N-arginine dibasic convertase (nardilysin) isoforms are soluble dibasic-specific metalloendopeptidases that localize in the cytoplasm and at the cell surface

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N-arginine (R) dibasic (NRD) convertase (nardilysin; EC 3.4.24.61), a metalloendopeptidase of the M16 family, specifically cleaves peptide substrates at the N-terminus of arginines in dibasic motifs *in vitro*. In rat testis, the enzyme localizes within the cytoplasm of spermatids and associates with microtubules of the manchette and axoneme. NRD1 and NRD2 convertases, two NRD convertase isoforms, differ by the absence (isoform 1) or presence (isoform 2) of a 68-amino acid insertion close to the active site. In this study, we overexpressed both isoforms, either by vaccinia virus infection of BSC40 cells or transfection of COS-7 cells. The partially purified enzymes exhibit very similar biochemical and enzymic properties. Microsequencing revealed that NRD convertase is N-terminally processed. Results of

immunocytofluorescence, immunoelectron microscopy and sub-cellular fractionation studies argue in favour of a primary cytosolic localization of both peptidases. Although the putative signal peptide did not direct NRD convertase into microsomes in an *in vitro* translation assay, biotinylation experiments clearly showed the presence of both isoforms at the cell surface. In conclusion, although most known processing events at pairs of basic residues are achieved by proprotein convertases within the secretory pathway, NRD convertase may fulfil a similar function in the cytoplasm and/or at the cell surface.

Key words: cell-surface biotinylation, extracellular endoprotease, M16 family, proprotein processing, testicular enzyme.

INTRODUCTION

Several members of the M16 family of metalloproteinases [1,2] have been implicated in the proteolytic processing of peptides or proteins. Examples include insulin-degrading enzyme (insulysin; EC 3.4.24.56 [3,4]), mitochondrial matrix-processing protease ([5], for a review see [6]) and chloroplast-processing endopeptidase [7,8], these two involved in the removal of the N-terminal transit peptide of nuclear-encoded proteins, yeast pro-a-factor-processing proteases, Axl1 and Ste23 [9,10], and N-arginine (R) dibasic convertase (NRD convertase, or nardilysin; EC 3.4.24.61).

First discovered in rat brain cortex [11,12], NRD convertase was purified from testis, where it is particularly abundant ([13], for reviews see [14,15]). *In vitro*, this metalloendopeptidase cleaves various peptides such as somatostatin-28 (S-28), dynorphin A and atrial natriuretic factor at the N-terminus of arginines within paired basic residues [13], canonical processing sites for secretory precursor proteins [16,17]. The analysis of the first NRD convertase cDNA obtained from rat testis [18] predicted an 1161-amino acid (aa)-long sequence containing a highly acidic stretch of 71 residues (79% Glu and Asp) and an HXXEH motif found in the catalytic site of several metallopeptidases [19,20]. Recently, a second NRD convertase cDNA was identified in human and rat testis [21]. The deduced aa sequence, NRD2 convertase, differs from the originally characterized NRD1 convertase by a

68-aa insertion between the acidic stretch and the HXXEH motif. Using a specific probe for each NRD isoform, Northern-blot analyses demonstrated that the NRD2 convertase isoform represents 50 and 10% of the total NRD convertase transcripts in human and rat testis, respectively [21]. However, since attempts to obtain rat NRD2 (rNRD2) convertase-specific antibodies remain unsuccessful, the ratio *in vivo* of the two protein isoforms in testis and other tissues could not be evaluated. In human, the gene encoding NRD convertase was localized to chromosome 1p32 [21].

Although NRD convertase expression was detected in most of the tissues analysed [13,21,22], testis remains its major expression site in the adult rat. In this tissue, NRD convertase synthesis is restricted to germ cells and is regulated during spermiogenesis [23]. While detected at low levels in young spermatids, the protein accumulates in the cytoplasm of elongated spermatids with a noticeable concentration at the level of two microtubular structures, the manchette and the axoneme [23]. Its regulated expression during spermatogenesis and its concentration in the flagellum suggest a role in the differentiation of the male germ cell. Furthermore, murine NRD convertase transcripts were detected on embryonic day 10.5, almost exclusively within neural tissues, suggesting that the enzyme plays specific roles in neuronal development as well [22]. NRD convertase mRNAs were also detected in all cell lines analysed [21]. Recently, this endopeptidase was rediscovered as a dynorphin-converting enzyme in

Abbreviations used: NRD convertase, N-arginine (R) dibasic convertase; rNRD convertase, rat NRD convertase; S-28, somatostatin-28; VV, vaccinia virus; IF, immunofluorescence; IEM, immunoelectron microscopy; ER, endoplasmic reticulum; aa, amino acid; NEP, neutral endopeptidase; D-PBS, Dulbecco's PBS; GFP, green fluorescent protein; β -Gal, β -galactosidase.

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the culture medium of the mouse thymus-derived cell line EL-4 [24]. Thus NRD convertase appears to be localized in the cytoplasm and/or to be secreted by some cell types, including the male germ cells [23]. Although the N-terminus of the protein shows some characteristics reminiscent of a signal peptide [25], its functionality remains to be established.

To study the potential differences existing between the two closely related NRD convertase isoforms, we overexpressed them by infecting BSC40 cells with recombinant vaccinia viruses (VVs). rNRD1 and rNRD2 convertases were then partially purified, and their biochemical and enzymic properties analysed. Their subcellular localization was investigated in transiently transfected COS-7 cells and/or infected BSC40 cells by immunofluorescence (IF), immunoelectron microscopy (IEM), subcellular fractionation and biotinylation of cell-surface proteins. The ability of the N-terminus of NRD convertase to function *in vitro* as a signal peptide was also assessed.

