuclear export sequence (NES) that spans part of helices 2 and 3 of the homeodomain of the homeoprotein EN2 (Maizel et al., 1999). The NES is required for efficient nuclear export (Maizel et al., 1999), but does not support efflux from the cell (Fig. 4P–R). This indicates that the N-terminus-appended NES may form a secondary structure different from its native configuration, and this might inhibit formation of the correct helix in its internal SS. The homeoprotein is delivered to caveola-like vesicles containing high levels of cholesterol and glycosphingolipid for secretion by an unidentified mechanism (Joliot et al., 1997). A possible molecular mechanism is that the homeoprotein is incorporated into an exosome to form a caveola-like vesicle, then externalized and dissociated in the extracellular space (Prochiantz, 1999; Prochiantz and Joliot, 2003). Nonclassical secretion does not necessarily require cell-type-specific machineries (Schafer et al., 2004). This characteristic may potentially confer universal application upon it, but a number of critical issues remain to be clarified: (1) the specificity and selectivity of the cargo and chaperone; (2) native versus denatured states of protein folding; (3) efficiency of the transport process (Arnoys and Wang, 2007).

What would happen if a cargo were co-driven by a classical signal peptide and a nonclassical secretion sequence? According to our observations, human catalase conjugated with both the bovine β-casein signal peptide and the mEN2 SS sequence showed severely impaired extracellular transport (Fig. 4N,O). The signal recognition factors belonging to the two different secretory pathways may possibly compete to bind to the N-terminus signal sequence, forming various complexes that are difficult to process by either pathway.

The combination of classical and nonclassical secretory signal peptides is less effective for extracellular translocation. Does the combination of classical signal peptide and cell internalization sequence work well? Our results show that the efficiency of protein secretion is increased when the bovine β-casein signal peptide is combined with the mEN2 IS sequence. The internalization sequence is a 16-residue peptide corresponding almost exactly to the third helix of the homeodomain, also known as Penetratin (Lindgren et al., 2000). It has been used as a vehicle for introducing cargoes into live cells. Translocation mediated by the internalization sequence is different from endocytosis. It is independent of energy and cell type and requires the tryptophan at position 48 in a basic environment (Prochiantz, 1999; Schafer et al., 2004). A potential mechanism involves binding of the peptide to the cell surface through charge interactions, and membrane destabilization caused by the insertion of the tryptophan into the lipid bilayer. This destabilization might promote the formation of inverted micelles, which would assist peptide translocation across the membrane and release into the cytoplasm (Derossi et al., 1996). Although the internalization sequence has been extensively used to deliver cargoes to cells, its use in directing the export of cargoes has rarely been reported. We assume that the internalization sequence following a classical signal peptide may facilitate vesicular secretion by membrane destabilization as in the internalization process, because the lumen of a secretory vesicle can be considered extracellular environment.

Although the exact mechanisms of nonclassical secretion and internalization remain to be elucidated, the short peptides conferring such capacities have potential industrial application in improving the production of therapeutic proteins by mammalian cell expression systems, and further in transgenic animals for enhanced production of valuable proteins in milk.

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