NRD convertase: a putative processing endoprotease associated with the axoneme and the manchette in late spermatids

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SUMMARY

N-arginine dibasic convertase is a novel metalloendopeptidase which selectively cleaves at the N terminus of arginine residues in paired basic amino acids. Although present in brain and several other tissues, NRD convertase is particularly abundant in testis, where its expression appeared to be restricted to germ cells. Low levels of both mRNA and its corresponding protein were detected early in spermatogenesis. However, a marked accumulation of the protein was observed during late steps (14 to 19) of spermiogenesis. By electron microscopy, the NRD convertase immunoreactivity was localized in the cytoplasm of elongating and elongated spermatids, with a noticeable concentration at the level of two microtubular structures, i.e. the manchette and the axoneme. These observations strongly support the hypothesis that NRD convertase is involved in processing events potentially associated with the morphological transformations occurring during spermiogenesis.

Key words: Testis, Spermiogenesis, Metalloendopeptidase, Proprotein processing

INTRODUCTION

Spermatogenesis is a complex differentiation process including cell proliferation, meiosis and structural metamorphosis (reviewed by de Kretser and Kerr, 1988). Mammalian spermatids leave the testis as functionally immature cells and acquire their motility during their transit through the epididymis (for a review, see Eddy, 1988). Proteolytic events have been implicated in both mobility and fertilization processes (de Lamirande and Gagnon, 1986; Farach et al., 1987; Cosson and Gagnon, 1988; Roe et al., 1988), but, at the present time, only a few relevant proteases have been purified and characterized (Klemm et al., 1991; Inaba et al., 1993). Whereas the morphological events accompanying spermatogenesis were precisely described (for reviews, see Eddy, 1988; Jégou, 1993), the detailed molecular mechanisms governing this extremely complex process remain to be determined.

N-arginine (R) dibasic (NRD) convertase (nordilysin, EC 3.4.24.61) was first isolated from rat brain on the basis of its ability to cleave in vitro somatostatin-28 at the N terminus of the Arg-Lys doublet (Gluschankof et al., 1987; for a review, see Cohen et al., 1993). Further processing of the extended form of somatostatin-14 generated in the in vitro assay by a copurifying aminopeptidase of the B-type leads then to the mature hormone (Cadel et al., 1995; Foulon et al., 1996).

Recently purified from rat testis, where it is particularly abundant, NRD convertase has been biochemically characterized (Chesneau et al., 1994) and its primary structure determined (Pierotti et al., 1994). This widely expressed metalloendopeptidase exhibits a strict cleavage selectivity for the N terminus of arginine residues in dibasic sites of various peptide substrates (Chesneau et al., 1994; for a review, see Cohen et al., 1995). Its cDNA corresponds to an mRNA encoding a 1,161 amino acid protein with a predicted molecular mass of 133 kDa. The primary structure reveals three major features: a putative signal peptide, a 71 residue acidic stretch (79% glutamic and aspartic acids) and the consensus HXXEH motif known to be involved in the fixation of the catalytic Zn2+ of several metalloproteases (Becker and Roth, 1992; Gehm et al., 1993). Moreover, this enzyme exhibits a significant degree of similarity with insulinase (insulysin; EC 3.4.24.55; Affholter et al., 1988; Kuo et al., 1990; Baumeister et al., 1993), and both endoproteases belong to the pitrilysin family of metallopeptidases in view of the presence of the HXXEH pentapeptide motif (Kuo et al., 1990; Pierotti et al., 1994). Interestingly, several members of this family have been shown to be implicated in the proteolytic maturation of precursors (Braun and Schmitz, 1995; Adames et al., 1995). Although these maturation events do not take place in the secretory pathway where only the proprotein convertases (PC) of the subtilisin/kexin
family (Van de Ven et al., 1993; Seidah and Chrétien, 1994; Halban and Irminger, 1994) have clearly been shown to participate in proteolytic processing. This observation further supports the hypothesis of an involvement of NRD convertase in similar processes.