EXPERIMENTAL

Cell culture

BSC40 and COS-7 cells were respectively maintained in minimal essential medium (Gibco-BRL, Life Technologies, Paisley, Scotland) and Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 25 µg/ml gentamicin (all from Gibco-BRL) at 37 °C in 5% CO₂.

Plasmid constructions

The 3'-untranslated region of the rNRD1 convertase cDNA in pBluescript II SK⁺ (pBlrNRD1 [18]) was shortened by replacing the *NsiI*–*HindIII* digestion product with a PCR fragment that inserts a *HindIII* restriction site 26 bp downstream of the stop codon (pBlrNRD1exp3'). This modified cDNA was then subcloned as a *SmaI*–*HindIII* fragment into the *EcoRV* and *HindIII* sites of the mammalian expression vector pcDNA3 (Invitrogen, Leek, The Netherlands). The removal of the 5'-untranslated region was achieved in two steps. The 3592-bp *SmaI*–*HindIII* fragment was first digested with *Bst*UI to remove the whole 5'-untranslated region plus 16 bp of the open reading frame. This was then ligated with a 24-bp-long linker composed of two annealed oligonucleotides, 5'-TCGACAGAAATGCTGAGGAGAGTCG-3' and 5'-CGACTCTCCTCAGCATTCTG-3', thereby introducing a *SalI* site 4 bp upstream of the initiation codon and a nucleotide context respecting Kozak's rules [26]. This ligation product was digested by *SalI* and subcloned into the VV transfer plasmid pMJ601 [27] to generate pMJ601-rNRD1. The pMJ601-rNRD2 and pcDNA3-rNRD2 vectors were obtained by replacing the *Bgl*II fragment (nt 439–1290) with a *Bgl*II cassette containing the 204-bp insertion of rNRD2 convertase.

For *in vitro* translation experiments, the *Bam*HI fragment of pBlrNRD1, containing part of the polylinker and nt 1–1348 of rNRD1 convertase cDNA, was cloned into the *Bam*HI site of the mutagenesis vector tg131 (Sculptor, Amersham Pharmacia Biotech, Uppsala, Sweden). In order to introduce a *HindIII* site at position 45, the underlined nucleotides shown here were mutagenized by the use of the oligonucleotide 5'-CTAGAGC-GGCCGCAAGCTTAGCGC-3'. The resulting *HindIII*–*Bam*HI fragment was subcloned in pBluescript II KS⁺ to generate pBl5'rNRD1Δ1-44. The vector containing the full-length cDNA of the rabbit neutral endopeptidase (NEP, or neprilysin; EC 3.4.24.11 [28]) under the control of the SP6 RNA polymerase promoter (pSP6NEP) was kindly provided by Dr G. Boileau (University of Montreal, Montreal, Quebec, Canada).

VV infections

The pMJ601 constructs were used to produce NRD convertase recombinant VV [27]. VV:mPC5* in which the active-site Ser³⁵⁴ of the proprotein convertase PC5 was mutated to Ala has been described previously [29]. VV:βGal expressed the *Escherichia coli* β-galactosidase (β-Gal). For enzyme preparation, subcellular fractionation and biotinylation, confluent BSC40 cells were infected at a multiplicity of infection of 5. Parallel plates were either not infected, or infected with the wild-type virus (VV: WT) or the recombinants VV:rNRD1, VV:rNRD2, VV:mPC5* or VV:βGal. Following 30-min infections at 20 °C in PBS containing 1 mM MgCl₂ and 0.01% BSA, the inoculum was replaced by minimal essential medium. Cells were incubated at 37 °C for either 16–18 h for enzyme preparation or 4–5 h for subcellular fractionation and biotinylation experiments. They were then scraped into a Dulbecco's PBS (D-PBS, Gibco-BRL) medium and collected by centrifugation (1000 g for 10 min) or treated with biotin.

Enzyme preparation

A single Wistar rat testis (Pelfreeze, Rogers, AR, U.S.A.) or pooled duplicate naïve or infected BSC40 cells (4 × 10⁷ cells) were homogenized in 50 mM Tris/HCl, pH 7.5, containing 5 mM β-mercaptoethanol, and centrifuged at 14000 g for 15 min. The supernatant (homogenate) was applied to a DEAE-Trisacryl M ion-exchange column (10 ml bed volume; BioSeptra, Villeneuve la Garenne, France) pre-equilibrated with 50 mM Tris/HCl, pH 7.5/150 mM KCl. After washing with 50 ml of the same buffer, the column was eluted with a 30-ml linear gradient from 150 to 400 mM KCl in 50 mM Tris/HCl, pH 7.5. Fractions (1 ml) were collected and assayed for enzyme activity. Pools of active fractions (post-DEAE pools) were concentrated and desalted on Ultrafree-30K (Millipore, Molsheim, France) and used in enzymic assays, analysis by SDS/PAGE (8% gel) and Western blotting or N-terminal sequencing. Purified NRD convertase was prepared from rat testis as described previously [13].

pH optima and inhibitor profiles

Routine enzyme assays were carried out by incubation of an aliquot of the enzyme preparation with either 5 µg of PRE-RKAGAKNY-NH₂ ([Ala¹⁷, Tyr²⁰]S-28-(10-20)-NH₂, residues 10–20 from S-28, the 17th (Ala) and 20th (Tyr) residues being different from the wild-type sequence [11]) in 50 mM Tris/HCl, pH 8.9 (buffer A), or 3.5 µg of dynorphin A (Sigma, St. Louis, MO, U.S.A.) in 250 mM Tris/HCl, pH 7.5 (buffer B), in a 100-µl final volume. The reaction was stopped by adding 10 µl of concentrated acetic acid and analysed by reverse-phase HPLC on a µ-Bondapak C₁₈ column, as described in [13]. For the inhibition study, enzymes were preincubated with the inhibitor for 15 min at 37 °C, prior to the addition of the substrate. A control incubation without inhibitor was defined as 100% endopeptidase activity. For pH-optima determinations, the substrate PRERKAGAKNY-NH₂ was digested in the following buffers: 50 mM sodium phosphate, pH 5.8–7.6, 50 mM Tris/HCl, pH 7.5–8.9, and 50 mM glycine/NaOH, pH 8.7–10.5.

