Molecular cloning and functional expression of bovine spleen ecto-NAD⁺ glycohydrolase: structural identity with human CD38

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Bovine spleen ecto-NAD⁺ glycohydrolase, an archetypal member of the mammalian membrane-associated NAD(P)⁺ glycohydrolase enzyme family (EC 3.2.2.6), displays catalytic features similar to those of CD38, i.e. a protein originally described as a lymphocyte differentiation marker involved in the metabolism of cyclic ADP-ribose and signal transduction. Using amino acid sequence information obtained from NAD⁺ glycohydrolase and from a truncated and hydrosoluble form of the enzyme (hNADase) purified to homogeneity, a full-length cDNA clone was obtained. The deduced sequence indicates a protein of 278 residues with a molecular mass of 31.5 kDa. It predicts that bovine ecto-NAD⁺ glycohydrolase is a type II transmembrane protein, with a very short intracellular tail. The bulk of the enzyme, which is extracellular and contains two potential N-glycosylation sites, yields the fully catalytically active hNADase which is truncated by 71 residues. Transfection of HeLa cells with the full-length cDNA resulted in the expression of the

expected NAD⁺ glycohydrolase, ADP-ribosyl cyclase and GDP-ribosyl cyclase activities at the surface of the cells. The bovine enzyme, which is the first 'classical' NAD(P)⁺ glycohydrolase whose structure has been established, presents a particularly high sequence identity with CD38, including the presence of 10 strictly conserved cysteine residues in the ectodomain and putative catalytic residues. However, it lacks two otherwise conserved cysteine residues near its C-terminus. Thus hNADase, the truncated protein of 207 amino acids, represents the smallest functional domain endowed with all the catalytic activities of CD38/NAD⁺ glycohydrolases so far identified. Altogether, our data strongly suggest that the cloned bovine spleen ecto-NAD⁺ glycohydrolase is the bovine equivalent of CD38.

Key words: ADP-ribosyl cyclase, CD157, cyclic ADP-ribose, ecto-enzyme, NAD⁺.

INTRODUCTION

Mammalian NAD⁺ glycohydrolases (NADases; EC 3.2.2.5 and 3.2.2.6) catalyse the hydrolytic cleavage of the nicotinamide-ribose bond of β -NAD(P)⁺ and, in their vast majority, transglycosylation reactions that give access to pyridinium analogues of NAD(P)⁺. They have been known and studied for many decades and constitute a fairly heterogeneous family of enzymes, e.g. in terms of apparent molecular masses and catalytic properties (for reviews, see [1,2]). NADases are chiefly membrane-associated proteins that are widely distributed in the organism, the richest sources being generally the spleen, brain and liver [1]. At a cellular level, it was first believed that these enzymes were constituents of the endoplasmic reticulum and could therefore play a role in the regulation of intracellular levels of the pyridine nucleotides. However, in organs that are rich in this enzyme, the catalytic activity is far too high to account for such a function, and it was established later that NADases are in fact overwhelmingly markers of the plasma membrane and ecto-enzymes [3], i.e. the active site of the enzyme facing the outer surface of the cells has no access to intracellular NAD⁺. Minor proportions of NADase activity could nevertheless be detected in intracellular compartments, such as lysosomes, nuclear envelopes [4] and mitochondria [5]. From a structural standpoint, the membrane-associated NADases are generally amphipathic proteins [6], and the earlier investigations on the structural and mechanistic properties of these enzymes were performed using their hydrosoluble domains, which contain the active site. These enzymically active truncated forms (hNADases) were generally obtained by digestion of microsomes or acetone powders with proteases or

steapsin followed by purification steps [7,8]. Access to the complete detergent-solubilized enzymes that were purified to homogeneity is much more recent [9,10]. Besides the limited cases where a glycosylphosphatidylinositol (GPI) anchoring has been clearly demonstrated [11], the mode of interaction of most NADases with membranes remains unknown.

Despite all the information that has accumulated on the cellular properties and the molecular mechanism of this class of enzyme, their physiological functions remained largely elusive. This situation has changed recently with: (i) the discovery of cyclic ADP-ribose (cADPR), a new metabolite of NAD⁺ thought to be an endogenous regulator of the Ca²⁺-induced Ca²⁺-release process mediated by the ryanodine receptor (for a review, see [12]); (ii) the finding that a purified canine spleen NADase is a multifunctional enzyme endowed with additional ADP-ribosyl cyclase and cADPR hydrolase catalytic activities [10]; and finally (iii) the report that CD38, a lymphocyte cell-surface antigen for which no biological activity was known, is also involved in the biosynthesis of this new mediator. Thus CD38, a 46 kDa type II transmembrane glycoprotein, in analogy with the canine NADase is also a multifunctional enzyme endowed with three different catalytic activities: NADase, ADP-ribosyl cyclase and cADPR hydrolase [13]. In sharp contrast with the invertebrate enzymes, such as *Aplysia californica* ADP-ribosyl cyclase, which convert NAD⁺ almost exclusively into cADPR [14], CD38 is predominantly an NADase, forming comparatively little of the cyclic metabolite (i.e. < 2% of reaction products). Similar activities were also found for BST-1/BP-3 (recently designated CD157), i.e. GPI-anchored bone marrow stromal cell-surface antigens that show high sequence identity with CD38 [15]. The

Abbreviations used: ADPR, ADP-ribose; cADPR, cyclic ADP-ribose; cGDPR, cyclic GDP-ribose; GPI, glycosylphosphatidylinositol; NADase, NAD⁺ glycohydrolase, hNADase, hydrosoluble form of NADase; NGD⁺, nicotinamide guanine dinucleotide.

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question arose as to whether or not all the 'classical' NADases are also multifunctional enzymes whose ADP-ribosyl cyclase and cADPR hydrolase activities had been overlooked. Accordingly, it was also found that bovine spleen ecto-NADase has similar catalytic activities to CD38 [16]. Because of the importance of CD38 in the biosynthesis of cADPR and in cellular signalling processes [17–19], it was of interest to determine whether the 'classical' ecto-NADases and CD38 are the same enzymes or if they constitute subclasses of a superfamily of enzymes that might differ by some aspects of their molecular mechanisms and/or structural properties. From previous mechanistic studies [20] and from the determination of NADase activity in different organs of transgenic CD38^{-/-} mice [21], we suggested that CD38 and the 'classical' ecto-NADases are the same.

We report here the molecular cloning of bovine spleen ecto-NADase, a 32 kDa glycoprotein, i.e. an archetypal 'classical' NADase and one of the most extensively studied members of this family of enzymes [1,2]. This first cloning of an NADase was obtained from information derived from purified enzyme (NADase and hNADase). The sequence comparison revealed striking sequence identity between the bovine enzyme and human and murine CD38. Interestingly, there are also some distinctive features that distinguish these enzymes; in particular, the bovine NADase lacks two of the conserved cysteine residues found in all other CD38 family members. Moreover, a single preferential cleavage site that gives rise to hNADase was found. The present results clearly indicate that we have cloned the bovine equivalent of human CD38, which is therefore also a 'classical' NADase. This finding has important consequences for the investigation of the physiological functions and catalytic properties of CD38.

