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Strategies for recombinant Furin employment in a biotechnological process: complete target protein precursor cleavage

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Abstract

Coagulation factors, amongst many other proteins, often require posttranslational endoproteolytic processing for maturation. Upon high yield expression of recombinant forms of these proteins, processing frequently becomes severely limiting, resulting in a hampered function of the protein. In this report, the human endoprotease Furin was used to achieve complete propeptide removal from recombinant von Willebrand Factor (rvWF) precursors in CHO cells. At expression beyond 200 ng rvWF/10^6 cells $\times$ day, processing became insufficient. Stable co- and overexpression of full length Furin resulted in complete precursor cleavage in cell clones expressing 2 μg rvWF/10^6 cells $\times$ day. Rather than occuring intracellularly, processing was found to be mediated by a naturally secreted form of rFurin, present in 100 fold higher concentrations than endogenous Furin and accumulating in the cell culture supernatant. Attempts to increase rFurin yield by amplification, in order to ensure complete rvWF precursor processing at expression rates beyond 2 μg rvWF/10^6 cells $\times$ day, failed. Truncation of the trans-membrane domain resulted in immediate secretion of rFurin and approximately 10 fold higher concentrations in the conditioned medium. In cases where these high rFurin concentrations are not sufficient to ensure complete processing, an *in vitro* downstream processing procedure has to be established. Secreted affinity epitope-tagged rFurin derivatives were constructed, the fate of which, at expression, was dependent on the size of the C-terminal truncation and the type of the heterologous epitope added. A suitable candidate was purified by a one step affinity procedure, and successfully used for *in vitro* processing. This allows complete proteolytic processing of large amounts of precursor molecules by comparably small quantities of rFurin. Complete precursor cleavage of a target protein at expression rates of up to approximately 200 ng, 2 μg, and 20 μg, as well as beyond 20 μg/10^6 cells $\times$ day can thus be anticipated to be accomplished by endogenous Furin, additional expression of full length rFurin, co-expression of truncated and hence secreted rFurin, and a protein-chemical *in vitro* procedure, respectively.

Abbreviations: aa – amino acid; arg – arginine; bp – base pair; kd – kilodalton; lys – lysine; MW – Molecular Weight; UTR – Untranslated Region

Introduction

Human von Willebrand Factor (vWF), a multimeric plasma glycoprotein, exhibits pivotal functions in hemostasis. It mediates the adhesion of platelets to the subendothelium at the site of injury, is involved in platelet–platelet interactions, and stabilizes Factor VIII in the blood (Furlan, 1996, for review). Individuals with quantitative or qualitative vWF defects suffer from von Willebrand disease (vWD), which clinically manifests heterogeneously in mild to severe bleeding.

Currently, vWD patients are treated either with 1-deamino-8-D-arginine vasopressin, where applicable, or with FVIII/vWF complex concentrates (Lethagen,

1995, for review). The latter, emphasizing FVIII quality, often lack those vWF molecular species particularly effective in hemostasis. Thus, vWF was recombinantly expressed (Fischer et al., 1994, 1995) in order to provide a candidate therapeutic agent particularly suited for the treatment of vWD. Upon extensive biochemical analysis, rvWF was shown to exhibit high molecular weight multimers of superior structural integrity compared to plasma derived vWF (Fischer et al., 1997, 1998). In appropriate animal models, rvWF preparations were subsequently demonstrated to function well *in vivo* (Turecek et al., 1997; Roussi et al., 1998).

vWF monomers are synthesized as precursor molecules harboring a 22 amino acid signal peptide, followed by a 741 amino acid propeptide and the 2050 amino acid mature monomer. Maturation of vWF requires proteolytic removal of the propeptide; its presence obviates the molecule's ability to bind to FVIII (Leyte et al., 1991; Wise et al., 1991).

In transient transfection experiments, it has been demonstrated that vWF propeptide removal can be performed by the mammalian endoprotease Furin (Van de Ven et al., 1990; Wise et al., 1990; Rehemtulla and Kaufman, 1992). Furin, a synonym of which is PACE (paired basic amino acid cleaving enzyme), is constitutively expressed in many different cell types and tissues (Seidah et al., 1994) and was shown to cleave a wide variety of precursor molecules including those of hormones, coagulation factors, viral antigens, and bacterial toxins C-terminal to the consensus site arg – x – lys/arg – arg (Denault and Leduc, 1996, for review).

The primary translation product of human Furin is 794 amino acids in length. Similar to vWF, it consists of a signal sequence, a propeptide, and the mature protein. Furin, whose catalytic domain is located at the N-terminus of the mature protein, followed by the so-called 'middle' or 'P' domain and a cysteine-rich region, is anchored in the trans-Golgi network by means of a trans-membrane domain close to the molecule's C-terminus. The C-terminal cytosolic tail of Furin has been shown to direct intracellular trafficking of the Furin molecules, which migrate between the trans-Golgi network, endosomal compartments and the cell surface (Jones et al., 1995; Takahashi et al., 1995; Schäfer et al., 1995; Chapman and Munro, 1994; Molloy et al., 1994).

High yield expression of desired biopharmaceuticals is routinely achieved by amplification of the foreign gene/cDNA copies in their host cell. Unfortunately, posttranslational modifications, such as γ-carboxylation, β-hydroxylation, or proteolytic processing, frequently required for maturation and/or exertion of functional activity, often become limiting at overexpression.

In this report, we describe different approaches taken to ensure complete precursor cleavage of rvWF, produced by permanently transfected CHO cells, at increasing rvWF expression levels. Similar *in vivo* and *in vitro* strategies employing stably expressed recombinant Furin (rFurin) and derivatives can be applied to the processing of other recombinant proteins, depending on the demand for and the yield of these desired target proteins. As a general concept for CHO cells, using rvWF precursor as the target protein to be processed and rFurin as the model protease, complete processing at expression below 200 ng, 2 μg, and 20 μg rvWF/10^6 cells $\times$ day is achieved by endogenous Furin only, additional expression with full length rFurin, and co-expression with truncated and hence secreted rFurin forms, respectively. Expression levels beyond 20 μg/10^6 cells $\times$ day require the establishment of an *in vitro* cleavage process, an efficient example of which is described, employing an affinity tagged rFurin derivative.

Material and methods

Expression vectors

For construction of the plasmids, cloning procedures according to standard protocols were used (Sambrook et al., 1989). Full length human Furin cDNA, encoding the entire 794 amino acids and containing approximately 1600 bp of the 3′ UTR and 120 bp of the 5′ UTR, was isolated as an EcoRI fragment from plasmid pA0308 (kindly provided by Wim van de Ven, Leuven University) and inserted into the EcoRI site of the multiple cloning site of pCMV-MCS V. The resulting plasmid was termed pCMV-rFurin(794aa)<long 3′UTR>.

For construction of pCMV-rFurin(794aa)<short 3′UTR>, the SmaI/AvrII Furin cDNA fragment was cloned into the SmaI/Avrll cleaved expression vector pCMV-MCS VII. The resulting plasmid comprises the 2382 bp Furin coding region as well as approximately 90 bp of the 5′ non-translated and 400 bp of the 3′ non-translated region.

For pCMV-MCS V, the β-galactosidase NotI fragment was removed from pCMV β (MacGregor and

Caskey, 1989). The religated vector was subsequently cut with SalI/HindIII, filled in with Klenow enzyme, and religated, yielding pCMV-Δ3′MCS. Thereafter, the annealed oligonucleotides 5′-TCGAATCGAT TGAATTCCCC GGGGTCCTCT AGAGTCGACC TGCAGAAGCT TAGTACTAGT AGGCCTAGGG CCCTA-3′ (sense) and 5′-TCGATAGGGC CC-TAGGCCTA CTAGTACTAA GCTTCTGCAG GTC-GACTCTA GAGGACCCCG GGGAATTCAA TCGAT-3′ (anti-sense) were inserted into XhoI digested pCMV-Δ3′MCS. The resulting plasmid was termed pCMV-MCS V.

Alternately, pCMV-Δ3′MCS was cleaved with NotI, and the annealed oligonucleotides 5′-GGCCAT-CGAT TGAATTCCCC GGGGTCCTCT AGAGTC-GACC TGCAGAAGCT TAGTACTAGT AGGCC-TAGGG CCCTA-3′ (sense) and 5′-GGCCTAGGGC CCTAGGCCTA CTAGTACTAA GCTTCTGCAG GTCGACTCTA GAGGACCCCG GGGAATTCAA TCGAT-3′ (anti-sense) inserted, yielding pCMV-MCS VII.