Preliminary immunocytochemistry and in situ hybridization studies have revealed that the enzyme and its mRNA are massively expressed around the lumen of seminiferous tubules (Chesneau et al., 1994; Pierotti et al., 1994). In the present study, to further investigate the onset of the mRNA and the protein expression profile in the male reproductive gonad, in situ hybridization and immunohistochemical studies were performed on rat testis and epididymis, and on testes obtained from wild type and mutant mice. In addition, extracts from isolated rat testicular cells were analyzed by northern and western blotting. Furthermore, subcellular localization in elongating and elongated spermatids was investigated by immunogold labeling. The present data show that NRD convertase expression increases markedly as spermiogenesis proceeds and that the protein is associated with the manchette and the axoneme, two essential structures of the late spermatid. It is therefore likely that this enzyme, previously described as a putative processing endopeptidase (Chesneau et al., 1994), could play, by interacting with the microtubular system, a crucial role in the final steps of germ cell morphological and functional differentiation.

MATERIAL AND METHODS

Immunohistochemistry (IHC) and in situ hybridization (ISH)

Animals

NRD convertase distribution in the male reproductive system was investigated in adult Sprague Dawley rats (90 days old), and in pubertal (27 days old) and adult (60 days old) C57BL mice. Three lines of sterile mutant mice, each of them presenting a different degree of alteration of the spermatogenetic process, were also analyzed. Mice, homozygotes for the ‘oïlotiche’ gene mutation (oïlot; Chubb, 1992), are characterized by a decreased number of spermatids; no spermatocytes and early spermatids were prepared by centrifugal elutriation and enriched populations of pachytene spermatocytes, early spermatids and residual bodies were prepared by centrifugal elutriation and disorganization of the middle piece of the flagellum. Both mutant strains were obtained from the Institut Pasteur (Paris, France). The third type of mutant used was an interspecific hybrid, named BC, which was obtained from a back-cross between F1 females (C57BL × Mus spretus) and a normal C57BL male (Matsuda et al., 1991). The testes of BC hybrids show a low count of spermatocytes, which rarely enter the second meiotic division, and thus generate very few round spermatids and no spermatozoa. The latter mutant was provided by the Laboratoire de Génétique, Neurogénétique et Comportement from the UFR Biomédicale des Saints Pères (Paris, France).

Tissue preparation

Tissues were fixed for 12 to 24 hours either in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, or ISH, or in Bouin’s liquid for IHC. They were then dehydrated in graded ethanol and embedded in paraffin wax. Sections (7 µm thick) were stained with toluidine blue.

Immunohistochemistry

The NRD convertase specific rabbit polyclonal antisera (Chesneau et al., 1994) was used at 1/850 (on rat) or 1/750 (on mouse) final dilutions. The corresponding preimmune serum was taken as control. The IHC procedure was previously described by Sibony et al. (1993). The Vectastain ABC-Elite kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine were used to perform the immunoperoxidase staining. Tissues were finally counterstained with Harris hematoxylin.

In situ hybridization

Sense and antisense 35 S-labeled riboprobes were obtained by in vitro transcription of a linearised plasmid bearing a 960 bp PstI fragment of the rat NRD convertase cDNA (Pierotti et al., 1994). In brief, the ISH protocol successively comprises pretreatment with proteinase K, overnight hybridization at 50°C, posthybridization washing under stringent conditions and RNase treatment (Sibony et al., 1993). Slides were then dipped in photographic emulsion (NTB2; Eastman Kodak, France), developed after one to two weeks exposure and counterstained with toluidine blue.

Ultrastructural immunocytochemistry

Mouse testes were fixed by intracardiac perfusion of 2% paraformaldehyde in 0.1 M PBS, pH 7.4, containing 0.5% glutaraldehyde, then immersed in the same solution for 1 hour at room temperature and finally rinsed for 1 hour in PBS containing 50 mM NH4Cl and 100 mM sucrose. Tissues were then postfixed for 1 hour in 5% uranyl acetate, dehydrated in cold methanol and embedded in Lowicryl K4M. Thin sections were treated according to the method of Weinman et al. (1986). Briefly, they were deposited on nickel grids, floated on phosphate-buffered saline containing 3% BSA and incubated in a humid chamber for 1 hour with the anti-NRD convertase serum at a 1/200 final dilution. After a quick rinse with PBS, they were incubated with the secondary gold-labeled antibody (10 nm particles; GAR-10, Janssen, Tébu, France), then washed several times with PBS and finally counterstained with uranyl acetate and lead citrate. The immunolabeling specificity was verified using either the preimmune serum or the secondary antibody alone.