EXPERIMENTAL

Materials

[adenosine-U-¹⁴C]NAD⁺ (567 Ci/mol) was from NEN (Boston, MA, U.S.A.). NAD⁺, nicotinamide guanine dinucleotide (NGD⁺), 1,*N*⁶-etheno-NAD⁺, lipase (type II; crude from pig pancreas), concanavalin A-agarose (type V-B) and *N*-lauryl-*N,N*-dimethylamine *N*-oxide were from Sigma (L'Isle d'Abeau Chesnes, France). Hydroxyapatite (Bio-Gel HTP) and Affi-Gel Blue were obtained from Bio-Rad Laboratories (Ivry-sur-Seine, France). Linear polyethylenimine Exgen500 was from Euro-medex (Souffelweysheim, France). Minimal essential medium (with Earle's salts), fetal calf serum, glutamine, penicillin/streptomycin and trypsin-EDTA were from Gibco BRL Life Technologies (Cergy Pontoise, France). cADPR and cyclic GDP-ribose (cGDPR) were prepared enzymically from NAD⁺ and NGD⁺ respectively using *Aplysia californica* ADP-ribosyl cyclase and purified by HPLC [16]. *Aplysia* cyclase was prepared using published procedures [14], and bovine spleen ecto-NADase was solubilized from microsomes with a non-ionic detergent and purified to homogeneity (specific activity 140 units/mg of protein) as described previously [9]. HeLa cells were from Dr. L. Monaco (Istituto San Raffaele, Milano, Italy). Edman N-terminal sequencing of peptides was carried out by Dr. J. D'Alayer (Department of Biotechnology, Pasteur Institute, Paris, France).

Purification of hNADase

hNADase was obtained by treatment of bovine spleen microsomes with steapsin and purified to apparent homogeneity by a combination of ion-exchange and (pseudo)affinity chromatography steps.

Solubilization

Crude pig pancreatic lipase (steapsin) was added to thoroughly washed calf spleen microsomes [9] resuspended in a 10 mM Tris/HCl buffer, pH 8.1, at a lipase/microsomal protein ratio of 2.5:1. The mixture was left under mild agitation for 90 min at 30 °C in the presence of 1 mM CaCl₂ and, after addition of PMSF (1 mM final concentration), was centrifuged at 100 000 *g* for 60 min. The supernatant containing the solubilized enzyme was immediately subjected to purification.

Purification

The first purification steps on ion-exchange gels were adapted from the procedure we developed for the intact form of the enzyme [9]. Briefly, step 1 [on DEAE-cellulose (DE-53)] and step 2 (on CM-Sephadex CL-6B) were carried out as in [9], except that no detergent was added. The following steps were then performed.

Step 3: a hydroxyapatite column (1.6 cm × 10 cm) was equilibrated for 12 h with a 10 mM potassium phosphate buffer, pH 7.4. After application of the sample, the gel was washed with the same buffer (about 2 vol.). Elution of the enzyme was performed at a flow rate of 0.5 ml·min⁻¹ with a linear gradient (2 × 50 ml) of potassium phosphate buffer (10–400 mM), pH 7.4. The fractions containing NADase activity were pooled and dialysed for 12 h against 1 litre of a 10 mM Tris/HCl buffer, pH 7.2, containing 0.4 M NaCl.

Step 4: partially purified hNADase was chromatographed on concanavalin A-agarose. Approx. 10 ml of gel, resuspended for 1 h at 4 °C in 100 ml of a 10 mM Tris/HCl buffer, pH 7.2, containing 10 mM each of CaCl₂, MgCl₂ and MnCl₂, was poured and the column was thereafter equilibrated for 12 h at 0.3 ml·min⁻¹ with the same buffer lacking the bivalent ions. After application of the enzyme fraction, at 0.1 ml·min⁻¹, the column was washed at a flow rate of 0.2 ml·min⁻¹ with 20 ml of a 10 mM Tris/HCl buffer, pH 7.2, containing 0.4 M NaCl, followed by 15 ml of the same buffer containing 0.05 M α -methylmannoside. Elution of the enzyme was performed at the same flow rate with 15 ml of 0.1 M α -methylmannoside followed by 15 ml of 0.5 M α -methylmannoside in the same buffer. The fractions containing the enzyme activity were pooled and dialysed for 12 h against 2 litres of a 10 mM potassium phosphate buffer, pH 7.4 (buffer A).

Step 5: approx. 1 ml of Affi-Gel Blue was washed at 0.2 ml·min⁻¹ with 10 ml of a 10 mM potassium phosphate buffer, pH 7.4, containing 500 mM NaCl to eliminate any unbound dye, and equilibrated for 12 h with buffer A. Elution of the enzyme was achieved with a linear gradient (2 × 10 ml) of NaCl (0–1.5 M) in the same buffer.

The protein concentration was determined using the BCA protein assay from Pierce, using BSA as a standard.

SDS/PAGE and in-gel detection of NADase activity

SDS/PAGE was performed according to classical methods and the gels were revealed with a silver staining kit based on the methodology of Heukeshoven and Dernick [22]. In-gel NADase activity was visualized fluorimetrically, using 1,*N*⁶-etheno-NAD⁺ as substrate, on samples that were analysed by SDS/PAGE in the absence of reducing agents and renatured essentially as described by Hagen and Ziegler [23].

CNBr cleavage of NADase

Purified NADase was subjected to CNBr cleavage in 0.1 M HCl using standard procedures [24]. The reaction mixture was then

subjected to SDS/PAGE, followed by a transfer on to a PVDF membrane (Problott; Applied Biosystems), and the stained peptide bands (Coomassie Blue) were excised and used for microsequencing.

In-gel NADase cleavage by endopeptidase treatment

The purified NADase was first denatured (guanidine hydrochloride), reduced (dithiothreitol) and alkylated with 4-vinylpyridine or acrylamide using standard procedures [25]. The modified protein was then isolated by SDS/PAGE and, after coloration with Coomassie Blue, the band was excised and subjected to in-gel digestion by endolysine C or sequencing-grade trypsin (Boehringer), as described in [24]. The peptides were extracted from the gel at 30 °C by incubation in 60 % (v/v) acetonitrile/0.1 % (w/v) trifluoroacetic acid and purified by HPLC on a 250 mm × 2.1 mm C₁₈ column (Vydac) operated at ambient temperature, at a flow rate of 1 ml/min. The peptides were eluted with a linear gradient of 2 % (v/v) acetonitrile/0.1 % (w/v) trifluoroacetic acid to 80 % (v/v) acetonitrile/0.1 % (w/v) trifluoroacetic acid in 120 min. The peaks were detected by their absorbance at 210 nm and collected manually for sequencing.