For cloning of plasmid phAct-ΔEcoRl upstream, phAct (Fischer et al., 1994) was partially cut with EcoRI, the ends filled in by Klenow enzyme, and the plasmid religated. Plasmid phAct-ΔEcoRI upstream differs from phAct by the lack of the EcoRI cleavage site 5′ to the human β-Actin gene promotor; the EcoRl cleavage site 3′ to the promotor and intron remains intact.

To prepare phAct-rFurinΔTM(707aa)-6xH, six histidine residues (His-tag) were linked to rFurin, C-terminal to amino acid 707, by PCR, using the primer pair 5′-GATAAGCTTG TCGACCATGG AGCTGAGGCC CTG-3′ and 5′-CTAGAATTCA ATGATGATGA TGATGATGCC CTGCGCGCAG CCGTTGCCCC-3′ and pCMV-rFurin(794aa)<short 3′UTR> as the template. After cleavage of the flanking SalI/EcoRl restriction sites, this fragment was inserted into SalI/EcoRl cut vector phAct-ΔEcoRI upstream, yielding phAct-rFurinΔTM(707aa)-6xH. The corresponding rFurin derivative has lost most of its trans-membrane domain and its cytosolic region, resulting in rapid secretion and potentially allowing affinity purification by the poly-histidine epitope.

In order to allow for increased steric flexibility of the epitope and the Furin domain, a 5-residue glycine spacer was inserted in between these two moieties. Thus, phAct-rFurinΔTM(707aa)-6xH was digested with BssHII/EcoRI, and the annealed oligonucleotides 5′-CGCGCAGGGG GAGGTGGGGG AGGTCAT-CAC CACCATCACC ATCATCATCA CCATT-3′ and 5′-AATTAATGGT GATGATGATG GTGATGGTGG TGATGACCTC CCCCACCTCC CCCTG-3′) inserted, thereby creating phAct-rFurinΔTM(707aa)-5xG-10xH.

pCMV-rFurinΔCys(577aa)-4xG-10xH was constructed by inserting the annealed synthetic oligonucleotides 5′-TGAGGGAGGT GGGGGAGGTC AT-CACCACCA TCACCATCAT CATCACCATT AAC-AATTGA-3′ and 5′-CTAGTCAATT GTTAATGGTG ATGATGATGG TGATGGTGGT GATGACCTCC CCCACCTCCC-3′ into the SauI/AvrII cleaved vector pCMV-rFurin(794aa)<short 3′UTR>. Also, two constructs with frameshifts were obtained due to individual nucleotides absent in the inserted oligos, encoding erroneous C-termini in the resulting expression constructs. Thus, pCMVΔCys(577aa)-4xG-8xH-4X (TINN) and pCMVΔCys(577aa)-4xG-12X (LTTITII-ITINN) contained, in sense, the inserted sequences 5′-TGAGGGAGGT GGGGGAGGTC TCACCACCAT CACCATCATC ATCACCATTA ACAATTGA-3′ and 5′-TGAGGGAGGT GGGGGAGGTC ATCACCAC-CA TCACCATCAT CACACCATTA ACAATTGA-3′, respectively (the two nucleotides adjacent to the skipped residue are underlined). The latter two constructs were included as controls in the course of the experiments.

pSV-rFurinΔCys(577aa)-4xG-10xH was constructed by means of PCR. Using primers 5′-TTGAAACTAA CAAGCCCGGG ACCATGGAGC TGAGGCCCTG GTTGCTATG-3′ and 5′-GAGGCGACGT GAAAGC-TTCC CGCGGCCTAG TCAATTGTTA-3′, and pCMV-rFurinΔCys(577aa)-4xG-10xH as the template, the resulting fragment was cleaved at the flanking SmaI/HindIII sites and inserted into SmaI/HindIII digested pSV-MCS VII (Schlokat et al., 1996).

For improved steric flexibility, the spacer located between the Furin encoding region and the His-tag was further enlarged: the annealed oligonucleotides 5′-TGAAGGTGGA GGCGGCGGTG GTG-GTGGGGG AGG-3′ and 5′-TCACCTCCCC CAC-CACCACC GCCGCCTCCA CCT-3′ were ligated into SauI cleaved pCMV-rFurinΔCys(577aa)-4xG-10xH, generating pCMV-rFurinΔCys(577aa)-9xG,E,5xG-10xH.

pCMV$\Delta\Delta$Cys(563aa)-E,5xG-10xH and pCMV-$\Delta\Delta\Delta$Cys(552aa)-5xG-10xH were constructed to contain even larger C-terminal truncations prior to the addition of the spacer and the affinity epitope. Both were constructed by inserting annealed oligonucleotides into pCMV-rFurinΔCys(577aa)-4xG-10xH that had been cleaved by MamI/SauI. In the case

of pCMV$\Delta\Delta$Cys(563aa)-E,5xG-10xH, 5′-GGACCC-CTCT GGCGAGTGGG TCCTCGAGAT TGAAAA-CACC AGCGAAGCCA ACAACTATGG GACGCT-3′ and 5′-TCAAGCGTCC CATAGTTGTT GGCTTC-GCTG GTGTTTTCAA TCTCGAGGAC CCACTCG-CCA GAGGGGTCC-3′ were used. For pCMV$\Delta\Delta\Delta$-Cys(552aa)-E,5xG-10xH, 5′-GGACCCCTCT GGC-GAGTGGG TCCTCGAGAT-3′ and 5′-TCAATCTC-GA GGACCCACTC GCCAGAGGGG TCC-3′ were annealed.

In all constructs, the length of the expressed Furin derivatives is indicated, in the one letter code, from amino acid No. 1 (i.e., the methionine) of the primary translation product, irrespective of the fact that the pre-pro sequence is absent from the secreted molecules. Construct inserts and boundaries were verified by sequencing.

Transfection, cell culture, and protein harvest

Dihydrofolate reductase (dhfr) deficient CHO (Urlaub and Chasin, 1980), and 293 HEK (human embryonic kidney fibroblasts; ATCC CRL 1573) cells were used for stable and transient transfection, respectively. Cells were routinely grown in DMEM/Ham's F12 (1:1) medium (Life Technologies, Cat. No. 31330-038) supplemented with 10 μg/ml adenosine, deoxyadenosine, and thymidine, and 10% fetal calf serum (full medium). For initial selection of stable transformants, DMEM/Ham's F12 (1:1) medium lacking glycine, thymidine and hypoxanthine (Life Technologies, Cat. No. 041-90163) but supplemented with 10% dialyzed fetal calf serum (10 kd MW cut off; Life Technologies, Cat. No. 10110-161) was used. For transfection, cells were grown to 40% confluency and transfected by the $CaPO_4$ technique (Van der Eb and Graham, 1980), slightly modified as described (Fischer et al., 1994). For amplification, cells were grown at incrementally increased concentrations of methotrexate, and arising clones isolated and screened for an expression increase at each cloning step.

Establishment of CHO-rvWF and CHO-rvWF/-rFurin (full length rFurin with short 3′UTR) clones with an intermediate expression level of 2 μg rvWF/10^6 cells $\times$ day were described previously (Fischer et al., 1994, 1995).

For the establishment of CHO-rFurinΔTM-6xH, CHO-rFurinΔCys-4xG-10xH, and low level CHO-rvWF (50–200 ng rvWF/10^6 cells $\times$ day) clones, 1 μg pSV-rdhfr (Fischer et al., 1994) was cotransfected with 20 μg either of pCMV-rFurinΔTM-6xH, pCMV-rFurinΔCys-4xG-10xH, or phAct-rvWF vector (Fischer et al., 1994) into CHO cells. For potential rvWF/full length rFurin (short 3′UTR) co-amplification, 10 μg of phAct-rvWF and pSV-rFurink (Fischer et al., 1995) each were used in combination with 1 μg pSV-rdhfr. Resistant clones were screened by western blotting of serum free supernatants with anti-vWF serum; high level rFurin expressing clones were identified by the absence of any rvWF precursor in 24 hr supernatants. For transient expression, 15 μg of the individual rFurin vectors were cotransfected with pCMV-rFIX, mediating expression of recombinant human Factor IX, as a control. For harvest of secreted recombinant proteins, confluent cells were thoroughly washed three times with PBS, and serum free full medium added. Complete medium changes were subsequently performed at intervals as indicated (i.e. routinely every 24 hr; at 8 hr where indicated). Harvested media were centrifuged in order to remove cells and debris, sterile filtered, and stored in aliquots at –80 °C. Recombinant protein yield was determined by a Furin activity assay (rFurin) and by ELISA (vWF; Asserachrom, Boehringer Mannheim).

