BENCHMARKS

Benchmarks are brief communications that describe helpful hints, shortcuts, techniques or substantive modifications of existing methods.

Construct for Assessment of Transfected Secretory Proteins Using an Independently Secreted Reporter Gene


Furin is a membrane-associated endopeptidase located in the trans-Golgi network that has the enzymatic capabilities to process a large number of precursors (6,11). Furin catalyzes the cleavage of a specific sequence \((R^6-X^5-R^4-X^3,R/Lys^2-R^1)\) with the added condition that there is no hydrophobic aliphatic amino acid at position +1 (12). Furin substrate sequence occurs in many proteins, including the secretory protein stromelysin 3 (10). It has been demonstrated previously that introduction of a 10 amino acid furin substrate sequence of stromelysin 3 into MMP1 (10), sandwiched between the pro and active segments of the enzyme, renders this protein, which is normally secreted in a latent form, to be secreted in an active form.

Furin is localized in the trans-Golgi network and catalyzes cleavage of various protein precursors. For example, proinsulin has been engineered to be processed to an active form by introduction of furin substrate sequence in the cleavage sites of the propeptide (7). Similarly, fusion of a secretory protein signal sequence allows expression of the Fc fragment of human IgG1 in COS-1 cells in a secretory form (8). Different vectors have been designed for expression of secretory proteins. Some require co-transfection of a second vector containing a reporter gene (5). Others produce fusion proteins that in many cases impair normal activity of the protein of interest and necessitate laborious proteolytic cleavage protocols after production (9).

We describe the design of a new expression vector for secretory proteins that eliminates some of the foregoing difficulties. To illustrate the strategy, we describe experiments using a construct containing bacterial RNase H as a reporter gene fused in-frame to progelatinase A, with a 30-bp furin substrate of stromelysin 3 sandwiched between them. Bacterial RNase H served as reporter gene for the transfection because its detection entails a simple and sensitive assay, and it is not expected to be secreted under normal conditions into the extracellular compartment. Also, the furin substrate sequence insertion between the gene of interest (gelatinase A) and the reporter gene (RNase H) renders the two genes to be secreted in separate forms with no need to further process the gene of interest.

Gelatinase A-furin substrate-bacterial RNase H (GFR; Figure 1) was constructed using polymerase chain reaction (PCR) in several steps.

![Figure 1. The gelatinase A (solid line)-furin (box)-RNase H (solid line) (GFR) construct used in this study. Numbers above the line indicate the base numbers. Arrows above the line indicate position of primers (bold numbers above arrows) used in PCR to generate two products that were cut with Apol (arrowhead) and ligated. The full-length product (GFR) was cut with NotI (arrowheads) and ligated into plasmid to yield pGFR (right orientation) or pRFG (reverse orientation). Arrows underneath the line indicate the position of the C terminus of gelatinase A without the TGA stop codon (No Stop) and the position of the Ala residue (Met→Ala) at the N terminus of RNase H just downstream of the furin substrate sequence.](image-url)
First, full-length gelatinase A containing furin substrate sequence at its 3' end with its stop codon deleted was constructed by blunt ligation of 2 PCR products derived from phosphorylated primers and the plasmid pGf (containing furin substrate sequence between the pro and active segments of gelatinase A) as template. This PCR product was then used as template for a subsequent PCR using sense primer 2415 (containing a NotI site for downstream ligation) (TCGAGCCGCCTCG-TAGAAGGCCTAAT) and antisense primer 1452 (GCTCGAGATTTTACTCGTTAAGAGCAUGCCTGTGA-CG) to construct a second full-length progelatinase A PCR product containing a NotI site at the 5' end and furin substrate at its 3' end plus 5' RNase H sequence. The antisense primer 1452 contained 15 bases of the 5' end of bacterial RNase H, including the Apol site. This primer also replaces Met with an Ala codon in the RNase H sequence because furin does not process its substrate with a hydrophobic aliphatic amino acid (12). Primer 1452 also contained 24 bases of the 3' end of furin substrate.

Second, similarly, bacterial RNase H was amplified using sense primer 3503 (CTTAAACAGGTAGAATTTTCATCGC) and antisense primer 141 (TGCAGCCGCGAGAATGCTGAAACCAGAG) with the plasmid pOPRSVIRH (2) as template. Primer 141 contained NotI sites for downstream ligation.

The PCR products obtained in the steps above were then cut with Apol and ligated to produce the full construct of GFR (Figure 1). Construct GFR was cut with NotI and ligated into dephosphorylated pOPRSV1 (Stratagene, La Jolla, CA, USA) in both orientations to yield pGFR (right orientation) or pRFG (reverse orientation). The same strategy was used for constructing pGFR (Met) by replacing sequence ACG in position 25 of primer 1452 with CAT. Bacterial RNase H cDNA alone was also introduced into the plasmid to yield pOPRSVIRH (2).

The plasmids were transfected into murine mesangial cells (MMC) using lipofection as previously described (2). Cells were selected for the transgene using 400 µg/mL Geneticin® (Life Technologies, Gaithersburg, MD, USA) for 2 weeks, plated and maintained in serum-free medium for 24 h. Conditioned medium was collected and spun to remove cell debris, and 0.02% (final concentration) Brij-35 and azide were added. Gelatinolytic activity, detection of RNase H activity in conditioned medium and reagents used in this study are described elsewhere (1–4).