Northern and western blot analyses of isolated testicular cell extracts

Cell isolation and conditioned media preparation

Germ cells were prepared from adult Sprague Dawley rat testes (Eleavage Janvier, Le Genest Saint Isle, France) by mechanical dispersion and enriched populations of pachytene spermatocytes, early spermatids and residual bodies were prepared by centrifugal elutriation with a purity of greater than 85% (Pineau et al., 1993). Pachytene spermatocytes and early spermatids were then incubated at 32°C for 4, 8 or 16 hours as previously described (Pineau et al., 1993). Viability of the cells at the end of the incubation periods was >95%, as judged by the trypan blue dye exclusion test.

Sertoli and peritubular cells were isolated from 20-day-old Sprague Dawley rats (Eleavage Janvier, Le Genest Saint Isle, France) as previously described (Skinner and Fritz, 1985). Sertoli cell suspensions were seeded at a density of approximately 1x10⁶ cells.ml⁻¹ in 75 cm² tissue culture flasks (NUNC, Copenhagen, Denmark). The cells were then incubated at 32°C in a humidified atmosphere of 5% CO₂ and 95% air in Ham’s F12/DMEM (Life Technologies, France) supplemented with insulin (10 mg.ml⁻¹), human transferrin (5 mg.ml⁻¹) and gentamycin (10 mg.ml⁻¹). Culture medium was replenished daily until the end of the experiment. On day 2 of the culture, Sertoli cells were exposed to a hypotonic treatment in order to eliminate the contaminating germ cells (Galdieri et al., 1981). Peritubular cells were seeded at a density of approximately 200,000 cells.ml⁻¹ in 75 cm² tissue culture flasks. The cells were then cultured for 5 days at 32°C in a humidified atmosphere of 5% CO₂ and 95% air in Ham’s F12/DMEM (v/v) supplemented with insulin (10 mg.ml⁻¹), human transferrin (5 mg.ml⁻¹), gentamycin (10 mg.ml⁻¹) and 10% fetal calf serum. When confluence was reached, cells were removed by treatment with PBS, pH 7.4, containing 0.05% trypsin and 0.5 mM...
EDTA, washed and seeded at 1/4 density in 175 cm² flasks. The subcultured cells were allowed to grow to confluence for an additional 4 to 5 days in the presence of 10% fetal calf serum. Finally, before collection, the cells were washed (3 × 2 hours) with fresh serum-free medium.

RNA extraction and northern blot analysis
Total RNA from isolated testicular cells and residual bodies was extracted using the TRIzol reagent (Life Technologies SARL, France) according to the manufacturer’s recommendations. Total RNA from testis was obtained by a guanidine isothiocyanate extraction followed by lithium chloride precipitation (Day et al., 1992). The northern blot transfer was performed according to the procedure described by Day et al. (1992). The 32P-labeled oligonucleotide probe corresponds to nucleotides 324 to 299 of the NRD convertase cDNA (Pierotti et al., 1992). The step-specific expression of NRD convertase during spermatogenesis was studied in rodents at both the transcriptional and translational levels. The spermiogenesis steps and the seminiferous epithelium stages were identified according to the morphological criteria proposed by Oakberg (1956) and Leblond and Clermont for the rat (1952). The term ‘stage’ refers to the following: at any given point in the tubule, several germ cell generations develop simultaneously from the base to the apex of the epithelium. The evolution of each generation of germ cells is strictly synchronized with the development of others and this leads over a certain segment of the tubule to the formation of defined cell associations or ‘stages of the seminiferous epithelium cycle’ (Jégou, 1993). In the rat, the classification of Leblond and Clermont (1952) defines 14 stages whereas that of Oakberg (1956) in the mouse defines 12 stages. Spermiogenesis, which refers to the morphological transformation of spermatids into spermatozoa, involves 19 steps according to Leblond and Clermont (1952) in the rat and 16 steps in the mouse (Oakberg, 1956). In this article the term late spermatids will be used to designate both elongating and elongated spermatids.