For the lysates, the cells were trypsinized and resuspended in full medium. The cells were counted and washed twice with PBS by gentle centrifugation and resuspension. Subsequently, the pellet was resuspended by the addition of 200 μl TE (10 mM Tris pH 7.0, 1 mM EDTA) and 70 μl 4x Loading Buffer (0.2 M Tris pH 6.8, 32% glycerol, 8% SDS, 20% β-mercaptoethanol) per 2×10^6 cells, and sonicated in a W-385 Sonicator (Heat Systems – Ultrasonics, Inc.).

The cell number was determined, after trypsinization and appropriate dilution in PBS, in a CASY counter with a 30 nm capillary (Schärfe Systems).

Visualization of vWF, Furin, and FIX

The samples were reduced, resolved by SDS PAGE on 4% stacking/5% separation (vWF) and 4%/8% (Furin) gels, and visualized by western blotting as described (Schlokat et al., 1996). For detection of vWF and Furin molecules, a rabbit anti-serum (Dakopatts, Cat. No. A 082) and a murine monoclonal antibody (MON 148, kindly provided by Wim van de Ven and John Creemers; Van Duijnhoven et al., 1992) were used. For FIX, a rabbit anti-serum was used also (Dakopatts, Cat. No. A 300). Alkaline phosphatase conjugated anti-rabbit (Promega, Cat. No. S 3731) and anti-mouse IgG goat sera (Sigma, Cat. No. A 4656) were used as the second antibodies.

Furin activity test

Furin activity was tested by a fluorogenic substrate cleavage assay as described (Schlokat et al., 1996). 1.8 ml buffer (100 mM Hepes pH 7.5, 1 mM $CaCl_2$) were incubated with 100 μl sample (conditioned medium or purified rFurin, appropriately diluted in buffer) and 20 μl of 5 mM t-Butyloxycarbonyl-Arg-Val-Arg-Arg-Methylcoumaryl-7-amide (Bachem, Cat. No. I-1645) at 30 °C. Reactions were stopped by the addition of 40 μl 50 mM $ZnCl_2$. Upon excitation at 380 nm, emission was monitored at 435 nm in a luminescence-spectrometer (Perkin Elmer LS 50B) in arbitrary units.

Affinity purification of rFurin derivative

CHO-rFurinΔCys-4xG-10xH cells were grown in roller bottles, and serum free conditioned medium (full medium) entirely replaced every 24 hr. 75 ml medium was mixed with 3 ml Ni^{2+} NTA agarose matrix (Qiagen, Cat. No. 30210), which had been previously equilibrated with unused serum free full medium for one hour at ambient temperature. Subsequently, the charged resin was filled into a column, assembled to a Pharmacia FPLC apparatus, and washed with 6 volumes buffer A (10 mM Hepes pH 7.0, 150 mM NaCl, 2 mM $CaCl_2$). Bound material was eluted with a 2.5 volume linear 0 to 200 mM imidazole gradient in buffer A, followed by a 2 volume step with 200 mM imidazole in buffer A. Subsequently, tightly bound material still retained on the column was eluted with buffer A plus 1 M imidazole. The eluates were collected in 0.5 ml fractions. Alternating fractions were resolved by SDS PAGE under reducing conditions, and visualized by silver staining (BioRad, Cat. No. 161-0449) according to the manufacturer's recommendations, as well as subjected to the Furin activity test. The fractions exhibiting highest activity and lowest contamination with other proteins were pooled and dialyzed against buffer A, 50% glycerol at 4 °C for 10 hr. Aliquots were shockfrozen and stored at –80 °C. Conditioned medium from genetically unaltered CHO cells underwent the same procedure, and corresponding fractions were used as a negative control.

rvWF precursor in vitro cleavage

Incompletely processed rvWF was produced and purified as described previously (Fischer et al., 1997).

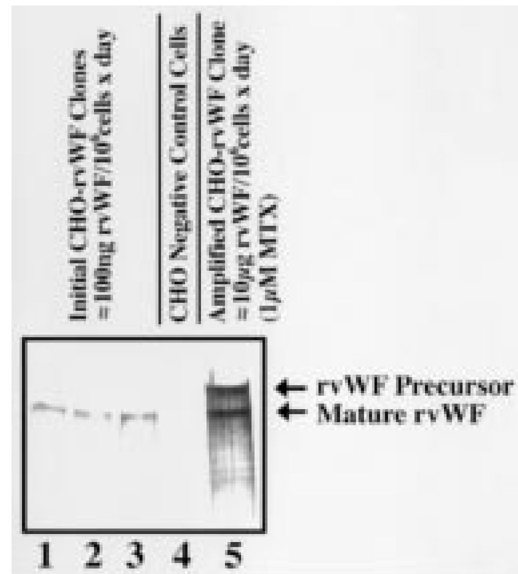

Figure 1. Limitation of rvWF precursor processing by endogenous Furin. rvWF molecules in conditioned media derived from three low yield, initial transfectant and one amplified CHO cell clones were visualized by western blotting. Lanes 1 to 3, low yield CHO-rvWF clones; 4, unmanipulated CHO cells; 5, amplified CHO-rvWF cell clone.

The cleavage reaction was assembled in a total volume of 600 μl, containing 10 μg purified rvWF and 1340 units purified rFurinΔCys-4xG-10xH in 20 mM Hepes pH 7.0, 1 mM $CaCl_2$, 1 mM β-mercapto-ethanol, and incubated at 37 °C. Samples were taken directly after mixing and a short spin in a table top centrifuge but prior to incubation, as well as after 4:35 hr, and shockfrozen. 15 μl of each reaction were used for western blotting.

Results

Limitation of rvWF precursor processing upon overexpression

CHO cell clones expressing rvWF were established; these clones secreted 50–200 ng/10^6 cells $\times$ day. Only completely processed, propeptide free rvWF molecules were detectable by western blotting of conditioned medium derived from individual cell clones (Figure 1, lanes 1 to 3). Upon dihydrofolate reductase/methotrexate mediated amplification (1 μM methotrexate), rvWF expression was increased to 10 μg/10^6 cells $\times$ day. However, at this 100 fold higher expression level, propeptide removal by the

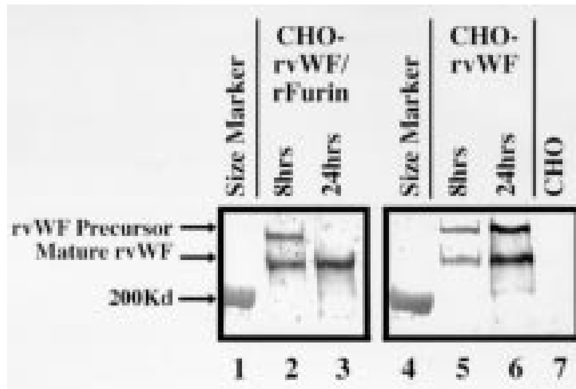

Figure 2. Proteolytic processing of rvWF precursor molecules alone and upon co-expression with wild-type rFurin. rvWF from a stable intermediate yield CHO-rvWF cell clone and from a successor cell clone, additionally transfected in order to express full length rFurin (CHO-rvWF/rFurin), was visualized by western blotting. The conditioned media were collected after 8 and 24 hr. Lane 1, molecular size marker; 2, 8 hr conditioned medium from CHO-rvWF/rFurin cells; 3, 24 hr CHO-rvWF/rFurin clone derived supernatant; 4, molecular size marker; 5, CHO-rvWF cell derived 8 hr conditioned medium; 6, 24 hr supernatant from a CHO-rvWF clone; 7, 24 hr conditioned medium derived from unmanipulated CHO cells.