Transfections of COS-7 cells

The pcDNA3, pcDNA3-rNRD1, pcDNA3-rNRD2 and pIRES2-EGFP (Clontech, Palo Alto, CA, U.S.A.) vectors were transfected into COS-7 cells using Superfect (Qiagen, Hilden, Germany). Briefly, 24 h before transfection, cells were grown on 100-mm plates (10⁶ cells), containing glass coverslips for IF

experiments, or on 35-mm plates for ultrastructural studies (10^5 cells). The Superfect-DNA precipitate, prepared using either 10 or 2 μg of DNA and 60 or 20 μl of Superfect depending on plate size, was incubated with COS-7 cells for 2.5 h at 37 °C. The cells were washed successively with D-PBS and fresh Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 25 $\mu\text{g}/\text{ml}$ gentamicin was added. Later (24 h), cells were either harvested for Western-blot analysis or subcellular fractionation, fixed for immunocytochemistry or treated with biotin.

Subcellular fractionation of transfected COS-7 cells and infected BSC40 cells

Transfected or infected cells were scraped in D-PBS, gently pelleted at 500 g , put on ice and washed twice with PBS. They were then resuspended in a buffer (5 mM Tris/HCl, pH 7.5/250 mM sucrose/1 mM MgCl_2) containing various protease inhibitors (2.5 mM EDTA/2 mM β -mercaptoethanol/100 $\mu\text{g}/\text{ml}$ PMSF/100 μM pepstatin/2 $\mu\text{g}/\text{ml}$ leupeptin and aprotinin) and broken by repeated passages through a 25-gauge needle. Nuclei and cellular debris were removed by centrifugation at 2000 g for 10 min at 4 °C. The resulting supernatant (S1) was centrifugated at 100 000 g for 30 min at 4 °C in a TLA 120-2 fixed-angle rotor (Beckman Instruments, Fullerton, CA, U.S.A.). The supernatant (S2), containing mainly cytoplasmic proteins, was collected. The pellet (P2), containing among others intact microsomes, was washed twice and resuspended in the above buffer after one freeze-thaw cycle. The proteins in the S2 and P2 fractions were analysed by Western blotting.

Antibodies

The calreticulin and PC5 polyclonal antisera used were directed against human recombinant calreticulin (both antigen and antibody were provided kindly by Dr L. Rokeach, University of Montreal) and the N-terminus of active mouse PC5 [30], respectively. For immunocytochemistry, the polyclonal NRD convertase antiserum [13] was purified as follows. Rat testis NRD convertase was electrophoresed on an 8% SDS/polyacrylamide gel and transferred on to a nitrocellulose membrane. After incubation with the anti-rNRD convertase immune serum, antibodies associated with the 140-kDa form were eluted from the membrane at 4 °C in 0.1 M glycine, pH 2.8, and neutralized with 2 M Tris/HCl, pH 8. Anti-green fluorescent protein (GFP) monoclonal antibodies and rabbit anti-*E. coli* β -Gal antibodies were purchased from Molecular Probes (Eugene, OR, U.S.A.) and Eppendorf-5Prime (Boulder, CO, U.S.A.) respectively.

IEM and IF

Transfected COS-7 were fixed in periodate/lysine/paraformaldehyde [31] for 2 h at room temperature, washed three times in PBS, preincubated for 30 min in 50 mM NH_4Cl , and permeabilized in PBS containing 0.2% gelatin/0.005% saponin for 1 h. After a 3-h incubation with purified anti-NRD convertase antibodies, cells were rinsed three times with PBS and incubated for 90 min with a goat Fab anti-rabbit IgG conjugated with peroxidase (Biosys, Compiègne, France) for IEM or with goat anti-rabbit IgG conjugated with FITC (Jackson Immuno-Research Laboratories, West Grove, PA, U.S.A.) for IF. For IEM, cells were postfixed in 1% glutaraldehyde and the peroxidase activity was visualized using 3,3'-diaminobenzidine tetrahydrochloride. They were then fixed in 1% osmium tetroxide, dehydrated and embedded *in situ* in epon [32]. Selected areas of

immunoreactive cells were sectioned as described previously [33]. Ultrathin sections were examined at the electron microscope without further staining. For IF, cells were washed and examined with an epifluorescence microscope (Leica Microscopy, Heerbrugg, Switzerland).

Western-blot analyses

Whole-cell extracts were prepared as follows. Cell pellets were resuspended in 50 mM Tris/HCl, pH 7.4/150 mM NaCl/0.02% sodium azide supplemented with the above cocktail of protease inhibitors. Crude extracts were then submitted to three cycles of freeze-thaw, homogenized through a 25-gauge needle, centrifuged at 12 000 g for 30 min and the supernatants analysed. Proteins from whole cell extracts, subcellular fractions or post-biotinylation immunoprecipitation were separated by 8 or 10% SDS/PAGE (SE250 apparatus; Hoefer, San Francisco, CA, U.S.A.) and transferred on to nitrocellulose membranes (Optitran BA-S 85; Schleicher and Schuell, Dassel, Germany) using a semi-dry TE270 unit (Hoefer). Anti-NRD convertase, anti-calreticulin, anti-PC5 and anti- β -Gal polyclonal sera were used at final dilutions of 1/2000, 1/5000, 1/500 and 1/1000, respectively. The antigen-antibody complexes were revealed using a donkey anti-rabbit IgG horseradish peroxidase conjugate (ECLPlus Western blotting detection, Amersham Pharmacia Biotech). Monoclonal anti-GFP was used at a 1/400 final dilution and revealed using a sheep anti-mouse IgG horseradish peroxidase conjugate (Amersham Pharmacia Biotech). Prestained molecular-mass markers were purchased from New England Biolabs (Hitchin, Herts., U.K.) or from Amersham Pharmacia Biotech. Protein concentration was estimated as described previously [34].