Generation of an NADase partial cDNA by PCR

Two degenerate primers based on internal amino acid sequences were synthesized (Genset): the sense primer 5'-AAYTGCCAGGCHATYMGSCAGGCHTTYATG-3' (NG-1), corresponding to amino acids 74–83 (NCQAIRQAFM), and the antisense primer 5'-ACYTCCCARAASACRSWSARRTARTTDGT-3' (NG-2), corresponding to amino acids 174–183 (TNYLSVFWEV). mRNA was prepared from fresh calf spleen obtained from a local slaughterhouse, by a modification of the method described in [26] using an RNA isolation kit (Tri-Reagent; Euromedex). First-strand cDNA synthesis was carried out with 5 µg of the mRNA and an oligo(dT) primer or the antisense degenerate primer NG-2 using recombinant Moloney murine leukaemia virus reverse transcriptase according to the procedure provided by the manufacturer (Appligene). An aliquot of the cDNA was amplified by PCR using 0.02 unit/µl *Taq* DNA polymerase (Appligene) with 1 µM of primers (NG-1 and NG-2) and 4.5 mM MgCl₂. The reaction mixture was heated for 5 min at 95 °C before thermal cycling at 95 °C for 1 min, 54 °C for 2 min and 72 °C for 3 min for 27 cycles, followed by an amplification cycle of 7 min at 72 °C. A PCR product of approx. 330 bp was obtained. One-tenth of the reaction mixture was subjected to 30 other PCR cycles. After gel purification (Low melting agarose; Nusieve FMC Products), the 330 bp partial cDNA fragment was subcloned into pBluescript SK(+) plasmid. The nucleotide sequence of the partial cDNA (NG-330) was determined using the T7 and T3 Bluescript universal primers and the T7 Sequenase v2.0 DNA sequencing kit (Amersham Life Science).

Isolation and sequencing of full-length cDNA

A λgt10 bovine cDNA library (bovine spleen 5'-Stretch Plus cDNA library; Clontech; 3.5 × 10¹⁰ plaque-forming units) was screened in *Escherichia coli* C600 Hfl host cells by plaque hybridization (2 × 10⁵ plaques) with probe NG-330, labelled by random priming with [α -³²P]dCTP. Lifts of 2 min were performed on nylon colony/plaque hybridization filters (Hybond N; Amersham Life Science). Filters were prehybridized for 1.5 h at 65 °C in 6 × SSC (1 × SSC = 0.15 M NaCl/0.015 M sodium cit-

rate, pH 7), 0.1 % SDS, 5 × Denhardt's solution (1 × Denhardt's solution = 0.02 % Ficoll/0.02 % polyvinylpyrrolidone/0.02 % BSA) and 100 µg/ml salmon sperm DNA. Hybridization was performed in the same solution with labelled probe (1.5 × 10⁶ d.p.m. per filter) overnight at 65 °C. Filters were washed once in 2 × SSC/0.1 % SDS at room temperature for 20 min, then in 1 × SSC/0.1 % SDS at room temperature for 20 min and finally in 0.1 × SSC/0.1 % SDS at 65 °C for 20 min. The filters were dried and exposed to Kodak X-Omat film for 48 h at -80 °C with intensifying screens. λDNA from a single purified clone was isolated, after amplification of the positive plaques in *E. coli* C600 Hfl, using the Wizard λ Preps DNA purification system (Promega). The size of each positive insert was estimated after PCR reaction on λDNA with Long Distance Insert Screening Amplimers sets (Clontech) and an Expand Long Template PCR System (Boehringer Mannheim). The reaction was performed according to the procedure provided by the manufacturer (Boehringer Mannheim). One-tenth of each reaction mixture was analysed on a 1 % (w/v) agarose gel. Southern blotting performed with [α -³²P]dCTP-labelled NG-330 probe showed that the isolated clones effectively contained the specific sequence. The longest insert (2500 bp) was purified by gel purification (Nusieve FMC Products) and subcloned into the *Eco*RI site of Bluescript SK(-). The full-length cDNA clone was sequenced first with T3 Bluescript primer and progressively, on both strands, with synthesized primers (Eurogentec).

The full-length cDNA clone was sequenced first with T3 Bluescript primer and progressively, on both the sense and antisense strands, with synthesized primers (Eurogentec). Sense primers were as follows: NG-3, 5'-AAAATCATCCAGGAGCGATGC-3' (nucleotides 157–177; amino acids 53–59); NG-4, 5'-AGAACCAACTATCTTTCTGTA-3' (nucleotides 517–537; amino acids 173–179); NG-5, 5'-AGGAAAGACTGCAGAACCAAC-3' (nucleotides 505–525; amino acids 169–175); NG-6, 5'-AAGTCCATCTTAGATGGAAGA-3' (nucleotides 757–777; amino acids 253–259). Antisense primers were: NG-7, 5'-GTTGTAGTCTTCCTTTGTCGC-3' (complementary to nucleotides 280–300; amino acids 94–100); NG-8, 5'-GAATACAGAAAGATAGTTGGT-3' (complementary to nucleotides 520–540; amino acids 174–180); NG-9, 5'-ATTTCTTCCATCTAAGATGGA-3' (complementary to nucleotides 760–780; amino acids 254–260); NG-10, 5'-CGAAAGTTTCCCCCAAAGTCC-3' (complementary to nucleotides 931–951).

C-terminal sequence confirmation

Single-strand cDNA synthesized from fresh calf spleen was used to verify the sequence surrounding the stop codon of the NADase sequence in the full-length cDNA clone found by screening the λgt10 bovine cDNA library. Two oligonucleotide primers (sense NG-6 and antisense NG-10) were used at 2 µM each in a PCR reaction using Vent polymerase at 0.02 unit/µl (Promega) and 2.5 µg of cDNA. Thirty amplification cycles were performed: 2 min at 95 °C, 1 min at 50 °C and 20 min at 70 °C. The PCR fragment was purified on a 1 % agarose gel. The corresponding gel slice was excised, frozen at -20 °C for 1 h, cut into small pieces and filtered by centrifugation at 5000 g and 4 °C on a 0.2 µm filter (Millipore). The PCR fragment was quantified by comparison with the Smartladder (Eurogentec) and sequenced directly using primers NA-4 and NA-8 on a 373A Applied Biosystems Sequencer.

Nucleic acid and amino acid sequence analysis

Sequence identity searches were conducted with the BLAST program [27] in the National Center for Biotechnology In-

formation data base. The deduced amino acid sequence was analysed on the ExPASy Swissprot Webserver (<http://www.expasy.ch>). The Swiss-Model program was used to model the NADase on the three-dimensional structure of *Aplysia californica* ADP-ribosyl cyclase. The program Protscale was used to determine the hydrophathy profile [28].