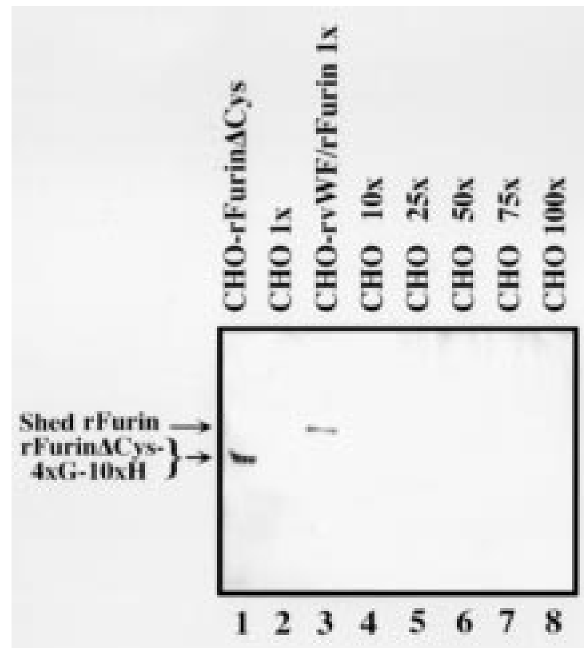

Figure 3. Quantification of endogenous and recombinant Furin expression. Naturally secreted, 'shed' Furin in 24 hr conditioned medium of a CHO-rvWF/rFurin clone and unmanipulated CHO cells is visualized by western blotting. In order to detect even low concentrations of shed Furin potentially present in the CHO cell derived supernatant, the latter was concentrated to different degrees by lyophylization of increasing volumes of conditioned medium as indicated. Lane 1, blot control with purified rFurinΔCys-4xG-10xH; 2, native, unconcentrated CHO conditioned medium; 3, unconcentrated CHO-rvWF/rFurin supernatant; 4 to 8, CHO cell derived conditioned medium concentrated 10, 25, 50, 75, and 100 fold.

endogenous proteolytic machinery had become insufficient. Approximately 50% of the secreted rvWF molecules retained the propeptide (Figure 1, lane 5). vWF immunoreactive material was not detected in the supernatant from unmanipulated CHO cells (Figure 1, lane 4).

Complete rvWF precursor processing extracellularly by naturally secreted 'shed' rFurin upon stable co-expression with recombinant human full-length Furin

As reported previously, stable co-expression of human full-length rFurin in an intermediate yield CHO-rvWF clone (2 μg rvWF/10^6 cells $\times$ day), which had exhibited significantly impaired propeptide removal (Fischer et al., 1994), resulted in the production of completely propeptide-free rvWF (Fischer et al., 1995; Schlokat et al., 1996).

A CHO-rvWF/rFurin cell clone exhibiting complete rvWF precursor processing was further characterized (Figure 2). While the precursor was completely absent from 24 hr supernatants (Figure 2, lane 3), significant amounts of rvWF precursor molecules were detectable at more frequent media changes, e.g. every 8 hr (Figure 2, lane 2). In contrast, the rvWF species present in 8 and 24 hr conditioned media derived from predecessor CHO-rvWF cells did not significantly differ; rather, the ratio rvWF precursor:mature form remained identical (Figure 2, lanes 5 and 6). The precursor:mature form ratio in the 8 hr conditioned media derived from the CHO-rvWF/rFurin clone (Figure 2, lane 2) closely resembled that exhibited by CHO-rvWF cells not expressing rFurin (Figure 2, lane 5). Thus, propeptide removal from rvWF precursors, beyond the degree already present in CHO-rvWF cells and mediated by endogenous Furin, occurred mainly, or even exclusively, extracellularly.

In the conditioned medium derived from CHO-rvWF/rFurin cells, a band was detected by western blotting employing MON148, a monoclonal antibody directed against human Furin (Figure 3, lane 3). This monoclonal antibody did not react with any material present in supernatants derived from unmanipulated CHO cells (Figure 3, lane 2).

Although Furin is anchored in the trans-Golgi network via its trans-membrane domain, a naturally secreted form termed 'shed' Furin has recently been

described upon recombinant expression of full-length rFurin (Vidricaire et al., 1993; Vey et al., 1994; Molloy et al., 1994).

100 fold higher rFurin compared to endogenous Furin expression by CHO-rvWF/rFurin cells

Endogenous Furin is expressed at a level lower than the detection limits of currently available quantification methods (Ayoubi et al., 1996). Thus, the amount of Furin specific mRNA is commonly accepted as an indirect means to measure the level of Furin expression (Seidah et al., 1994). In order to quantify rFurin overexpression compared to endogenous Furin expression directly, CHO cell derived conditioned medium was concentrated to different degrees by lyophylization (Figure 3). However, despite up to a 100 fold concentration of conditioned medium derived from unmanipulated CHO cells, immuno-reactive material could not be detected (Figure 3, lanes 4 to 8). The Furin specific monoclonal antibody MON148, which was used for detection, had been mapped to an epitope located between amino acids 16 to 189 of mature human Furin, i.e. specifically the catalytic domain (van Duijnhoven et al., 1992). This antibody has been shown to crossreact with mouse Furin, which differs from the human molecule by two amino acid substitutions in this region of the molecule. CHO cell Furin is identical to murine Furin in the region interacting with this antibody (Spence et al., 1995). Therefore, it is expected that MON148 should react with this molecule also. This finding shows that rFurin expression by CHO-rvWF/rFurin cells exceeds endogenous Furin levels by more than a factor of 100, based on the quantification of shed Furin. In CHO-rvWF cells secreting 2 μg rvWF/10^6 cells $\times$ day, endogenous Furin, which is present only in trace amounts in the cell, is able to remove the propeptide from approximately 60% of rvWF precursor molecules (Figure 2). Since more than a 100 fold rFurin overexpression is necessary for complete processing of the remaining 40% of rvWF precursors (within 24 hr), endogenous Furin exhibits a higher proteolytic activity than shed rFurin. Intracellular rFurin does not seem to contribute significantly to cleavage.

In CHO cells, rvWF expression levels of up to 20 μg/10^6 cells $\times$ day can be achieved by amplification. However, a variety of attempts to co-amplify independently established initial CHO-rvWF/rFurin co-transformants in order to ensure complete propeptide removal of increasing concentrations of rvWF by simultaneously increased rFurin levels failed. Although the yield of rvWF could be increased, processing became limiting and full length rFurin expression uncoupled. The inability to further amplify rFurin resulted in incomplete processing of rvWF at higher levels of expression; increased yields of rvWF did not result in increased yields of processed rvWF, but led to an increase of the precursors.

C-terminal truncation as a means to further increase rFurin expression

In order to investigate whether the CHO cells' tolerance of recombinant full length Furin expression is limited by the intracellular or extracellular rFurin concentrations reached, a rFurin deletion mutant was constructed, the C-terminal 87 amino acids of which had been replaced by a short tail harboring a six histidine residue affinity tag. This rFurin derivative, rFurinΔTM-6xH, lacks the cytosolic and trans-membrane domains. rFurin molecules without the trans-membrane domain have previously been reported to be immediately released into the cell culture supernatant rather than retained in the trans-Golgi network (Wasley et al., 1993; Rehemtulla and Kaufman, 1992; Rehemtulla et al., 1992; Creemers et al., 1993).

In the conditioned medium derived from permanently transfected CHO-rFurinΔTM-6xH clones, anti-Furin antibody reactive material was detectable by western blotting (Figure 4a, lanes 5 to 7). Compared to shed rFurin in a CHO-rvWF/rFurin cell derived supernatant (Figure 4a, lane 4), significantly larger quantities of rFurin were present in the conditioned medium. At closer inspection, the immunoreactive material from CHO-rFurinΔTM-6xH cells resolved as a doublet (Figure 4b). The smaller molecular species (Figure 4a, lanes 5 to 7) migrated at a position identical to the shed rFurin band in the CHO-rvWF/rFurin supernatant (Figure 4a, lane 4).