Transcription-translation analyses *in vitro*

The plasmids pBl5'rNRD1 Δ 1-44 and pSP6NEP were linearized with *Ssp*I and *Bst*XI, respectively, and transcribed *in vitro* using an RNA transcription-translation kit (Promega, Madison, WI, U.S.A.). RNAs (5 μg) were translated *in vitro* in the presence or absence of rough microsomes and [^{35}S]methionine. Where indicated, trypsin and Triton X-100 were added to 5 $\mu\text{g}/\text{ml}$ and 1% final concentrations respectively. The translation products were analysed on SDS/PAGE (15% gel) followed by fluorography (Amplify, Amersham Pharmacia Biotech).

Biotinylation of cell-surface proteins

Transfected COS-7 and infected BSC40 cells were washed three times with ice-cold PBS with 1 mM MgCl_2 and incubated for 1 h at 4 °C with 0.5 mg/ml sulphydryl-NHS-LC-Biotin (Pierce, Rockford, IL, U.S.A.). Then, cells were washed three times with ice-cold PBS containing 1 mM MgCl_2 , lysed in a buffer (20 mM Tris/HCl, pH 8.0/150 mM NaCl/1% sodium deoxycholate/1% Triton X-100) containing protease inhibitors (protease-inhibitor-cocktail tablets, Amersham Pharmacia Biotech) and centrifuged at 12 000 g for 15 min at 4 °C. The supernatants were immunoprecipitated with antibodies directed against NRD convertase (1/250), β -Gal (1/200) or GFP (2 μg). Immunoprecipitated proteins corresponding to 2×10^6 transfected COS-7 or 10^6 infected BSC40 cells were resolved by SDS/PAGE (10% gel) and transferred on to nitrocellulose membranes as described above. Biotinylated proteins were revealed with a streptavidin-horseradish peroxidase conjugate (Amersham Pharmacia Biotech).

RESULTS

Comparison of the biochemical properties of the two recombinant rNRD isoforms

Expression of enzymically active rNRD1 and rNRD2 convertases

To study the possible influence of the 68-aa insertion [21] on the catalytic properties of NRD2 convertase, we first expressed the two rat isoforms either by VV infection of BSC40 cells or by

Figure 1 Overexpression of rNRD1 and rNRD2 convertases in infected BSC40 cells and transfected COS-7 cells

Western-blot analysis of crude protein extracts from (A) BSC40 cells infected with the wild-type VV (V, 9 µg), the recombinant VV:rNRD1 (3 µg) and VV:rNRD2 (9 µg); (B) and COS-7 cells transfected with pcDNA3 vector (V, 15 µg), pcDNA3-rNRD1 (5 µg) and pcDNA3-rNRD2 (15 µg) were separated by SDS/PAGE on an 8% polyacrylamide gel. Purified rat testis NRD convertase (rNRD; 0.67 µg) and crude testis extract (T; 10 µg) were also analysed.

Table 1 Inhibition profiles of rNRD convertase isoforms and rat testicular enzyme

Results are expressed as percentage inhibition, taking the control incubation as 0% inhibition. Quantities of enzyme introduced in the assay were adjusted for each enzyme preparation to obtain a 50% cleavage of the substrate in 15 min at 37 °C in the absence of inhibitor. Conditions were as follows: (A) 50 mM Tris/HCl, pH 8.9, and 5 µg of PRERKAGAKNY-NH₂; (B) 250 mM Tris/HCl, pH 7.5, and 3.5 µg of dynorphin A. The enzyme was preincubated with the inhibitor for 15 min at 37 °C and the reaction initiated by addition of the substrate. After 15 min of incubation, the reaction was stopped by addition of 10 µl of glacial acetic acid. The final volume of the assay was 100 µl.

(A)	Inhibition (%)		
	Testicular rNRD	rNRD1	rNRD2
EDTA (100 µM)	32 ± 5	56 ± 2	46 ± 4
1,10-Phenanthroline (500 µM)	59 ± 4	100	100
N-Ethylmaleimide (1 mM)	18 ± 4	11 ± 4	0
Amastatin (200 µM)	25 ± 3	86 ± 1	100
Bestatin (500 µM)	0	0	0
(B)	Inhibition (%)		
	Purified rNRD	rNRD1	rNRD2
EDTA (100 µM)	100	100	100
1,10-Phenanthroline (500 µM)	100	100	100
N-Ethylmaleimide (1 mM)	100	100	100
Amastatin (200 µM)	33 ± 2	0	0
Bestatin (500 µM)	0	0	0

transient transfection of COS-7 cells. The expression of the recombinant proteins was monitored by Western-blot analyses of crude cell extracts (Figure 1). In both expression systems, the recombinant rNRD1 convertase migrated with an apparent molecular mass of 140 kDa, similar to that of rNRD convertase

Figure 2 pH dependence of PRERKAGAKNY-NH₂ cleavage by recombinant rNRD convertase isoforms or the rat testis purified enzyme

The percentage of peptide cleavage was determined at various pH values (5.8–10.5) using the following buffers: 50 mM sodium phosphate (▲), 50 mM Tris/HCl (□) and 50 mM glycine/NaOH (■). The extent of cleavage at the pH optimum (8.9) was taken as 100%.