Construction of plasmids

The NADase full-length cDNA (2500 bp) was subcloned directly into the *Eco*RI site of the transient expression vector pSG5 (Clontech), and the two constructions (sense NADpSG5 and antisense NADpSG5ANTI) were amplified into *E. coli* DH5 α F' and purified.

Cell culture and transient expression in HeLa cells

HeLa cells were maintained in monolayer in minimal essential medium supplemented with 10 % (v/v) fetal calf serum, 2 mM L-glutamine, 0.01 % (v/v) penicillin/streptomycin and 0.225 % (w/v) sodium bicarbonate. The transfection was performed with linear polyethylenimine (22 kDa) according to Boussif et al. [29] using cells that were grown up to 70 % confluency in 10 cm diameter Petri dishes. The transfected HeLa cells were detached under very mild conditions, to ensure maximal cell integrity, by a brief treatment (5 min) with 1.5 ml of a 10-fold diluted (PBS) solution of trypsin-EDTA supplemented with EDTA (0.2 mg/ml final concentration). Cells were then spun down at 200 *g* for 3 min, resuspended in 500 μ l of PBS/1 mM CaCl₂/0.5 mM MgCl₂ and monitored for NADase expression and ecto-enzyme activities (see below). Cell viability (> 97 %) was assessed with a Trypan Blue exclusion test.

Enzyme assays

NADase activity was routinely determined with NAD⁺ by the cyanide addition assay [8] or by a sensitive continuous fluorimetric method using 1,*N*⁶-etheno-NAD⁺ as substrate [3]. To measure both ADP-ribosyl cyclase (formation of cADPR) and NADase [formation of ADP-ribose (ADPR)] activities, the enzyme fraction was assayed in the presence of [*adenosine*-¹⁴C]NAD⁺ (final concentration 20–50 μ M) as described previously [16,30]. GDP-ribosyl cyclase activity was measured by a continuous fluorimetric assay using NGD⁺ as substrate [16,31] and the reaction products were further analysed by HPLC as described below. For the cADPR hydrolase assay, given concentrations of cADPR were incubated at 37 °C in a 50 mM potassium phosphate buffer, pH 7.4 (50 μ l final volume), in the presence of 1 milliunit of NADase. At different time points, analysis of the reaction products was performed by HPLC. Kinetic analyses were performed using a non-linear regression program (Prism; GraphPad). Nucleotide pyrophosphatase activity was checked by a fluorescent assay using 1,*N*⁶-etheno-pyridine adenine dinucleotide as substrate [32] and by HPLC [16].

Analysis of reaction products by HPLC

Aliquots of the reaction medium were centrifuged at 10000 *g* for 8 min at 4 °C in a Microcon instrument (10 kDa cut-off) (Amicon), and the reaction products were analysed by HPLC on a 300 mm $\times$ 3.9 mm μ Bondapak C₁₈ column (Waters). The compounds were eluted isocratically with a 10 mM ammonium phosphate buffer, pH 5.5, containing 1.2 % (v/v) acetonitrile, detected by their UV absorbance at 260 nm and/or by radio-detection (Flo-one; Packard Instruments) and identified by co-elution with authentic samples, essentially as described previously [16,30].

RESULTS

In order to clone the bovine spleen ecto-NADase, we first sought to obtain structural information by microsequencing the purified enzyme. This approach was chosen because of the existence within certain cells of multiple proteins endowed with NADase activity, and also to avoid any bias by using probes deduced from conserved regions of CD38. The N-terminal amino group of the enzyme was found to be blocked, and only a very weak signal could be obtained, yielding tentatively the following sequence: XDCTTPRRKL. It was therefore necessary to generate internal peptides by proteolysis and/or by chemical cleavage with cyanogen bromide.

Sequencing of internal peptides generated from NADase

As noted previously [6], ecto-NADase proved to be very resistant to proteolytic digestion. The best results were obtained after thorough reduction of the enzyme with dithiothreitol followed by S-alkylation with 4-vinylpyridine or acrylamide and in-gel digestion [24] with proteases. Thus analysis by HPLC of the peptides obtained by limited proteolysis with endoproteinase Lys-C yielded the peptide (K)EDYNSLINLAPPTVPCGQQVF, which presents 45 % identity with human CD38. Similarly, two peptides obtained with trypsin were microsequenced: (R)MXTLEDTLLGYLAD and (R)TNYLSVFWVLSEK. The first peptide has a sequence that is highly conserved in all CD38 proteins (93 % identity) and can be considered to belong to the signature domain of this family of enzymes. The second peptide has about 54 % identity with CD38. Treatment of NADase with CNBr yielded two major bands of 29 kDa and 21 kDa. The latter polypeptide, after transfer to a PVDF membrane, gave by microsequencing the sequence (M)SAFISKDPCKATKEDYNSL (71 % identity with CD38), the last six residues of which overlap with the peptide obtained with endoproteinase Lys-C. From these preliminary data it appears that bovine ecto-NADase and CD38 are closely related enzymes.

Purification of the soluble ecto-domain of NADase

Because of the N-terminal blockage in the intact form of ecto-NADase and the difficulties encountered in obtaining internal peptide sequences, we prepared in parallel hNADase, i.e. a truncated and hydrosoluble form of the enzyme. The fully catalytically active hNADase, which originates from the membrane-bound form of NADase by removal of the hydrophobic membrane-anchoring domain and presumably the cytoplasmic domain, corresponds to the extracellular domain of the enzyme [6]. hNADase was originally obtained by digestion of calf spleen acetone powders with a crude pancreatic lipase (steapsin) [8], and we have previously shown that, although we could not reproduce this solubilization by limited proteolysis using a variety of proteases, the formation of the hydrosoluble form results most probably from a proteolytic processing of the membrane-bound NADase [6]. Thus we reasoned that, if bovine ecto-NADase is, by analogy with CD38, a type II transmembrane glycoprotein, the N-terminus of hNADase should be free and amenable to sequencing. The purification procedure for hNADase that was devised two decades ago [8] yielded an enzyme preparation which on SDS/PAGE was apparently homogeneous; however, this preparation was proved by sequencing to be heavily contaminated with trypsinogen, originating from steapsin, that co-purifies with the enzyme. We have therefore reassessed the original purification scheme and introduced new chromatographic steps.

Table 1 Purification of the steapsin-solubilized form of bovine spleen NADase

hNADase obtained by treatment of bovine spleen microsomes with crude pig pancreatic lipase (steapsin) was used for the purification procedure (see the Experimental section). One unit of NADase activity is defined as the amount of enzyme that hydrolyses 1 μ mol of NAD⁺ per min at 37 °C under standard conditions (50 mM sodium phosphate buffer, pH 7.4, and 2 mM NAD⁺).