These findings suggest that truncation C-terminal to amino acid 707 did not remove the intramolecular cleavage site leading to shed rFurin. Intramolecular cleavage does not require prior membrane anchorage. Since, in addition, the cleaved molecule is almost indistinguishable in size from rFurinΔTM-6xH, the cleavage site lies N-terminal to and in the immediate vicinity of amino acid 708.

rFurin yield from CHO-rFurinΔTM-6xH conditioned medium was approximately 5 to 10 fold higher than that of CHO-rvWF/rFurin supernatants, as determined both by antigen (Figure 4a, lanes 5 to 6 vs. lane

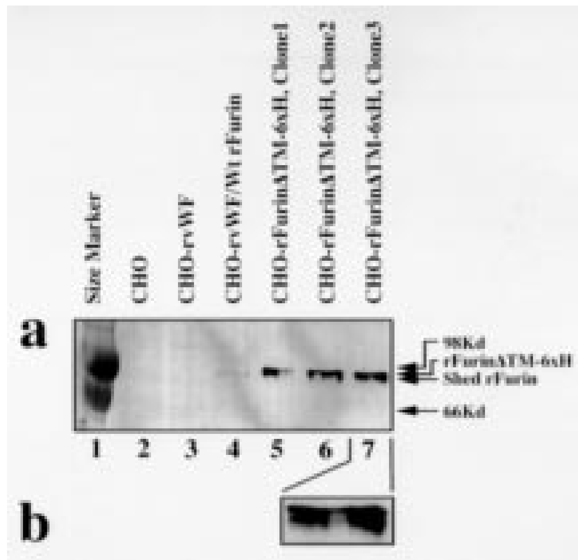

Figure 4. Improvement of stable rFurin expression by truncation of its C-terminus. rFurin expression from CHO clones transfected either with full length rFurin (CHO-rvWF/rFurin) or truncated rFurinΔTM-6xH (CHO-rFurinΔTM-6xH) was determined by western blotting of conditioned media. a) Investigation of different cell clones. Lane 1, molecular size marker; 2, unmanipulated CHO cells; 3, CHO-rvWF clone; 4, CHO-rvWF/full length rFurin clone; 5 to 7, three different CHO-rFurinΔTM-6xH cell clones. b) Enlargement of lane 7 showing the resolution of the double band present, representing the smaller shed rFurin and the larger rFurinΔTM-6xH molecular species.

4) as well as by a fluorogenic substrate cleavage test (not shown).

Complete propeptide removal is successfully accomplished upon co-expression with full-length rFurin only up to an expression level of approximately 2 μg rvWF/10^6 cells $\times$ day. Since rFurin primarily or even exclusively acts extracellularly and 10 fold higher yields of functional rFurin in the conditioned medium can be achieved by rFurin mutant molecules lacking the trans-membrane domain, up to approximately 20 μg rvWF/10^6 cells $\times$ day may be anticipated to be completely processable by truncated rFurin. Therefore, complete precursor processing of a desired target protein by co-expression of a trans-membrane deleted rFurin may be accomplished in those cases when full-length rFurin co-expression is not sufficient. In addition, the host cells' tolerance to extracellular rFurin levels that are approximately 10 fold higher than those achieved with full length/shed rFurin suggests that the latter's resistance to further expression improval may be due to an adverse effect caused by intracellular rFurin.

Epitope-tagged rFurin derivatives as tools for the establishment of an efficient in vitro *cleavage process*

For recombinant Factor X (rFX), expression levels of 120 μg/ml $\times$ day (78 μg/10^6 cells $\times$ day), have been reported (Himmelspach et al., 1998). Similarly, recombinant Factor IX (rFIX) could be expressed to 188 μg/ml (Kaufman et al., 1986). Both of these proteins require endoproteolytic processing for maturation. However, co-expression with trans-membrane deficient rFurin molecules does not yield a high enough rFurin concentration to accomplish complete processing of these excessive quantities of target protein precursors within a reasonable period of time. Rather, the establishment of an *in vitro* cleavage process is required. The aim thereby is to achieve complete proteolytic cleavage of large amounts of desired protein precursor molecules with comparably small amounts of the protease. Thus, Furin derivatives lacking the trans-membrane domain, which should be secreted, and carrying heterologous affinity epitopes, to facilitate subsequent purification, were constructed.

A panel of C-terminally truncated rFurin molecules was created. In order to allow efficient one step affinity purification using the Ni^{2+} NTA chelating method (Crowe et al., 1994), poly-histidine tails were added, partly linked to the residual Furin sequence by means of short flexible amino acid hinges of varying lengths. In order to compare expression of the individual molecules, they were transiently expressed in 293 HEK cells, as shown in Figure 5. The results are summalized schematically in Figure 6.

As an internal control for transfection efficiency, a vector mediating expression of rFIX was cotransfected, and the presence of roughly equal amounts of rFIX in the cell lysates demonstrated comparable transfection efficiencies (Figure 5c). The individual rFurin constructs, however, exhibited differences as to intracellular concentration (Figure 5a) and, strikingly, secretability (Figure 5b).

Expression of full length rFurin from the cDNA containing the entire 3′ untranslated region, the latter of which is approximately 1.6 kb in length (rFurin<long 3′UTR>), yielded very little intracellular Furin (Figure 5a, lane 4), and only trace amounts of shed rFurin were present in the conditioned medium (Figure 5b, lane 4). Removal of 1.2 kb of the 3′UTR from the 3′ end (rFurin<short 3′UTR>) led to an increase in both intracellular (Figure 5a, lane 5) and extracellular (Figure 5b, lane 5) Furin concentrations. The deleted part of the 3′UTR thus may encode