Figure 3 N-termini of rNRD convertase species

Western-blot analyses of post-DEAE concentrated pools from rat testis (T, 5 μ g) and from BSC40 cells infected by VV:rNRD1 (1 μ g) or VV:rNRD2 (2 μ g) are shown. N-terminal sequencing of 140-kDa (rNRD and rNRD1) or 110-kDa (rNRD and rNRD2) species identified Val⁷⁷ and Gly⁸¹, and Asp¹⁸⁸ and Gly²⁷⁵, as N-termini, respectively. These are positioned on a schematic representation of the NRD convertase sequence below. The white box represents the 71-aa acidic stretch while the elevated hatched one represents the specific rNRD2 convertase 68-aa insertion.

purified from rat testis. rNRD2 convertase showed a slightly slower migration ($\approx$ 145 kDa) in accordance with its additional 68 aa. Although rNRD2 convertase transcripts represent 10% of the total NRD convertase mRNA in testis [21], we could not detect rNRD2 convertase in testicular extracts (Figure 1, rNRD and T). Since to date rNRD2 convertase-specific antibodies are not available, it is difficult to conclude whether this isoform is absent or scarcely present in this tissue. The 110-kDa immunoreactive protein detected in the rat testis crude extract and the

purified enzyme preparation was identified as a degradation product of NRD convertase (see below). Also, neither rNRD1 nor rNRD2 convertases could be detected in culture media of either cell line (results not shown), suggesting that they are not secreted by these cells.

After partial purification on a DEAE-Trisacryl M ion-exchange column, both recombinant proteinases were shown to cleave the model peptide PRERKAGAKNY-NH₂. This synthetic peptide mimicking the dibasic processing site of pro-somatostatin had been previously used routinely to monitor the purification of NRD convertase [11,13]. As with the endoprotease isolated from rat testis, isoforms 1 and 2 produced the same proteolytic fragment (RKAGAKNY-NH₂). Finally, comparable percentages of cleavage were obtained when the same quantity of each enzyme was used (as estimated by silver staining of SDS/acrylamide gels). Control extracts from BSC40 cells not infected or infected by the wild-type VV, as well as those from COS-7 cells transfected with pcDNA3, were totally devoid of activity towards the model peptide. To further analyse the biochemical properties of each isoform, the VV overexpression system was chosen because of its higher expression rate.

pH optima and inhibition profiles

Using the same model substrate as above, an optimal pH of 8.9 was determined previously for the purified rat testis enzyme [13]. Since at this pH the optimal concentration in Tris was found to be 50 mM (results not shown), three 50 mM buffers covering a pH range from 5.8 to 10.5 were used. As seen in Figure 2, the same major optimal pH of 8.9 was obtained for the three enzyme preparations (done in triplicate). In addition, the three curves were superimposable. A similar shoulder was observed for each enzyme preparation around neutral pH. However, rNRD2

Figure 4 IF localization of rNRD convertase isoforms

Transfected COS-7 cells with either pcDNA3-rNRD1 (A) or pcDNA3-rNRD2 (B) or untransfected cells (C) were immunolabelled with rNRD convertase-purified antibodies (A, B) or anti-calreticulin (C). Negative controls consisted of either COS-7 cells transfected with pcDNA3 and revealed with anti-rNRD convertase (E) or COS-7 cells expressing rNRD1 convertase and labelled with rNRD convertase preimmune serum (D). Scale bar, 10 μ m.

Figure 5 IEM

NRD convertase immunoreactivity in COS-7 cells transfected with pcDNA3-rNRD1 (**A**) or pcDNA3-rNRD2 (**B**) was visualized indirectly by peroxidase activity. In (**B**), note the presence of transfected (upper) and untransfected (lower) cells present on the same section. Scale bar, 1 μ m.

convertase seemed to conserve a higher activity at acidic pH.

To detect any differences between the enzymic properties of the two isoforms, various reagents reported to inhibit NRD convertase activity [13] were tested in two types of assay (Table 1A and 1B). The first one (Table 1A) was performed in 50 mM Tris/HCl, pH 8.9, with the model substrate PRERKAGAKNY-NH₂, whereas the second (Table 1B) was carried out in 250 mM Tris/HCl, pH 7.5, with dynorphin A as a substrate, as described previously [13]. In both assays, the two isoforms showed very similar inhibitor sensitivities. However, except for 1,10-phenanthroline, the amounts of inhibitors needed to achieve 100% inhibition in assay B were 10 times higher than reported previously [13]. This discrepancy may be explained by the lower degree of purity of the enzyme preparation used here. The same explanation stands for the higher inhibitions observed for rNRD1 or rNRD2 convertases than for the rat testis enzyme (except in the case of *N*-ethylmaleimide). Indeed, the latter exhibits a lower specific activity. Curiously, whereas testis rNRD convertase showed comparable inhibitions by amastatin in both assays, the

two isoforms were either not inhibited (assay B) or completely inhibited (assay A). Note that 500 μ M bestatin, representing a 10-fold higher concentration than previously used, failed to inhibit any of the three enzymes.