NRD convertase expression in male germ cells

**RESULTS**

The step-specific expression of NRD convertase during spermatogenesis was studied in rodents at both the transcriptional and translational levels. The spermiogenesis steps and the seminiferous epithelium stages were identified according to the morphological criteria proposed by Oakberg for the mouse (1956) and Leblond and Clermont for the rat (1952). The term ‘stage’ refers to the following: at any given point in the tubule, several germ cell generations develop simultaneously from the base to the apex of the epithelium. The evolution of each generation of germ cells is strictly synchronized with the development of others and this leads over a certain segment of the tubule to the formation of defined cell associations or ‘stages of the seminiferous epithelium cycle’ (Jégou, 1993). In the rat, the classification of Leblond and Clermont (1952) defines 14 stages whereas that of Oakberg (1956) in the mouse defines 12 stages. Spermiogenesis, which refers to the morphological transformation of spermatids into spermatozoa, involves 19 steps according to Leblond and Clermont (1952) in the rat and 16 steps in the mouse (Oakberg, 1956). In this article the term late spermatids will be used to designate both elongating and elongated spermatids.

**NRD convertase in adult rat testis and epididymis**

In adult rat testis, our ISH study showed a stage-specific labeling with maximal ad-luminal mRNA levels at stage XI-XII (Fig. 1). In agreement, the protein levels of NRD convertase estimated by IHC revealed a stage-dependent ad-luminal expression within the seminiferous tubules (Fig. 2A-E). The staining was first observed in rat spermatids at steps 8-9 of spermiogenesis which correspond to the beginning of the elongation process, and increased to reach a maximum at steps 14-18 (Fig. 2B-E). Both cytoplasm and flagellum of elongated spermatids were immunoreactive (Fig. 2B-E). The residual bodies, which correspond to the portions of the cytoplasm of elongated spermatids that are shed at the time of spermiation, were also strongly stained. In addition, spermatozoa released into the lumen of the seminiferous tubules still reacted positively, especially in the flagellum area (Fig. 2D). Since, in testis, artefacts were reported to occur by immunoperoxidase staining, the above observations were confirmed by immunofluorescence labeling which displays an identical pattern (data not shown). In the head segment of the epididymis, the enzyme immunoreactivity was still present in both the flagellum and cytoplasmic droplets of immature spermatozoa (Fig. 2F). In contrast, the protein was no longer detected in spermatozoa present in the

![Fig. 1. In situ hybridization study of NRD convertase in rat testis. The cellular distribution of NRD convertase transcripts was investigated using 35S-labeled antisense cRNA probes. Bright-field (A) and dark-field (B) of the same testis section allowed us to correlate the density of the silver grains with the stages (roman numerals) of the epithelium (×199).](image-url)
caudal region of this organ (Fig. 2G). In both testis and epididymis, no NRD convertase could be detected in somatic cells.

**NRD convertase in mouse testis**
As an excellent immunological cross-reactivity was observed between the mouse and rat enzymes, we first looked at the NRD convertase localization in the wild-type mouse testis, as a prelude to the analysis of its expression in mutant mice. In a similar manner to the rat protein, mouse NRD convertase was only detected in late spermatids (Fig. 3A-C): staining was observed starting from steps 9-10 and reaching a maximal intensity at the latest steps of spermiogenesis (steps 15-16).
The oligotriche (olt) and ébouriffé (ebo) mutant mice, in which spermiogenesis was disrupted, are characterized by an abnormal development of the flagellum. They were thus chosen to assess the possible correlation between the defective spermiogenesis and a dys-expression of NRD convertase. Our results show that the enzyme is expressed in these two sterile mutants (Fig. 3D,E). A strong cytoplasmic staining was observed in the rare elongated spermatids of olt mutants (Fig. 3D). This increased spermatid staining, when compared to the corresponding steps in the wild-type mouse, suggests that NRD convertase could even be either overexpressed or accumulated in the olt mutant, especially in degenerating cells. In ebo
mutants, staining was also observed in late spermatids, in the rare flagella present in the lumen of the seminiferous tubules, as well as in the residual bodies and degenerating cells (Fig. 3E). As in the adult interspecific hybrid BC spermatogenesis is mostly interrupted just after the second meiotic division, the seminiferous epithelium is almost totally devoid of post-spermatocyte cells. In these animals, in contrast to both preceding mutants, no NRD convertase could be detected (Fig. 3F), except in a few degenerating cells. The lack of NRD convertase expression in BC mice is most probably related to the absence of spermatids and thereby confirms that the observed labeling was restricted to haploid cells. In contrast, in seminiferous tubules of a normal 27-day-old pubertal mouse, exhibiting the appearance of first elongating spermatids, a clear staining is observed in the cytoplasm and flagellum of this cell type (Fig. 3G).