Step	Total activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
DEAE-cellulose	156	82	1.9	100	1
CM-Sepharose	80.6	12.5	6.5	52	3.4
Hydroxyapatite	50.4	2.95	17.1	32	9
Concanavalin A	18	0.96	18.8	11.5	9.9
Affi-Gel Blue	12.3	0.125	98.4	7.9	52

The hNADase was obtained by treatment of calf spleen microsomes with steapsin; the solubilization yield was dependent on the commercial batch used and on the steapsin/microsomal protein ratio. Under optimal conditions (see the Experimental section), more than 80–90 % of NADase activity could be routinely released from the membranes as a hydrosoluble form. Importantly, a similar yield was obtained on preparation of NADase by treatment of the microsomes with detergents [6,9], indicating that hNADase represents the truncated form of the bovine ecto-NADase and not a minor isoform more susceptible to proteolytic cleavage.

hNADase was purified according to the scheme outlined for the NADase [9], but in the absence of detergents and with some modifications. Thus an affinity chromatography step on immobilized concanavalin A was included to separate the hNADase, a glycoprotein [9], from trypsinogen, followed by a final step on Affi-Gel Blue, which strongly retains the enzyme and allows the elimination of any lectin leaching from the previous affinity gel. A representative purification scheme is given in Table 1. SDS/PAGE gave for the purified enzyme, revealed both by silver-staining and, after renaturation, by in-gel fluorimetric detection of its activity using 1,*N*⁶-etheno-NAD⁺ as substrate [23], single bands migrating at the same apparent molecular mass of 29 kDa (results not shown); this is somewhat higher than the molecular mass published earlier for hNADase (i.e. 24 kDa), as determined by gel filtration [7,8]. Analysis of the hNADase by matrix-assisted laser-desorption ionization/time of flight MS revealed a broad pattern characteristic of a glycoprotein with a major molecular peak at 29690 (results not shown).

ADP-ribosyl cyclase and cADPR hydrolase activities of hNADase

The 'classical' reactions catalysed by bovine hNADase have been examined in detail in previous studies (reviewed in [1,2]). However, because of the recent evidence that the intact form of the enzyme is, in analogy with CD38, a multifunctional enzyme involved in the metabolism of cADPR [16], the question arose as to whether the truncated form of NADase has retained its ADP-ribosyl cyclase and cADPR hydrolase activities. NGD⁺, which is a convenient surrogate substrate to study the formation of cyclized compounds by the ADP-ribosyl cyclases [31], was found to be efficiently converted into cGDPR by hNADase. The cyclization reaction was established both by measuring the increase in fluorescence at 410 nm and by analysing the reaction products by HPLC; we could thus determine that the cGDPR/GDPR ratio of 3:1 observed with the hNADase-catalysed reaction was similar to that found with NADase [16]. We have

also confirmed the ability of hNADase to catalyse the formation of cADPR from NAD⁺. Using [¹⁴C]NAD⁺ and an on-line radioactivity detector [16], time-dependent formation of [¹⁴C]cADPR was observed; the maximal amount of cADPR formed was about 1 % of the reaction products, i.e. a value very similar to that found with the intact form of the enzyme [16] or with CD38 [13]. Moreover, this cyclization reaction did not vary with pH, and the same amount of cADPR was obtained at pH values from 5.0 to 8.0. cADPR was also found to be a substrate for the hNADase, which hydrolyses it into ADPR. The kinetic parameters were determined and a *K_m* of 990 ± 110 μ M was found, which is slightly less than the value found for the complete enzyme [16]. The *V_{max}* was 26 μ mol · min⁻¹ · mg of protein⁻¹ for this particular enzyme preparation.

N-terminal sequence of hNADase

In contrast with NADase, hNADase could be microsequenced, indicating that its N-terminus was not blocked. Interestingly, a single sequence of 35 residues, i.e. SRNCQAIRQAFMSAFISKDPCKATKEDYNSLINLA, was obtained, which presents 50 % identity with human CD38. This sequence was found to overlap and exactly match the sequences of two internal peptide fragments originating from the NADase: (i) complete overlap with the 18-residue sequence obtained with CNBr, and (ii) a 10-residue overlap with the peptide produced with endoproteinase Lys-C (see above). These results confirm that hNADase corresponds to a truncated form of ecto-NADase; they also suggest that the hydrosoluble form was generated from the membrane-anchored protein by cleavage at a single site, or alternatively that hNADase represents the molecular form of the enzyme that is the most resistant, under the experimental conditions used, to the action of the protease(s) present in steapsin.

Cloning and structure of NADase

The nucleotide and deduced amino acid sequences of bovine spleen ecto-NADase are shown in Figure 1. The N-terminal amino acid sequence of hNADase (amino acids 74–83: NCQAIRQAFM) and the amino acid sequence of an internal tryptic peptide obtained from the native NADase (amino acids 174–183: TNYLSVFWEV) were used to synthesize two degenerate oligonucleotides. These sequences were chosen because of their weaker degree of identity with CD38 compared with the other internal peptide sequence (amino acids 135–147: MTLEDTLGLYLAD), which had a very high identity with human CD38. A reverse transcriptase reaction was carried out on template cDNA derived from bovine spleen mRNA, and in a specific manner with the antisense primer derived from the amino acid sequence of the tryptic peptide, and with template cDNA derived from bovine spleen mRNA. Both samples were subjected to a PCR reaction using the sense and antisense primers, and both reactions produced a fragment of approx. 330 bp, which was subcloned into pBluescript SK(+). The nucleotide sequence of the insert, called NG-330, was determined and revealed an amino acid sequence (residues 74–183) with 70 % identity with human CD38. This PCR fragment was then used for screening a bovine spleen cDNA library. Eleven clones were obtained, one of which contained the full-length cDNA coding for NADase. It contained an 834 bp open reading frame, encoding a 31 532 Da protein. The environment of the first ATG codon (GACCCCATGG) encountered in the cDNA sequence matches with a Kozak sequence [33], and the next one is located 325 bp downstream in an unfavourable context (TTCATGA). This observation led us to conclude that the open reading frame of bovine spleen NADase starts with this first ATG codon