N-terminal heterogeneity of the different NRD convertase immunoreactive species

Western-blot analysis of the partially purified enzyme preparations revealed a noticeable difference in the stability of the respective isoforms (Figure 3). While rNRD1 convertase remained relatively stable as a 140-kDa protein for several days at 4 °C, rNRD2 convertase was transformed rapidly into a 110-kDa protein. The disappearance of the 145-kDa rNRD2 form seemed to be concomitant with the loss of enzyme activity. Whereas the partially purified rNRD1 convertase samples showed comparable specific activities 2 or 10 days post-homogenization, rNRD2 convertase activity, which was easily measurable at day 2, could no longer be monitored at day 10. In addition to the major 140-kDa and minor 110-kDa forms, another minor 120-kDa immunoreactive protein is also observed in Figure 3 (lane NRD1). Only in BSC40 cell extracts did we observe similar levels of the 110- and 120-kDa forms. In testicular extracts, we rarely see the 120-kDa form. The nature of this intermediate is yet to be determined. The possible presence in the rNRD2 convertase preparation of a contaminant responsible for its degradation, and its absence in the rNRD1 convertase preparation, was ruled out by mixing the partially purified enzymes in various ratios. Indeed, the 140-kDa rNRD1 convertase remained stable in the presence of various quantities of rNRD2 convertase preparation. We thus hypothesize that rNRD2 convertase offers a specific fragile site. Concerning the rat testis enzyme, the formation of an inactive 110-kDa degradation product mainly occurred during the last hydroxyapatite purification process [13].

N-terminal sequencing of the 140-kDa species revealed that rat testis NRD convertase and recombinant rNRD1 convertase start at Val⁷⁷ and Gly⁸¹, respectively (Figure 3; sequence accession no. L27124). Due to the instability of the 145-kDa rNRD2 convertase, its N-terminus could not be elucidated. Sequence analysis of the 110-kDa forms of rNRD2 and testicular rNRD convertases showed that they start at Gly²⁷⁵ (i.e. within the 68-aa insertion; accession no. X93208) and at Asp¹⁸⁸ (i.e. within the last third of the acidic stretch), respectively (Figure 3). The identification of a rNRD2-specific residue (Gly²⁷⁵) as the N-terminus of the 110-kDa rNRD2 species reinforces the above hypothesis of a fragile site specific to rNRD2 convertase. Whether the corresponding cleavage, which occurs during the purification process, may have relevance *in vivo* or not remains to be determined.

Subcellular localization of recombinant rNRD1 and rNRD2 convertases

IF microscopy

To determine the intracellular localization of the rNRD convertase isoforms, an IF microscopy study was conducted in transiently transfected COS-7 cells. Similar staining patterns, consisting of a dense non-uniform perinuclear staining that extends up to the cell periphery, were observed in rNRD1 or rNRD2 convertase-expressing cells (Figures 4A and 4B). To check if this pattern indicates an endoplasmic reticulum (ER)-like localization, cells were also stained with an antiserum directed against calreticulin (Figure 4C), a soluble protein localized mainly

Figure 6 Distribution of overexpressed rNRD1 and rNRD2 convertases isoforms in subcellular fractions

Membrane (P2) and soluble (S2) fractions (15 μ g) from BSC40 cells infected with the wild-type VV (V), VV:rNRD1, VV:rNRD2, VV:PC5* (see text) or 20 μ g of fractions P2 and S2 of transfected COS-7 cells with pcDNA vector (V), pcDNA3-rNRD1 and pcDNA3-rNRD2 were analysed by Western blotting using anti-rNRD convertase or anti-PC5 polyclonal sera. BSC40 cell extracts were also probed with anti-calreticulin (antiCaR) antibodies. Lanes C consist of a mixture of 0.67 μ g of purified testicular rNRD convertase and 0.1 μ g of human recombinant calreticulin. Black arrowheads show NRD convertase species in either BSC40 or COS-7 subcellular fractions (left-hand upper and lower panels), whereas white arrowheads indicate either to PC5-specific species (upper right panel) or calreticulin-specific species (middle left and lower right panels).

in ER [35]. Although rNRD convertase also presents a punctuate labelling, its staining is more diffused when compared with that of calreticulin. The two negative controls consisting of either rNRD1 convertase-transfected cells incubated with preimmune serum (Figure 4D) or COS-7 cells transfected with pcDNA3 (Figure 4E) did not show any labelling.

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At the ultrastructural level (Figure 5), cells expressing either rNRD convertase isoform were characterized by the same dense labelling throughout the cytoplasm, whereas non-transfected cells were totally devoid of staining (Figure 5B). These data support the primary location of the enzyme as being within the cytoplasm.

Subcellular fractionation

Infected BSC40 cells and transfected COS-7 cells were homogenized in an isotonic buffer (0.25 M sucrose), and the resulting extract submitted to consecutive low- and high-speed centrifugations. Aliquots of the resulting high-speed pellets (P2) and supernatants (S2) were analysed by Western blotting (Figure 6). The fraction P2 was expected to contain primarily intact organelles and membrane-associated proteins. In both cell types, rNRD1 and rNRD2 convertases were found predominantly in the soluble S2 fractions. The ratio of either isoform in the soluble fraction to that in the membrane (S2/P2) was about 2 in BSC40 cells and 4 in COS-7 cells. As a control, the 54-kDa calreticulin

was detected mainly in the P2 fractions of BSC40 cells. The calreticulin immunoreactive 58-kDa species, only observed in P2 fractions, were not identified. In order to eliminate the possibility that overexpression causes missorting of proteins, BSC40 cells were also infected by VV:mPC5*, encoding a catalytically inactive mutant of the proprotein convertase PC5 [29], which is retained as a zymogen in the lumen of the ER since it is unable to autocatalytically cleave its pro-segment (N. G. Seidah, unpublished work). As expected, mPC5* immunoreactivity was detected exclusively in the P2 fraction (Figure 6). Thus when compared with the absence of mPC5* or the limited amount of calreticulin in S2 fractions, the above data suggest that rNRD1 and rNRD2 convertases were present predominantly in soluble fractions, even though a significant portion was also present in membrane fractions.