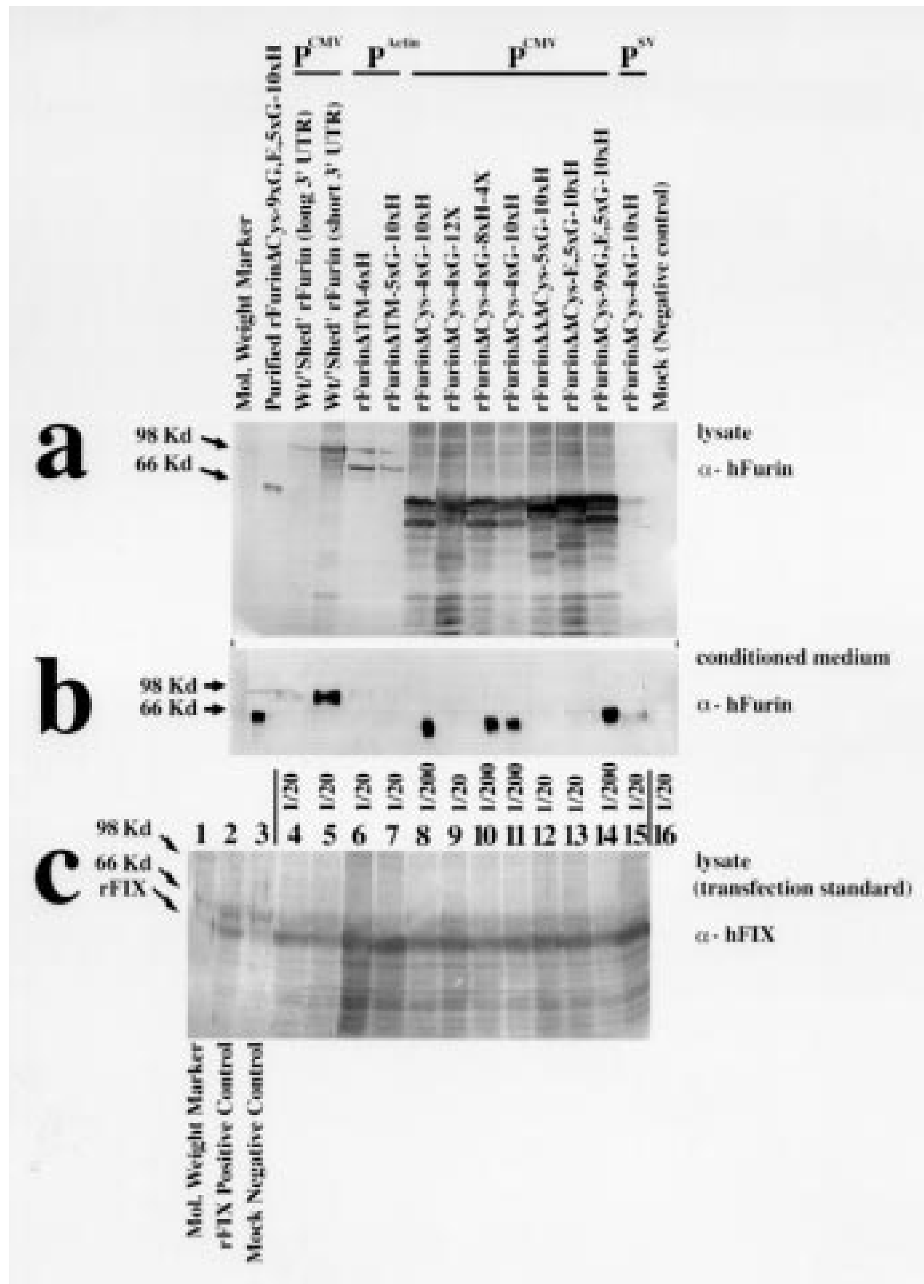

Figure 5. Intra- and extracellular concentration of rFurin derivative molecules upon transient expression. Different vectors mediating the expression of full length rFurin and several C-terminal truncation mutants were transiently transfected into 293 HEK cells. 24 hr supernatants were collected three days after transfection, and the cells harvested for lysis. Equal amounts of a recombinant Factor IX (rFIX) expression vector were included in each transfection reaction in order to ensure corresponding transfection efficiencies. Equal amounts of cell lysate (a, c), but 1/20 or 1/200 of the conditioned medium (b) from individual transfections were applied to each lane and visualized by western blotting employing antibodies directed against Furin (α-hFurin) and FIX (α-hFIX) as indicated. For the 1/20 samples, the medium was concentrated 10 fold by lyophylization; the significant ion concentration difference between the 1/20 and 1/200 samples resulted in a horizontal contraction of the 1/200 lanes in the gel. The promoters of the individual expression vectors are indicated at the top of the figure. a) Material in cell lysates reacting with the antibody directed against Furin; b) rFurin molecules detectable in the conditioned media; c) material in cell lysates reacting with the antibody directed against FIX. Lane 1, molecular weight marker; 2, rFIX blot control (c) and molecular weight marker (a, b); 3, mock transfection (negative control, c) and rFurin blot control (a, b); 4, full length rFurin transfection with the construct containing the entire 3′UTR of the cDNA; 5, full length rFurin transfection with the construct retaining only approximately 400 bp of the Furin 3′UTR; 6, rFurinΔTM-6xH; 7, rFurinΔTM-5xG-10xH; 8, rFurinΔCys-4xG-10xH; 9, rFurinΔCys-4xG-12X; 10, rFurinΔCys-4xG-8xH-4X; 11, rFurinΔCys-4xG-10xH; 12, rFurinΔΔΔCys-5xG-10xH; 13, rFurinΔΔCys-E,5xG-10xH; 14, rFurinΔCys-9xG,E,5xG-10xH; 15, rFurinΔCys-4xG-10xH; 16, mock transfection (negative control).

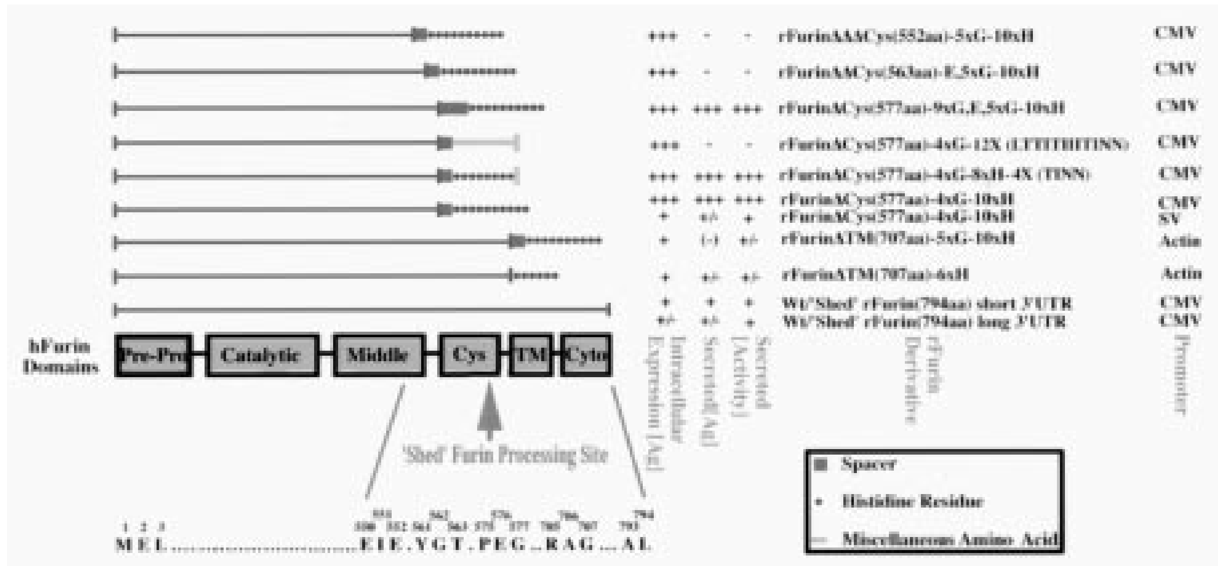

Figure 6. Schematic representation of the results from the transient expression of different rFurin derivatives in 293 HEK cells. The results from the rFurin construct expression in Figure 5 are schematically summarized. The structural domains of human Furin are indicated: Pre-Pro, signal sequence and propeptide; Catalytic, catalytic domain; Middle, middle domain; Cys, cysteine rich domain; TM, trans-membrane domain; Cyto, cytosolic tail. The number of N-terminal amino acid codons of the original Furin sequence present in the derivative molecules are indicated in parentheses and always refers to the translational initiation methionine irrespective of the actual presence or absence of the pre-pro-sequence. Additional amino acids are indicated by the one letter code. The lower panel depicts the environment of those amino acids after which the heterologous epitopes were added. The diagram is not drawn to scale. +++, strong expression; +, low expression; +/-, expression just detectable; -, no expression; (-), expression not detectable by western blotting but by the functional test.

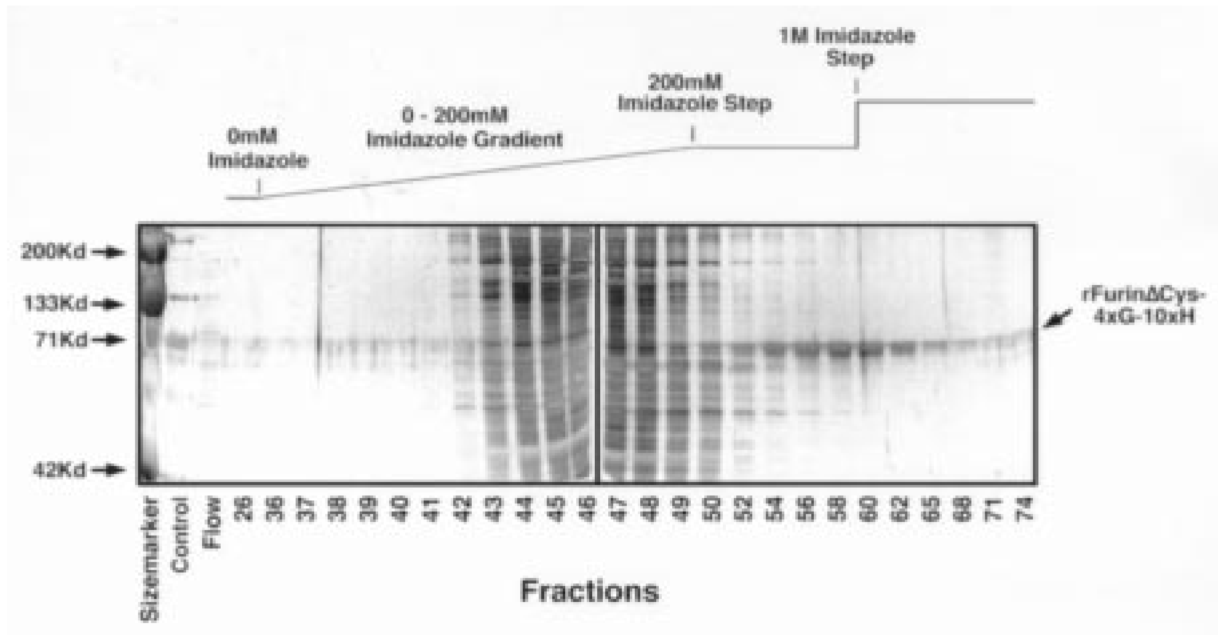

Figure 7. Affinity purification of a rFurin derivative carrying a heterologous epitope. CHO-rFurinΔCys-4xG-10xH cell clone derived serum free conditioned medium was loaded onto a Ni^{2+} NTA matrix column. The column was washed and bound material gradient and step eluted by imidazole as shown at the top of the figure. The proteins present in individually collected fractions were visualized by silver-stained SDS-PAGE. The molecular size marker, a previously (semi-) purified rFurinΔCys-4xG-10xH fraction as a control, and the flow through material (i.e. not binding to the Ni^{2+} NTA resin) are indicated.

a sequence that interferes with transcription and/or translation, the removal of which results in improved expression.