RNase H is an intracellular enzyme, and under normal circumstances, detectable RNase activity outside the cell is not expected. Consequently, any RNase H activity measured in conditioned medium implies secretion of RNase H and, in the case of the GFR construct, secretion of gelatinase A. When the GFR construct was prepared without changing Met to Ala at the start of the bacterial RNase H sequence just downstream of the furin sequence, we observed a high-molecular-weight band of gelatinolytic activity at about 85 kDa, suggesting that furin cleavage had not occurred and that the GFR product was unprocessed. However, RNase H activity was still evident under these circumstances (data not shown). Therefore, Met amino acid of the bacterial RNase H was replaced by an Ala amino acid. RNase H activity was present in a conditioned medium of cells transfected with the plasmid pGFR (1372 ± 116 [mean ± standard deviation; n = 3] disintegrations per minute [dpm]/50,000 cells) but not the control plasmid pRFG. When RNase H was transfected alone into cells using pOPRSVIRH, no RNase H activity was demonstrable in the conditioned medium. This confirms that the observed RNase H activity was caused by secretion and not simply a result of cell lysis upon processing with consequent release of endogenous transfected bacterial RNase H into the medium. To verify that the measured RNase H activity does not originate from an unprocessed GFR fusion protein, gelatinolytic activity was monitored in the conditioned medium. As shown in Figure 2, enhanced activity in an approximately 68-kDa clearing band was observed in a conditioned medium of cells transfected with pGFR compared with pRFG. Because endogenous gelatinase A and the processed transgenic gelatinase A are both expected to migrate around 68 kDa, the enhanced activity in GFR-transfected cells suggests that the gelatinase A of the transgene was processed by furin. Also, there was no high-molecular-weight clearing band at 85 kDa in the GFR lane corresponding to the unprocessed GFR fusion protein, further indicating that the fusion protein was cleaved endogenously before secretion. Note that the 68-kDa activity corresponds to the activity of latent gelatinase A (4). Taken together, the RNase H and gelatinolytic activity profile in conditioned medium suggest that the GFR fusion protein was produced by MMC, processed intracellularly and secreted as two distinct proteins into the conditioned medium.

The expression plasmid described in this study allows monitoring of secretozyme proteins (such as progelatinase A) in conditioned medium with no need to lyse cells or further process the secretozyme gene of interest. It uses bacterial RNase H as a reporter gene. The assay for RNase H activity in the conditioned medium is easy to perform, highly
sensitive, accurate and reproducible. With complete DNA sequences becoming available for various organisms, including those from the human genome project, increasing emphasis is being placed on determining the function of unknown genes. A fundamental prerequisite in assigning function is to establish whether a protein is secreted or remains inside the cell. This can now be readily accomplished by replacing gelatinase A in the GFR construct described above with the unknown gene to be characterized. One qualifying comment should be made. Secretory RNase H activity will also be observed in the case of plasma membrane proteins in which the C terminus is extracellular.

REFERENCES


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On-Line Microdialysis for Mass Spectrometry

New ionization techniques such as matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) allowing the examination of large biomolecules by mass spectrometry have catapulted the method to the center of microbiological and biochemical research. However, common contaminants in biological samples (polyethylene glycol [PEG], buffer salts, diocyl phthalate etc.) present background that often results in a partial or complete loss of signal from the molecule of interest. Several off-line dialysis devices are available, providing dialysis or ultrafiltration of microliter samples prior to mass spectrometric analysis. These include dialysis cassettes (Pierce Chemical, Rockford, IL, USA), floating membranes (Millipore, Bedford, MA, USA) and centrifugal filters (Orbital Biosciences, Topsfield, MA and Chemicon International, Temecula, CA, USA). However, they do have some disadvantages with regard to mass spectrometry in terms of dialysis rate and recovery, and most are not easily reusable. Direct, on-line dialysis overcomes these problems and is still desirable for many applications. Flow microdialysis devices have been available for several years for sampling low-molecular-weight compounds from living systems (Harvard Apparatus, Holliston, MA, USA). Wu et al. (4) have applied the reverse of this principle to on-line desalting of proteins for ESI mass spectrometry. Their idea is based on the use of only one of the 176 fibers of the Fleaker® Hollow Fiber Concentration System (Spectrum Medical Industries, Laguna Hills, CA, USA). These fibers consist of regenerated cellulose and have a molecular weight cut-off of 13,000 Da. The fiber is connected on both ends to fused silica tubing (100-µm inner diameter [id]/186-µm outside diameter [od]) and placed inside of a flexible Teflon® tube with Swagelok® T Connectors (Swagelok, Solon, OH, USA) at both ends, arranged to allow buffer to flow on the outside of the fiber. The same laboratory also described a similar microdialysis junction for capillary electrophoresis mass spectrometry (2).

Impressed by this work, we constructed the device described in Reference 4 for use in conjunction with a Model TSQ 700 Triple Quadrupole Mass Spectrometer (Finnigan MAT, San Jose, CA, USA), and we can confirm its great effectiveness in dialyzing proteins on-line. However, we found that construction of the device, although straightforward, is somewhat difficult in that rotation of the epoxy-glued joints occurs as the compression nuts on the T connectors are tightened. As a result, the fragile connections tend to break or cut through the cellulose. In