cg gta gcg ctc tcc tcc ccg gcc gct cag act cca gcg gcg gct tct aca aat tgg tcc	-22
ggg aga ccc tca tgc gac ccc atg gcc gac tgc tgc acg ccg aga aag aag ctt tgc tgg	39
M A D C C T P R K K L C W	13
tgc gtt ctt ctg ttc ttc ctg gtg gcg ggg atc acc ggg ggg gcg atc gga atc tac aag	99
C V L L F F L V A G I T G G A I G I Y K	33
tgg cac tac tcc ggg ctc aac agg tgg cac ggg gca gcc agc acc gcc gat ttt cag aaa	159
W H Y S G L N R W H G A G S T A D F Q K	53
atc atc cag gag cga tgc gac acc tac acc cag acg atc ccg ccg ggt tcc aga agc aga	219
I I Q E R C D T Y T Q T I R P G S R S R	73
aac tgc caa gcg atc agg cag gca ttc atg agt gcc ttc att tcc aag gat ccc tgc aaa	279
N C Q A I R Q A F M S A F I S K D P C K	93
gcg aca aag gaa gac tac aac tcc ctg atc aac ctg gca cct ccc act gtg cct tgt ggc	339
A T K E D Y N S L I N L A P P T V P C G	113
cag caa gtc ttc tgg agc aaa acg aag gag ctg gct cat gaa tac gcg aaa agg ccg cgc	399
Q Q V F W S K T K E L A H E Y A K R R R	133
ctg atg act ctg gag gac acg ctg ctg ggt tac ctt gcg gat ggc ctc agg tgg tgt gga	459
L M T L E D T L L G Y L A D G L R W C G	153
gag cca ggc tct tct gat ttg aac att tgg tct tgt cca gac tgg agg aaa gac tgc aga	519
E P G S S D L N I W S C P D W R K D C R	173
acc aac tat ctt tct gta ttc tgg gaa gtg ctg tct gaa agg ttt gca gaa agt gcc tgt	579
T N Y L S V F W E V L S E R F A E S A C	193
aat aca gtc ccg gtg gtg ctc aat gga tgc ttg gag aac gcc ttt gat agc atg agc ata	639
N T V R V V L N G S L E N A F D S M S I	213
ttt gga cga gtg gaa gcc cct aac ttg agg cca cag gtt gaa cta gag gct tgg ctg gta	699
F G R V E A P N L R P Q V E L E A W L V	233
cat gac act gga aaa cct ccc agt gac tca tgc tct ggc tcc tct ata agg aaa ctg aag	759
H D T G K P P S D S C S G S S I R K L K	253
tcc atc tta gat gga aga aat gtc aaa ttt aga tgc atg gat aat ctc agc cgt gac cag	819
S I L D G R N V K F R C M D N L S R D Q	273
ttt ctt caa aga tcc tga gga ttc atc ttg cag atg ggt cag tca gca tgg ctg agc cct	879
F L Q R S *	278
ggc agt gca gat caa cac aca cga ttc agg aga cat gct ggt gga aag ttg gga ctt tgg	939
ggg aaa ctt tgc	951

Figure 1 Nucleotide sequence and deduced amino acid sequence of bovine spleen ecto-NADase

The nucleotides are numbered on the right from the beginning of the open reading frame of the cDNA. The translated amino acid sequence, numbered in *italics*, is shown below the nucleotide sequence in single-letter code. The location of the putative transmembrane segment (residues 11–31) is indicated by solid boxes. The boxed tripeptides (broken lines) correspond to consensus sequences for putative asparagine N-glycosylation sites. hNADase, the truncated and hydrosoluble form of NADase, starts at residue Ser-72. The emboldened amino acids indicate the sequences that were confirmed from the two forms of purified NADase by microsequencing: the N-terminus of hNADase (residues 72–106), and internal peptides derived from the ecto-NADase by chemical degradation (CNBr; residues 84–102) or by partial digestion with trypsin (residues 134–147 and 174–187) or endoproteinase Lys-C (residues 97–117).

(Figure 1). The deduced amino acid sequence of this protein includes all the sequences found by microsequencing on both purified forms of the enzyme (Figure 1).

Confirmation of the C-terminal sequence of NADase

The alignment of the sequence of NADase with the known CD38 sequence (Figure 2) revealed an interesting feature: the bovine enzyme was globally shorter. For that reason, it was necessary to confirm the NADase C-terminal sequence in another system than our bovine spleen cDNA library. Thus we used cDNA synthesized from freshly prepared bovine spleen mRNA and, by PCR reaction with primers localized on both sides of the stop codon (NG-6 and NG-10), we confirmed that KSILDGRNV-KFRCMDNLSRDQFLQRS is the correct sequence of the final 26 amino acids of bovine ecto-NADase.

Analysis of the tryptic peptides obtained from bovine NADase by post-source decay–matrix-assisted laser-desorption ionization

MS also allowed the identification of the peptide DQFLQR ($M = 806.4$), in agreement with the expected sequence in the C-terminal region between residues 272 and 277. Therefore the occurrence of this specific peptide rules out the presence of a cysteine residue at position 277 which is strictly conserved in the other known CD38 proteins.

Transient expression of NADase in HeLa cells

To determine if the isolated full-length cDNA encodes a catalytically active ecto-NADase, we transfected HeLa cells with the cDNA subcloned in the mammalian transient expression vector pSG5 (NADpSG5). In control experiments, the cells were transfected with the plasmid containing the antisense sequence (NADpSG5ANTI). The day after transfection, the adherent cells were detached with a brief and mild trypsin treatment; these conditions were found not to affect cell viability or NADase activity.

The intact cells transfected with NADpSG5 expressed ecto-NADase activity, as assessed fluorimetrically using the non-permeant substrate 1,*N*⁶-etheno-NAD⁺ (Table 2). In contrast, very little activity could be detected in cells transfected with the antisense cDNA. Similarly, the wild-type cell line did not express measurable NADase or nucleotide pyrophosphatase activities. The comparative NADase and ADP-ribosyl cyclase activities were obtained by incubating the transfected HeLa cells in the presence of [¹⁴C]NAD⁺. Analysis of the reaction products by radio-HPLC revealed, only in the case of sense-transfected cells, the appearance of [¹⁴C]cADPR, which amounted to approx. 0.5 % of the products formed. This cADPR/ADPR ratio is very close to that measured with purified bovine NADase [16]. Using NGD⁺ [31], the ecto-GDP-ribosyl cyclase activity of the sense-transfected cells could also be detected fluorimetrically (formation of cGDP) and by HPLC (Table 2). The relative proportions of NADase, ADP-ribosyl cyclase and GDP-ribosyl cyclase (approx. 200:1:200) in HeLa cells transfected with NADpSG5 were within the range of those measured, under the same experimental conditions, with purified bovine ecto-NADase [16]. Interestingly, the levels of expression found in the present study were within the range of those in cells transfected with CD38 or in cells that constitutively express that activity ([34] and references therein).

The catalytic activities of cDNA-transfected HeLa cells were assayed on intact cells. Data were obtained in a representative experiment which was repeated three times and gave similar results. The substrate concentration used was 25 μ M in each case. ND, not detectable.

DISCUSSION

Very little is known about the molecular and structural characteristics of NADases, which were discovered in animal tissues more than five decades ago. Thus, although purified catalytically active hydrosoluble domains of NADases were obtained in the past [1], no molecular biological approaches were carried out. Moreover, purification to apparent homogeneity of hydrophobic untruncated forms of this class of enzymes was achieved only

recently in a few cases [9,10]. We have reported the purification of bovine spleen ecto-NADase [9], a glycoprotein overwhelmingly expressed at the surface of splenocytes [3] and which presents catalytic properties similar to those of CD38 [16]. The present work, which reports the molecular cloning of this enzyme, has yielded the first primary sequence of a ‘classical’ ecto-NADase, as well as information on its mode of association with cell membranes and its structural identity with CD38.