Northern and western blot studies of isolated testicular cell extracts

In order to further extend the above histological studies, more sensitive analyses were performed by northern and western blotting on isolated testicular cell extracts. First of all, the absence of signal in peritubular and Sertoli cells confirmed that, in seminiferous tubules, NRD convertase expression appears to be restricted to germ cells both in terms of mRNA (Fig. 4) and protein levels (Fig. 5A). The analysis of sub-populations of germ cells revealed that, although at a relatively low level, NRD convertase is already expressed in pachytene primary spermatocytes (SPC; Figs 4, 5A). As expected, a stronger signal was obtained for round spermatids (SPT). The fact that the presence of NRD convertase in these two cell types was not revealed by IHC, but only by western blotting, is unlikely due to a contamination of the spermatocyte and spermatid purified fractions by more differentiated germ cells. Indeed, their degree of purity was estimated to be of about 85% and the few heads of late spermatids present in these samples cannot account for the intensity of the specific labeling. As shown in Fig. 5A, the strongest signal was obtained for residual bodies. Finally, immature and mature spermatozoa, collected from the head and the caudal segments of the epididymis, respectively, were both immunoreactive, although the protein appeared degraded when analyzed by western blotting (data not shown).

Since NRD convertase was isolated as a putative proprotein processing enzyme and may thus be secreted, its possible presence in culture media conditioned by either spermatocytes or early spermatids was also investigated. In these experiments, the NRD convertase secretion level was analyzed after 4, 8 or 16 hours. As shown in Fig. 5B, the protein was released in a time-dependent manner by both cell types and in much higher amounts by spermatids. Since 95% of the cells were found viable after 16 hours of culture, lysis cannot account for such a strong signal.

The size heterogeneity of the detected transcripts among the different isolated fractions (Fig. 4) probably reflects a reproducible migration artefact, as indicated by migration of xylene cyanol and bromophenol blue or by control hybridization (not shown). On the other hand, the same protein pattern was obtained in all cell types analyzed as well as in the culture media (Fig. 5). Indeed, whereas the enzyme was purified from rat brain cortex and testis as two isoforms of 110 and 140 kDa (Chesneau et al., 1994), the present western blot analysis shows that the largest form is in all cases predominant. This
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observation reinforces the hypothesis that the 110 kDa isoform is likely a degradation product of the 140 kDa isoform.

Subcellular localization of NRD convertase in mouse late spermatids

In accordance with light microscopy observations, a diffuse labeling was present throughout the cytoplasm of late spermatids (Fig. 6A,B). Furthermore, the gold particles were found to be remarkably abundant at the level of two fundamental microtubular structures: the manchette (Fig. 6A,B) and the axoneme (Fig. 6C,D). Fig. 6A and B shows two longitudinal sections of late spermatids at steps 10 and 13 of spermiogenesis (stages X and XI of the seminiferous epithelium, respectively) in which the manchette surrounding the nucleus is strongly labeled. This structure, transiently expressed during spermiogenesis (acrosomal and maturation phases), forms a tube extending from the caudal pole of the nucleus and terminating freely in the caudal cytoplasm. In step 16 (stages VII-VIII), the immunoreactivity was mostly located on the transverse sections of flagella (Fig. 6C), in the vicinity of the microtubular components of the axoneme (Fig. 6D). More specifically, this labeling was observed at the level of the flagellum of the middle or principal piece.

DISCUSSION

NRD convertase selectively cleaves peptide substrates at the N terminus of arginine residues in dibasic sites. The present study shows that, in the testis, the enzyme is only detected in germ cells and that its expression increases during spermiogenesis, reaching a maximum in elongated spermatids. Moreover, the enzyme, which is still present in mature spermatozoa, is found in the cytoplasm of late spermatids, accumulating at the level of two important structures: the manchette and the axoneme. The marked increase of NRD convertase mRNA and protein levels in elongated spermatids, compared to the elongating ones, and in residual bodies may result either from an increased expression or, alternatively, from a concentration of both mRNA and protein. Indeed, during the last steps of spermiogenesis, a dramatic reduction of the spermatid cytoplasm occurs as a consequence of a loss of cellular water via the
formation and processing of the tubulobulbar complexes by the Sertoli cell (for a review, see Jégou, 1993).