Expression of rFurinΔTM-6xH (Figure 5a, lane 6) and rFurinΔTM-5xG-10xH (Figure 5a, lane 7), both of which retain the first 707 amino acids of Furin and additionally contain either a six histidine or a five glycine/ten histidine tag, resulted in intracellular Furin immuno-reactive material in yields comparable to rFurin<short 3′UTR>, although both derivatives were under the control of a weaker promoter (the human β actin gene rather than the Cytomegalovirus immediate early promoter, which is particularly efficient transactivated in 293 HEK cells due to Adenovirus E1A/E1B gene product transactivation therein). Unexpectedly, only trace amounts of rFurin were detectable in the conditioned medium (Figure 5b, lanes 6 and 7), thus contrasting with the results obtained with permanently expressed full length rFurin<short 3′UTR> and rFurinΔTM-6xH in CHO cells described above. Therefore, cell specific differences as to the expression of individual rFurin constructs may occur.

Removal of the cysteine rich region, in addition to both trans-membrane and cytosolic domains, resulted in significantly enhanced intracellular concentrations of all derivatives (ΔCys,ΔΔCys, ΔΔΔCys versions), but striking differences in their secretion (Figures 5a and b). rFurin molecules retaining the 577 N-terminal amino acids and carrying different heterologous C-terminal peptide sequences thereafter (rFurinΔCys-...; Figure 5b, lanes 8–11, 14) were very efficiently secreted, with one exception. rFurinΔCys-4xG-12X, which contains the sequence leu-thr-thr-ile-thr-ile-ile-ile-thr-ile-asn-asn at its C-terminus, was not detectable in the conditioned medium (Figure 5b, lane 9) despite strong intracellular expression (Figure 5a, lane 9). In contrast, the rFurin derivative rFurinΔCys-4xG-8xH-4X containing his-his-his-his-his-his-his-his-thr-ile-asn-asn differs in only eight amino acid residues but yielded high amounts of rFurin in the supernatant (Figure 5b, lane 10). Thus, very few amino acids at the C-terminus may be determinative for the fate of the molecules. SV40 promoter driven rFurinΔCys-4xG-10xH expression, due to this promoter's repression in 293 HEK cells, yielded only minute amounts of the rFurin derivative intra- and extracellularly (Figures 5a and b, lane 15), compared to that of rFurin derived from expression mediated by the CMV promoter (Figures 5a and b, lane 8).

rFurin molecules with deletions extending amino-terminal of residue 577, and thus affecting the 'middle' domain (Figures 5 and 6, rFurinΔΔCys and ΔΔΔCys versions), were not detected in the conditioned medium (Figure 5b, lanes 13 and 12). However, high amounts of immuno-reactive material were found in the cell lysates (Figure 5a, lanes 13 and 12). The intracellular concentration of these molecules was equal to that shown by the readily secretable versions of rFurinΔCys. The lack of extracellular accumulation of the rFurinΔΔCys and ΔΔΔCys molecules, similar to rFurinΔCys-4xG-12X, suggests that these molecules are most likely degraded late but prior to release into the tissue culture supernatant.

The most efficiently secreted derivatives were rFurinΔCys-4xG-10xH, -4xG-8xH-4X, and -9xG,E,5xG-10xH. Due to their 10 residue histidine tags, rFurinΔCys-4xG-10xH and rFurinΔCys-9xG,E,5xG-10xH can be anticipated to be the most promising candidates for affinity purification.

rvWF precursor propeptide removal by purified rFurin derivative in vitro

Permanent CHO-rFurinΔCys-4xG-10xH cell clones were established, and rFurinΔCys-4xG-10xH molecules were purified from serum free conditioned medium by means of the Ni^{2+}-NTA chelate technology (Crowe et al., 1994). The serum free conditioned medium was applied to the Ni^{2+}-NTA matrix, the bound molecules were eluted by a 0–200 mM imidazole gradient, and individual fractions collected (Figure 7). Functional activity was determined by the Furin specific fluorogenic substrate test (not shown) and correlated with the predominant protein band in fractions 52 to 62 as visualized by silver staining; fractions 58 to 60 were pooled, since rFurin was the only protein band detectable therein and thus exhibited near homogeneity.

The pooled rFurinΔCys-4xG-10xH fractions were dialyzed and mixed with purified, incompletely processed rvWF molecules under appropriate, defined minimal conditions. Directly after mixing and a short spin in the centrifuge but prior to incubation (Figure 8, lane 1), an aliquot of the reaction solution was frozen, as was the remainder after the incubation step (Figure 8, lane 2). Comparison of the amount of rvWF precursor in these two samples demonstrated successful rvWF precursor cleavage upon incubation. A mock-purified rFurin preparation from unmanipulated CHO cells did not mediate rvWF precursor processing. In fact, rvWF precursor cleavage was very efficient, since the short exposure of rvWF precursor

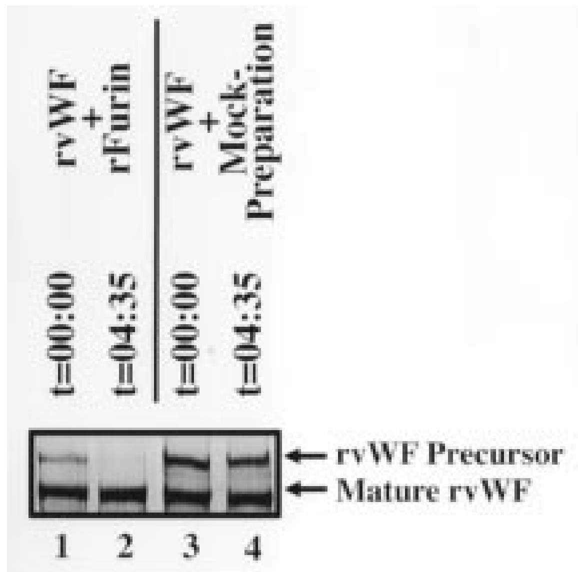

Figure 8. In vitro maturation of incompletely processed rvWF precursor by purified rFurin. Purified rFurinΔCys-4xG-10xH (lanes 1 and 2), as well as a mock preparation, derived from unmanipulated CHO cells and purified identically (lanes 3 and 4), were mixed with a purified partially propeptide containing rvWF preparation under appropriate conditions. Samples were taken upon mixing (and a short spin in the centrifuge; lanes 1 and 3) as well as after a 4 hr 35 min incubation at 37 °C (lanes 2 and 4).

to the purified rFurin derivative during the mixing and spin, prior to the actual incubation, led to a significant reduction in the amount of rvWF precursor already (compare Figure 8, lanes 1 and 3).

Discussion

At low expression levels, i.e. approximately 100 ng rvWF/10^6 cells $\times$ day, stable CHO-rvWF cell clones secreted only mature rvWF molecules. Upon amplification, yields of 10 μg rvWF/10^6 cells $\times$ day were achieved. However, in these high yield CHO-rvWF cell clones, removal of the propeptide from rvWF precursors was incomplete. Full length rFurin co-expression in intermediate yield CHO-rvWF cells, expressing 2 μg rvWF/10^6 cells $\times$ day, led to complete rvWF propeptide removal within 24 hr. Processing was found to be performed predominantly, if not entirely, extracellularly by a naturally secreted form of rFurin, termed 'shed' rFurin. In contrast to previous reports suggesting the intramolecular cleavage occurs between the middle and cysteine rich domains (Rehemtulla et al., 1992), the data presented here suggest the cleavage site is located amino-terminal to and in close vicinity of amino acid 708. Full length rFurin, located intracellularly, seems to be largely inactive. Based on the quantification of shed rFurin, full length rFurin expression in permanently transfected CHO cells was estimated to exceed endogeneous Furin expression by more than 100 fold. Similar overexpression was suggested for full length rFurin expression in transient transfection experiments (Rehemtulla and Kaufman, 1992) as well as in the mammary glands of transgenic animals (Paleyanda et al., 1997). Endogenous Furin, present only in trace amounts (compared to rFurin), mediated propeptide removal in 60% of the rvWF precursors at intermediate rvWF expression, implying that endogenous Furin exhibits a significantly higher functional activity than does shed rFurin.