Cloning and sequence identity with CD38

The cDNA coding for bovine NADase was obtained by screening a bovine spleen cDNA library with sequence information obtained after microsequencing of the purified protein. The authenticity of the clone was validated by sequence analysis of the purified NADase and of its truncated form, hNADase. Thus the sequences obtained by Edman microsequencing of the N-terminus of hNADase and/or of internal peptide fragments obtained from NADase by partial digestion with endoproteases or by cleavage with CNBr were found to be identical to the deduced amino acids at positions 72–117, 134–147 and 174–187 (Figure 1), i.e. approx. 25% of the cDNA deduced amino acid sequence could be confirmed by microsequencing of the purified enzyme. Moreover, transient heterologous expression of the clone in HeLa cells confirmed the appearance of the NADase at the cell surface, as shown by the determination of NADase, ADP-ribosyl cyclase and GDP-ribosyl cyclase activities on intact cells.

Computer-assisted comparisons of the deduced amino acid sequence of bovine ecto-NADase with protein and nucleic acid databases revealed a high degree of identity of the enzyme with CD38; thus 48% and 38% identity was established with murine and human CD38 respectively (Table 3). A significant level of identity was also found with other enzymes known to be involved in the metabolism of cADPR, such as the surface-expressed GPI-anchored membrane protein BST-1 (CD157) and the soluble invertebrate ADP-ribosyl cyclases (Table 3). This identity with CD38 stretched over the entire sequence, including the presence of a single transmembrane spanning segment at the N-terminal domain, typical of type II transmembrane glycoproteins, and a marked conservation of cysteine residues. It was documented previously [35] that about 40 residues are strictly conserved in the eight enzymes involved in the metabolism of cADPR and whose structures are known (listed in Table 3), but

Figure 3 Functional structure of bovine ecto-NADase

The cytoplasmic tail (residues 1–10) and the ectodomain are represented by open boxes separated by the transmembrane segment (residues 11–31; black box). hNADase starts at Ser-72. The eight cysteine residues that are strictly conserved in NADase and in all enzymes involved in the metabolism of cADPR (see Table 3) are indicated by Cys (normal type); the three additional cysteine residues that are conserved in NADase and CD38 are shown in bold; the broken lines denote the potential disulphide bonds; the solid diamonds indicate putative N-glycosidation motifs. HA, putative hyaluronate binding site.

the bovine enzyme presents some minor differences. Thus five of these residues are different (positions 56, 114, 199, 226 and 232), three of which are conservative changes (positions 114, 119 and 226), and two usually conserved cysteine residues are missing (see below).

Structure analysis

Analysis of the bovine ecto-NADase primary sequence reveals the presence of a single putative transmembrane-spanning segment near the N-terminus of the enzyme, from amino acid 11 to 29 or 31 depending on the algorithm used (e.g. [28]), and a large extracellular domain that contains the active site (Figure 3). This topological arrangement, classical for ecto-enzymes that are usually attached to the cell membrane by a single protein segment, predicts that bovine NADase is a type II transmembrane glycoprotein. It appears also that this enzyme, by analogy with CD38, is in this respect clearly different from GPI-anchored proteins such as CD157, which possess a short C-terminal hydrophobic region [36].

The transmembrane domain of NADase, which is characterized by high hydrophobicity typical of apolar α -helices, contains two potentially cross-linkable cysteines. In relation to this observation, we have demonstrated recently that homooligomerization of NADase, via the formation of a disulphide bond, can be observed; it is, however, a slow process that occurs for the purified form of the enzyme under oxidative conditions [37]. The prevalent presence in integral proteins of tyrosine and

Table 3 Comparison of the extent of identity and similarity of amino acids in primary sequences of bovine ecto-NADase and related proteins

The sequences are available in databases (Genbank-NCBI/NIH) with accession numbers as follows: human CD38, M34461/D84276; rat CD38, D30795; mouse CD38, L11332; human BST-1 (CD157), D21878; rat BST-1, D49955; mouse BST-1, D31788/L32812; *Aplysia californica* ADP-ribosyl cyclase, M85206; *Aplysia kurodai* ADP-ribosyl cyclase, D30048. The extent of identity and similarity were calculated with a BLAST P program [27]. Values represent the percentage of identical amino acids; values in parentheses represent the percentage of similar amino acids.

Enzyme	Identity (similarity) (%)							
	Bovine NADase	Human CD38	Rat CD38	Mouse CD38	Human CD157	Rat CD157	Mouse CD157	<i>A. californica</i> cyclase <i>A. kurodai</i> cyclase
Bovine NADase	100 (100)	39 (56)	47 (60)	48 (62)	37 (53)	40 (52)	37 (53)	30 (45)
Human CD38		100	55 (60)	54 (62)	36 (53)	34 (52)	34 (53)	31 (45)
Rat CD38			100	79 (83)	37 (55)	34 (52)	35 (53)	35 (49)
Mouse CD38				100	36 (54)	33 (52)	35 (51)	34 (48)
Human CD157					100	75 (86)	73 (84)	32 (35)
Rat CD157						100	89 (91)	32 (52)
Mouse CD157							100	32 (51)
<i>A. californica</i> cyclase								100
<i>A. kurodai</i> cyclase								100

tryptophan residues in regions corresponding to membrane surfaces, where they develop favourable interfacial interactions [38] and references therein), could also be extended to the bovine NADase; thus residues 32 and 34 next to the membrane surface are Tyr and Trp respectively. Moreover, the occurrence of a tryptophan at position 34 is also in agreement with the known propensity of this amino acid for the extracellular face of membrane proteins [39].

The short N-terminal cytoplasmic tail of the bovine NADase of 9–10 amino acids, which is about half the length of the cytoplasmic domains of the other CD38 proteins, has a rather unremarkable sequence. It contains no consensus motifs for post-translational modifications that could mediate interactions with other signalling proteins; this contrasts with CD38 species, which contain in their intracellular domains two conserved serine residues within sites recognized by cGMP-dependent protein kinases [18]. Besides a conserved Cys residue in the N-terminal domain, there is little sequence overlap in either the cytoplasmic tail or the transmembrane sequences between CD38 and the NADase. These regions do not seem to play a major role in the catalytic activity of these enzymes. Accordingly, it was shown recently in mouse CD38 that replacement of these domains by those of unrelated integral proteins did not affect the catalytic activity of this enzyme, or its signalling properties when transfected in lymphoma cells [19].