In contrast to NRD convertase mRNA which is already detected in pre-meiotic germ cells by ISH, the protein itself was not observed by IHC before the beginning of the elongation phase of spermatids. The lack of detection of this enzyme in spermatocytes and early spermatids is probably due to the low sensitivity of the IHC technique since the western blot analysis revealed unequivocally the presence of the enzyme in both cell types.

By electron microscopy, NRD convertase was localized in the cytoplasm of late spermatids, but a similar study performed on rat pituitary endocrine cells demonstrated its association with the secretory machinery (C. Tougard, personal communication). This finding is in keeping with a number of reported data which strongly argued in favor of the involvement of NRD convertase in proteolytic processing within the secretory pathway. These include: (i) the presence of a putative signal peptide at the N terminus of its polypeptide chain; (ii) its in vitro specificity for dibasic sites, canonical prohormone and maturation signals; (iii) the fact that several members of the pitrilysin family of metallopeptidases were shown to participate in prohormone processing (Adames et al., 1995) and mitochondrial maturation of nuclear encoded protein precursors (Braun and Schmitz, 1995; Brunner and Neupert, 1995). Moreover, an NRD convertase-like enzyme was implicated in processing of the human recombinant protein C precursor within the secretory pathway of transgenic swine mammary cells (Lee et al., 1995). We have now additionally demonstrated that significant amounts of the enzyme are liberated in both spermatocyte- and spermatid-conditioned media. Whether both localizations, i.e. the cytoplasm and the secretory pathway, coexist in round spermatids remains to be determined. The fact that no signal could be obtained in round spermatids by immunogold staining is probably due to an insufficient level of protein expression at the corresponding steps of spermiogenesis. Additionally, the specialization and reorganization of the secretory pathway occurring during the morphological evolution of spermatids may render difficult the detection of the enzyme in this pathway.

The detection of large quantities of NRD convertase in the cytoplasm of late spermatids raises the question of its implication in proprotein processing in the latter compartment. The close association of the protein with the microtubules of both the manchette and the axoneme of the germ cell suggests that some proteolytic processing events may take place at the level of these two dynamic structures. However, only a few of their components have been biochemically characterized and so far, to our knowledge, none has been shown to be proteolytically processed at basic sites. The immunohistochemical analysis of the seminiferous epithelium of olt mutant mice, characterized by a total absence of sperm tails, indicates that NRD convertase expression is not dependent upon the formation of the flagellum. However, in this mutant, spermatids were shown to contain a manchette (Chubb, 1992). Although the flagella of ebo mutants show a severe disorganization, NRD convertase appears to be still able to interact with some constituents of the axoneme. Further experiments on appropriate systems, including endocrine and motile cells, should be undertaken to identify the nature of these constituents and to determine whether or not this association with the microtubules is restricted to the germ cell.

A large amount of NRD convertase not associated with microtubules was also detected. Although this may only reflect its future accumulation at the level of the microtubular structures, the possibility cannot be excluded that the free enzyme is active in this compartment. Despite the correlation between the up-regulation of the enzyme expression and the morphological evolution of the spermatid suggesting its implication in the elongation process, the biochemical events underlying such a process remain to be elucidated.

Finally, endoproteases have been implicated in the spermatozoan motility (Cosson and Gagnon, 1988). Indeed, transient inhibitory effects of various synthetic peptide substrates and protease inhibitors suggest that a serine protease with Lys and Arg ester bond specificity is involved in the control of sperm motility. By IHC, NRD convertase is detected in non-motile spermatozoa localized in the caput epididymis but not in the motile ones, present in the caudal portion of this organ. This could be argued against the involvement of NRD convertase in the direct control of sperm motility. However, an immunoreactive product that presents a lower apparent molecular mass can be observed by western blotting in both immature and mature spermatozoa extracts. Whether this reduced mass is the result of a degradation occurring during the extraction process or has a physical meaning remains an open issue. Conversely, it could also be hypothesized that NRD convertase is involved in the cancellation of a motility inhibitory mechanism. The identification of the physiological substrate(s) of NRD convertase during germ cell differentiation remains a prerequisite to a better understanding of its role in germ cell activity.

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