Attempts to co-amplify rFurin and rvWF in order to establish cell clones providing yields of completely processed rvWF in excess of 2 μg rvWF/10^6 cells $\times$ day failed. While rvWF yields of up to 20 μg rvWF/10^6 cells $\times$ day could be achieved, rFurin expression could not be amplified to a level sufficient to ensure complete propeptide removal of such high levels of rvWF. Thus, 2 μg rvWF/10^6 cells $\times$ day represented an approximate upper limit for complete processing within 24 hr by full length rFurin co-expression. The inability to increase full length rFurin expression beyond a certain level has also been observed by Creemers (1994) and may be attributed to potential toxicity of larger amounts of full length Furin to its host cell. Interference of full length rFurin with cell viability suggests the molecules are functional; on the other hand, and for reasons yet to be determined, rFurin that is overexpressed intracellularly is severely hindered, or even blocked, from contributing to rvWF propeptide removal.

The enzyme might be miscompartmentalized, and thus be unavailable for rvWF propeptide removal even though functional. Furin is known to be modified by phosphorylation at its C-terminal cytosolic tail, thereby undergoing intracellular trafficking between the trans-Golgi network, endosomal compartments and the cell surface (Jones et al., 1995; Takahashi et al., 1995; Schäfer et al., 1995; Chapman and Munro, 1994; Molloy et al., 1994), and to be routed to lysosomes upon accumulation and aggregation in the trans-Golgi network (Wolins et al., 1997). Upon transfection with an expression vector encoding full length rFurin, Ayoubi et al. (1996), established CHO cell clones, in some of which rFurin was found to be present as functionally inactive forms lacking the

C-termini, and was located at erroneous sites. Most plausibly, these forms result from alterations at the DNA level, e.g. by truncation prior to concatemerization and integration into the host cell chromosomes, a fate common to transfected DNA molecules (Razzaque et al., 1982; Ashman and Davidson, 1985; Calos et al., 1983).

Rather than being located in an unfavorable microenvironment, some additional factor not mandatory for functional activity per se, but crucial specifically to rvWF precursor cleavage may be missing or limiting. Transient co-expression of rvWF with an inactive full length rFurin mutant had been reported to impair rvWF propeptide removal compared to that observed upon rvWF expression without the rFurin mutant (Rehemtulla and Kaufman, 1992). Competition for an auxiliary factor required for rvWF precursor processing may explain the impaired cleavage efficiency of the endogenous enzyme in the presence of additional molecules of an inactive analogue. If quantities of the auxiliary factor are already limiting endogenous Furin activity, this may explain why any additional full length rFurin expression fails to mediate intracellular rvWF precursor processing.

The findings observed from stable rvWF/rFurin expression in CHO cells contrast with evidence gathered from transient transfection experiments in COS cells. While rvWF propeptide removal, upon rFurin (PACE) co-expression, was improved in either case, the site where the cleavage occurs as well as the rFurin derivative molecules capable of mediating processing at a given location differed. Full length PACE and an experimentally truncated and secreted molecule termed SOL PACE were found to only mediate rvWF precursor cleavage intracellularly (Wise et al., 1990; Rehemtulla and Kaufman, 1992). Possibly, these discrepancies may be attributed to host cell specific effects as well as the transient nature and/or the low yield of expression in COS cells. SOL PACE, expressed transiently by COS or stably by CHO cells, was found to mediate the cleavage of a synthetic substrate and rFIX precursor *in vitro* but to be incapable of performing rvWF propeptide removal under analogous conditions (Rehemtulla et al., 1992; Wasley et al., 1993). Retaining the 715 N-terminal amino acids, SOL PACE is larger than shed rFurin. Therefore, access to the cleavage site in the bulky rvWF multimer may be sterically obviated to SOL PACE but may be possible for the shed rFurin and smaller derivatives. Successful intracellular rvWF processing by SOL PACE may, however, be mediated by the aforementioned auxiliary factor. This factor could assist, in a chaperone like fashion, in bringing the rvWF precursor into a favorable conformation, rendering the cleavage site more accessible to SOL PACE.

The presence of auxiliary factors specifically aiding the cleavage of individual target proteins is also suggested by experiments involving rFX cleavage. Endogenous Furin in CHO cells performs only single chain precursor to light/heavy chain cleavage. *In vitro*, shed rFurin exhibits relaxed specificity, also mediating rFX propeptide removal. Intracellularly, an endoprotease different from Furin is reponsible for this reaction (Himmelspach et al., 1998).

Due to the rapid secretion and significant extracellular accumulation, approximately 10 fold higher yields of functional rFurin molecules in the conditioned medium, compared to that of shed rFurin, were achieved by truncation of the trans-membrane domain. This suggests that complete cleavage of correspondingly higher yields of the target protein precursor, i.e. up to 20 μg rvWF/10^6 cells $\times$ day, can likely be met by co-expression with one of these rFurin derivatives. The hypothesis, that a higher degree of proteolytic maturation of a desired target protein can be achieved upon co-expression with truncated rFurin, is supported by rFIX expression experiments. While PACE co-expression was sufficient for complete precursor cleavage at 4.5 μg/10^6 cells $\times$ day, SOL PACE was used in cell clones exhibiting higher rFIX expression (Wasley et al., 1993).

In cases where the precursor of the secreted target protein is fairly stable and not prone to potentially erroneous and, hence, undesired proteolytic degradation or damage by other cellular proteases accumulating in the conditioned medium, complete processing may ultimately be achieved by extended exposure of the desired protein precursor to rFurin in the cell culture supernatant, i.e. by prolonged incubation of the conditioned medium prior to harvest.

Cleavage susceptibility of protein precursor molecules depends on the individual target sequence therein. While only a minor fraction of rFIX molecules at 4.5 μg rFIX/10^6 cells $\times$ day contained the propeptide (Wasley et al., 1993), 40% of rvWF molecules were present as precursors at 2 μg rvWF/10^6 cells $\times$ day. It may be possible to modulate cleavage by Furin to some extent by mutating either the Furin target sequence itself or, where tolerable, the amino acid(s) directly following the cleavage site, as has successfully been demonstrated for recombinant Renin

(Takahashi et al., 1994) and FIX precursors (Meulien et al., 1990).

If the DNA encoding a desired target protein is readily amplifiable beyond 20 μg/10^6 cells $\times$ day, as has been reported for rFX (Himmelspach et al., 1998) and rFIX (Kaufman et al., 1986), complete precursor processing may not be feasible even by co-expression with truncated rFurin derivatives. Rather, the establishment of an *in vitro* process, performing the proteolytic cleavage, is required. Replacement of the membrane anchor by an affinity epitope allowed efficient purification of rFurin derivatives in a one step procedure. Conventional purification procedures have been quite laborsome for full length and truncated Furin molecules (Stieneke-Gröber et al., 1992; Hatsuzawa et al., 1992).

A variety of C-terminally truncated and, hence, secreted, epitope-tagged rFurin derivatives were constructed and expressed for this purpose. The extent of the truncation and the amino acids constituting the new C-terminus determined the fate of the rFurin derivatives upon expression and their individual properties. Truncation beyond amino acid No. 564 of the wild type sequence towards the amino-terminus resulted in intracellular degradation of the rFurin molecule, whereas rFurin derivatives retaining the 577 N-terminal amino acids generally yielded secreted molecules (with one exception). This suggests that the region from amino acid 564 to 577 harbors an element crucial to the stability of Furin, the absence of which leads to degradation. However, the 'stabilizer's beneficial effect could be overcome by the addition of an inappropriate heterologous epitope, rendering the molecule prone to degradation.

By choice of a suitable epitope-tagged rFurin candidate, this strategy allows for the complete processing of excessive amounts of rvWF precursor with comparably small amounts of rFurin in processing reactions consisting solely of defined components. Upon complete cleavage, the rFurin derivative may be recaptured from the processing reaction by means of the epitope tag, and subsequently reused. Finally, successful cleavage of a Furin-specific fluorogenic substrate preliminarily suggests the feasibility of the establishment of an industrial scale *in vitro* processing procedure based on rFurin derivatives remaining bound to the column matrix. Currently, the suitability of this approach for *in vitro* processing of rvWF precursors is investigated.

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