In contrast with human CD38, which exhibits four potential N-glycosylation sites [40], the bovine ecto-NADase has only two consensus sequence Asn-Xaa-Thr(Ser) sites, located at Asn-201 and Asn-268. Comparison of the four sequences (Figure 2) indicates that only one asparagine (Asn-209 and Asn-201 in human CD38 and NADase respectively) is strictly conserved in a consensus sequence (Figure 2). The presence of such sites is in agreement with the previously demonstrated glycoprotein nature of the bovine enzyme [9]. Human and mouse CD38 were also found to contain several binding sites for ligands such as hyaluronates. In human CD38, for example, one such putative motif was located in the cytoplasmic domain (residues 13–21) and two others in the ectodomain (residues 121–129 and 268–276) [41]. Such binding sites are not conserved in the bovine enzyme, in which only one putative site could be found, at K³³WHYSGLN^R⁴¹ in the extracellular juxtamembrane domain, which is lost in the hNADase (see below).

It is particularly striking that, of the 12 cysteine residues that are present in the ectocellular domains of CD38 and are also strictly conserved in all the other enzymes involved in the metabolism of cADPR, 10 are also found in bovine NADase (Figure 3). The cysteines that are lacking (e.g. corresponding to Cys-287 and Cys-296 in human CD38) are part of the 12-amino-acid sequence that is missing from the C-terminal domain of the NADase. By analogy with previous work by the group of Stout [35], we have modelled the ecto-domain of the bovine enzyme on the three-dimensional structure of *Aplysia californica* ADP-ribosyl cyclase. As expected, the overall protein architectures are quite similar and predict, in the NADase, the formation of disulphide bridges between Cys-59 and Cys-75, Cys-92 and Cys-172, Cys-152 and Cys-165, and Cys-244 and Cys-265 (Figure 3). By analogy with CD38 [35], the occurrence of an additional disulphide bridge can also be suggested in the hinge region between Cys-112 and Cys-193. Site-directed mutagenesis of these latter cysteines in human CD38 (e.g. C119K) resulted in an enzyme that had lost its NADase activity and became an ADP-ribosyl cyclase [42]. It should be noted, however, that a similar mutation in mouse CD38 resulted in activation of its catalytic activity which led to a small increase in cyclase activity without loss of NADase activity [19].

Relationship between structure and catalytic properties

Bovine ecto-NADase, when subjected to treatment with steapsin, yields hNADase, i.e. a hydrosoluble and catalytically active form of the enzyme [8]. This cleavage occurs apparently at one defined site, Arg-71, which corresponds to a trypsin-like cleavage site. Our data show for the first time that the transmembrane-spanning segment of the NADase has no regulatory effect on enzyme activity and that a N-terminal region as large as 71 residues (i.e. about 40 amino acids of the juxtamembrane domain) is not necessary for the maintenance of a fully catalytically active enzyme. This observation has now been extended to the metabolism of cADPR by hNADase. We have thus shown in the present work that the truncated form of the bovine NADase is also an ADP-ribosyl cyclase and a cADPR hydrolase, with kinetic parameters very similar to those of the intact form of the enzyme. It should be mentioned here that hNADase also lacks a putative disulphide bridge between Cys-59 and Cys-75 which exists in the juxtamembrane domain of all CD38 species and in the intact form of NADase. It seems, therefore, that such a bridge is not indispensable for maintaining a catalytically active conformation. Altogether, these results indicate that the hydro-soluble fragment obtained by the loss, within the N-terminal domain, of about 25% of the total residues represents a conformationally independent domain that maintains the full catalytic properties of NADase. It was similarly shown recently that replacement of the transmembrane domain of mouse CD38 with those of unrelated integral proteins did not affect the catalytic activity of this enzyme, or its signalling properties when transfected in lymphoma cells [19]. This contrasts with other ectoenzymes, such as ectoapyrase (CD39), whose enzymic activity and quaternary structures are regulated by their transmembrane domains [43].

The possibility of preparing a fully active truncated form of bovine NADase which represents the extracellular domain of the enzyme is also reminiscent of the naturally occurring soluble forms of human CD38. These soluble CD38, which are present *in vivo* in normal and pathological fluids, can also arise upon ligation in several cell lines of the surface-expressed CD38 by specific anti-CD38 monoclonal antibodies [44]. Unfortunately no structural data are yet available for soluble CD38, such as, for example, identification of the bond that has been cleaved to release the soluble form. This shedding of the ecto-domain of CD38 is a characteristic feature shared by numerous other cell-surface-associated membrane proteins involved, for example, in signalling (for a review, see e.g. [45]).

Analysis of the multiple sequence alignment of CD38 species and bovine NADase (Figure 2) indicates the presence of a highly conserved region between Thr-136 and Gly-153 (NADase numbering), representing the 'signature' of this class of multifunctional enzymes and containing residues involved in catalytic activity [19,35,46]. All the other residues that have been shown in the literature to be of some importance in CD38 catalytic activity are conserved in bovine ecto-NADase. These include, in human CD38, Cys-119/Cys-201 [42], Cys-275 [47] and Lys-129/Leu-131 [48] and, in mouse CD38, Cys-123 [19]. Finally, the R140W mutation in CD38, which was found to be associated with type II diabetes mellitus and which moderately affects catalytic activity [49], also concerns a residue that exists in the bovine enzyme (Arg-132).

We have also shown that bovine ecto-NADase is naturally truncated at the C-terminus by 12 amino acids which are highly conserved residues in the other known CD38 proteins. Moreover, this missing sequence contains two cysteines (Cys-287 and Cys-296 according to human CD38 numbering) that are strictly

conserved in all eight known enzymes related to ADP-ribosyl cyclase (i.e. invertebrate ADP-ribosyl cyclases, CD38 and CD157). The deletion of these 12 residues, however, has no apparent consequences for the catalytic activity of the bovine enzyme, a result that is in agreement with recent data obtained by Hoshino et al. [47] with human CD38. Altogether, the data obtained in the present study indicate that a polypeptide as short as 207 amino acids, and which lacks two potential disulphide bridges compared with CD38, represents the smallest functional domain that is endowed with all the multifunctional catalytic properties that characterize CD38.

Conclusions

The present results on the molecular cloning of bovine spleen ecto-NADase indicate that this enzyme is most probably the bovine equivalent of CD38. This conclusion fully conforms with our previous work on comparison of the catalytic properties of this enzyme and CD38 [16,20] and on the tissue localization of NADase in CD38^{-/-} mice [21]. This 'classical' NADase shares very high structural identity with CD38, as well as multifunctional catalytic properties related to the hydrolytic turnover of NAD(P)⁺ and the metabolism of cADPR. Finally, this identity has some important bearing with regard to the investigation of the physiological functions of these enzymes. The finding that the spleen ecto-NADase is the bovine equivalent of CD38 means that many other NADases, whose properties and localization are known [1], could also be CD38 proteins. This enlarges the scope of these latter enzymes, which were originally associated with a much narrower range of cellular/tissue localization. Moreover, it is now evident that, when delineating the functions of CD38, one should also take into account the accumulated knowledge on animal NAD(P)⁺ glycohydrolases that deals with the important, and singular, structural (e.g. molecular masses) and catalytic heterogeneity that characterizes this class of enzymes, and with their diversified tissue and subcellular distribution.

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