Translation *in vitro* of rNRD1 convertase mRNA

The presence of NRD convertase prompted us to check the ability of its putative signal peptide to direct it to the ER lumen. Transcription *in vitro* of the 5' region of rNRD1 (up to nt 1174 [18]) or NEP (up to nt 1004 [29]) cDNAs was performed and the resulting RNAs were used in an *in vitro* translation assay. In the absence of canine microsomes, the protein products showed apparent molecular masses of 42 and 36 kDa, respectively, in agreement with the sizes expected from each construct (Figure 7, lane 1). In the presence of canine microsomes, an increase in the size of the NEP product was observed (Figure 7, lane 4). This is in accordance with the presence of an N-terminal hydrophobic

Figure 7 Translation and translocation assay *in vitro* of an N-terminal rNRD1 convertase polypeptide

In vitro-synthesized RNAs encoding N-terminal regions of rNRD1 convertase or NEP were translated *in vitro* using a rabbit reticulocyte lysate system in the absence (lanes 1–3) or presence (lanes 4–6) of dog pancreas microsomes. Aliquots of the translated products were assayed for sensitivity to trypsin digestion (lanes 2, 3, 5 and 6) in the absence (lanes 2 and 5) or presence (lanes 3 and 6) of Triton X-100.

signal peptide/membrane-anchor motif allowing an ER luminal location and N-glycosylation of NEP translation products. Under the same conditions, no apparent molecular-mass change was observed for the rNRD1 convertase products, in spite of the presence of two putative N-glycosylation sites (compare lanes 1

and 4 in Figure 7). In addition, whereas NEP translation products were sensitive to trypsin digestion only in the presence of Triton X-100, which lyses microsomes, rNRD1 convertase translation products were digested with trypsin even in the absence of Triton X-100 (Figure 7, lanes 2, 3, 5 and 6). With the caveat that this assay was conducted with a truncated form of rNRD1 convertase to facilitate the detection of potential changes in molecular mass, these results suggested that the N-terminus of NRD convertase did not function as a signal peptide and reinforce the proposal of a primary cytoplasmic location for the enzyme.

Biotinylation of the cell-surface proteins

The inability *in vitro* of NRD convertase to enter into microsomes was surprising, since it was reported to be secreted by spermatids [23] and EL-4 cells [24]. However, as mentioned above, it could not be detected in culture media of COS-7 or BSC40 cells, even when rNRD1 or rNRD2 convertases were overexpressed. Since the subcellular P2 fraction may also contain plasma membranes, the possible presence of the enzyme at the cell surface was assessed. Intact COS-7 or BSC40 cells overexpressing either rNRD1 or rNRD2 convertases, or control proteins, were biotinylated and then lysed. Immunoprecipitated proteins were analysed subsequently by Western blotting and revealed by either streptavidin (Figure 8, upper panel) or specific antibodies (Figure 8, lower panel). In either expression system, biotinylated rNRD1 and rNRD2 convertases were detected. On the other hand, the two control cytoplasmic proteins overexpressed by either infection of BSC40 cells (β -Gal, $\approx$ 110 kDa) or transfection of COS-7 cells (enhanced GFP, $\approx$ 28 kDa) did not show any streptavidin labelling, but were immunoprecipitated effectively (Figure 8, lower panel). Thus these results indicate clearly that both NRD convertase isoforms are present at the cell surface and that this localization cannot be attributed to their overexpression. No labelling of the cell surface in the immunohistochemical study (Figures 4 and 5) could be observed but this may be due to the fixation conditions and/or to a low cell-surface/cytoplasm ratio of the enzyme. However, it is worth noting that the pH

Figure 8 Biotinylation of cell-surface proteins

BSC40 and COS-7 cells overexpressing either rNRD1 or rNRD2 convertases, β -Gal or enhanced GFP (EGFP) were biotinylated. Immunoprecipitated proteins (see the Experimental section) were revealed by either streptavidin or specific antibodies. WT and V indicate that the cell extracts were obtained by wild-type VV infection or pcDNA3 transfection, respectively. Arrows indicate the expected positions of the different proteins analysed.

optimum of the enzyme is in favour of its activity being in the extracellular compartment.

DISCUSSION

Since the discovery of NRD convertase 10 years ago, its cellular localization has been unambiguously defined only in the testis, its major expression site, where it is associated with two microtubular structures of late spermatids [23]. Until now, the physiological function of NRD convertase has remained unresolved. In addition, it was recently shown that human and rat NRD convertases exist as two isoforms, NRD1 and NRD2 convertases [21]. They differ by the presence in NRD2 convertase of a 68-aa insertion close to the active site, which possibly confers a specific function to this isoform. In this report, we aimed at comparing the biochemical and enzymic properties as well as the subcellular distribution of overexpressed rNRD1 and rNRD2 convertases.

Purification of each recombinant enzyme following VV infections of BSC40 cells allowed the comparison of their properties *in vitro*. The data did not reveal any significant differences in their optimal pH towards a somatostatin-related undecapeptide substrate (Figure 2). Furthermore, their enzymic activities towards dynorphin A or the above peptide exhibited comparable sensitivities to inhibitors (Table 1). Parallel analyses showed that both recombinant enzymes behaved mostly like native NRD convertase purified from rat testis. Accordingly, this study did not allow us to establish significant variation between the enzymic properties of the two isoforms. The major difference observed resided in the instability of rNRD2 convertase, which was converted readily into a 110-kDa species (Figure 3). The corresponding cleavage occurs upstream of Gly²⁷⁵, which lies within the 68-aa insertion. The physiological relevance *in vivo* of this observation is uncertain since the 110-kDa species could not be detected in fresh cellular extracts.

N-terminal sequencing of the purified testis enzyme and recombinant rNRD1 convertase revealed that the first ≈ 80 residues predicted from the cDNA sequence were missing. Indeed, the unique N-terminus identified for the testis NRD convertase suggested a cleavage at GlyArg↓Val⁷⁷, whereas that of the recombinant rNRD1 convertase isolated from BSC40 cells revealed processing at ArgLeu↓Gly⁸¹ (Figure 3). Alignment of the sequences surrounding these N-termini did not reveal any striking similarity and the candidate enzyme(s) involved remains to be identified. Such N-terminal variability may reflect a protease-sensitive region processed in a cell-specific manner *in vivo* or during the purification procedure. Future studies will address the question of the role of the N-terminal sequence and its effect on the activity of NRD convertase.

Whereas NRD convertase has been localized in the cytoplasm of spermatids [23], several elements prompted us to check for the possible localization of the enzyme, or one of its isoforms, in the secretory pathway: (i) detection of the enzyme in conditioned [23] or culture media [24]; and (ii) a cleavage selectivity reminiscent of that of the secretory proprotein convertases [36].

The present study shows that both NRD convertase isoforms localize primarily within the cytoplasm of BSC40 and COS-7 cells, although a significant fraction of these proteins is present at the cell surface. This was based on a compendium of results obtained by immunocytochemical analyses at both the light (Figure 4) and electron (Figure 5) microscopy levels, subcellular fractionations (Figure 6), translation *in vitro* (Figure 7) and biotinylation of cell-surface proteins (Figure 8). Altogether, these data are consistent with those obtained in the testis, which show that NRD convertase is present in the cytoplasm of spermatids and exported out of these cells [23].

It is thus revealing that an enzyme, which was isolated originally based on its ability to specifically cleave model peptides at the N-terminus of arginines in dibasic sequences [12,13], turns out to be primarily a cytosolic proteinase. Processed dibasic and monobasic sequences are normally found in secretory proteins including pro-hormones and proproteins [36,37]. A survey of the literature revealed that although several cytosolic proteinases have been studied extensively, such as those involved in the calcium-dependent (calpains) and ubiquitin/proteasome-dependent pathways [38,39] and the pro-apoptotic caspases [40], none of them presented a selectivity for pairs of basic residues. Moreover, to our knowledge, no cytosolic precursors were reported to be processed specifically at dibasic sites, although, for most of them, the processing mechanism(s) as well as the precise cleavage sites remain to be elucidated. This is the case, for example, of the polyglutamine-containing proteins involved in neurodegenerative disorders such as huntingtin in Huntington's disease [41], and atrophin-1 in dentatorubropallidolusian atrophy (DRPLA [42]). It remains to be seen if NRD convertase is involved in the metabolism of these and whether it may represent a new target for therapeutic intervention in some degenerative pathologies.

The presence of NRD convertase at the cell surface opens new perspectives concerning its biological function. NRD convertase's nearest neighbour, insulin-degrading enzyme, is the only M16 family member that has been reported to be possibly present at the cell surface, where it would degrade insulin [43]. Several members of other families of metalloproteinases have been involved in either post-secretory peptide inactivation, such as neprilysin [44] or meprin [45], or activation such as the more selective endothelin-converting enzyme [46]. An example of a dibasic processing event occurring at the cell surface is the cleavage at Arg-Arg¹⁸ of glucagon-generating miniglucagon (19-29) [47]. However, if NRD convertase was implicated in such a cleavage, the presence of a surface aminopeptidase B-like activity [48] would be required to trim out the N-terminal arginine(s).

No transmembrane domain or canonical site for membrane anchorage was predicted from the primary structure, except the presence of an N-myristoylation site starting at Gly⁸¹ [49]. Since Gly⁸¹ constitutes the N-terminus of rNRD1 convertase overexpressed in BSC40 cells, this isoform, and possibly rNRD2 convertase, may thus be myristoylated in this cell type. It was reported recently that this type of acylation localized signalling-model proteins to specific intracellular membranes [50]. We may thus consider that, independently of its presence at the cell surface, NRD convertase may be myristoylated in the cytoplasm and then attached to intracellular membranes. This would be in agreement with the non-uniform perinuclear staining (Figure 4) and the rather high levels of NRD convertase (Figure 6) in BSC40 membrane fractions (about 30%). Note, however, that the testis enzyme shows a different N-terminus (Val⁷⁷) incompatible with its myristoylation, suggesting that myristoylation of NRD convertase, if it occurs, may be cell-type dependent.

The export/secretion mechanism of NRD convertase is still obscure, and further studies are needed in order to elucidate this process. In that context, several proteins lacking conventional signal peptides and unambiguously localized outside the cell were described, without a clear-cut mechanistic explanation, e.g. interleukin-1 α or β [51], basic fibroblast growth factor [52] or metalloendopeptidase EC 3.4.24.15 [53].

In conclusion, the present studies revealed for the first time that a dibasic-specific protease can be found both in the cytosol and at the cell surface. The identification of the cognate substrates of NRD convertase will surely open new avenues in our understanding of the limited proteolysis of precursors within

these cellular compartments. In addition, it is also conceivable that NRD convertase may have another function, independent from its enzymic activity, as reported for the M16 family member Ax11 [9].

We thank Claude Tougard for her advice and microscope facilities. We are very grateful to Marsha Rosner for her logistical support of V.C., Guy Boileau for welcoming A.P. in his laboratory for the *in vitro* translation experiments and Luis Rokeach for the generous gifts of calreticulin and corresponding antibodies. This work was supported in part by CNRS and the University Pierre et Marie Curie (to UMR 7631) and by a Medical Research Council (Canada) program grant no. PG-11474. V.H. and V.C. were recipients of the Association pour la Recherche sur le Cancer. V.H. was also supported by the Office Franco-Québécois pour la Jeunesse and A.B. was a CNRS post-doctoral fellow.

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Received 20 January 2000/24 March 2000; accepted 5 May 2000