CD38-dependent ADP-ribosyl cyclase activity in developing and adult mouse brain

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CD38 is a transmembrane glycoprotein that is expressed in many tissues throughout the body. In addition to its major NAD^+-glycohydrolase activity, CD38 is also able to synthesize cyclic ADP-ribose, an endogenous calcium-regulating molecule, from NAD^+. In the present study, we have compared ADP-ribosyl cyclase and NAD^+-glycohydrolase activities in protein extracts of brains from developing and adult wild-type and *Cd38*$^{-/-}$ mice. In extracts from wild-type brain, cyclase activity was detected spectrofluorimetrically, using nicotinamide–guanine dinucleotide as a substrate (GDP-ribosyl cyclase activity), as early as embryonic day 15. The level of cyclase activity was similar in the neonate brain (postnatal day 1) and then increased greatly in the adult brain. Using [^{14}C]NAD^+ as a substrate and HPLC analysis, we found that ADP-ribose is the major product formed in the brain at all developmental stages. Under the same experimental conditions, neither NAD^+-glycohydrolase nor GDP-ribosyl cyclase activity could be detected in extracts of brains from developing or adult *Cd38*$^{-/-}$ mice, demonstrating that CD38 is the predominant constitutive enzyme endowed with these activities in brain at all developmental stages. The activity measurements correlated with the level of CD38 transcripts present in the brains of developing and adult wild-type mice. Using confocal microscopy we showed, in primary cultures of hippocampal cells, that CD38 is expressed by both neurons and glial cells, and is enriched in neuronal perikarya. Intracellular NAD^+-glycohydrolase activity was measured in hippocampal cell cultures, and CD38-dependent cyclase activity was higher in brain fractions enriched in intracellular membranes. Taken together, these results lead us to speculate that CD38 might have an intracellular location in neural cells in addition to its plasma membrane location, and may play an important role in intracellular cyclic ADP-ribose-mediated calcium signalling in brain tissue.

Key words: ADP-ribosyl cyclase, brain, CD38, cyclic ADP-ribose, NAD^+, nicotinamide–guanine dinucleotide.

INTRODUCTION

CD38 is a 45 kDa transmembrane glycoprotein that is distributed extensively on haematopoietic cells and is also present on an array of adult cells and tissues, including liver, kidney, skeletal and cardiac muscle, and brain [1–3]. The protein is present at the surface of cells, and now has also been demonstrated to have an intracellular localization [4,5]. In addition to serving for many years as an antigenic marker for discriminating between different subsets of haematopoietic cells, CD38 also possesses signalling and catalytic properties.

The assignment of catalytic activity to CD38 has emerged from its sequence similarity with the ADP-ribosyl cyclase from the mollusc *Aplysia californica*, cloned in 1991 [6]. Using recombinant CD38, it was recognized as a multifunctional enzyme, able to hydrolyse NAD^+ to ADP-ribose (ADPR) and nicotinamide (NAD^+-glycohydrolase activity), to cyclize NAD^+ in order to form cyclic ADPR (cADPR) (ADP-ribosyl cyclase activity) and also to hydrolyse cADPR into ADPR and nicotinamide (cADPR hydrolase activity) [7,8]. CD38 is the prototypic mammalian member of a protein family that includes BST-1 (bone marrow stromal cell antigen-1, which was discovered in bone marrow stromal cell lines derived from patients with severe rheumatoid arthritis, and is also called CD157) and *Aplysia* ADP-ribosyl cyclase. These proteins share a common gene organization, suggesting that they arose from a common progenitor gene [9,10], and the same enzymic reaction mechanism for the conversion of NAD^+ into ADPR and cADPR [11,12].

It has been postulated that ADP-ribosyl cyclases regulate cell signalling, as cADPR was shown to mobilize intracellular calcium in sea urchin eggs and to regulate the calcium-induced calcium release phenomenon that occurs via ryanodine receptors in mammalian cells (for reviews, see [13,14]). In brain, cADPR has been shown to be abundant [15,16] and to be implicated in many neural processes, such as synaptic transmission, neurotransmitter release and calcium events of rhythmic bursting [17–19]. Even if cADPR is clearly involved in regulating signalling in neural cells, it is not known whether CD38, BST-1 or another unknown cyclase is responsible for cADPR production in these cells.

The ADP-ribosyl cyclase activity of CD38 has been shown to regulate calcium mobilization in non-neural mammalian cells [16,20,21]. In haematopoietic cells, in which the protein has been best studied, data indicate that CD38 has enzyme-dependent and -independent functional roles. CD38 was shown to operate as a receptor in lymphocytes, independently of its enzyme activity, to control proliferation, differentiation, apoptosis, activation

Abbreviations used: ADPR, ADP-ribose; BST-1, bone marrow stromal cell antigen-1; cADPR, cyclic ADP-ribose; cGDPR, cyclic GDP-ribose; E15, embryonic day 15; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; GDPR, GDP-ribose; HBSS, Hanks balanced salt solution; NGD^+, nicotinamide–guanine dinucleotide; PN1, postnatal day 1; RT-PCR, reverse transcription–PCR; SH medium, saline hippocampus medium; WT, wild type.

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and adhesion [22–24]. However, in other haematopoietic cells, CD38 regulates calcium signalling through chemotactic receptors via the production of cADPR [16].

Although it has been demonstrated that CD38 is expressed in brain tissue isolated from rat and human [2,3], it is not known whether CD38 functions as an enzyme, a receptor, or both an enzyme and a receptor in this tissue. In the present paper, we have studied ADP-ribosyl cyclase activity in the developing and adult mouse brain. We demonstrate, by comparing extracts prepared from wild-type (WT) and *Cd38*$^{-/-}$ mice, the presence of CD38-dependent ADP-ribosyl cyclase activity, as well as NAD^+-glycohydrolase activity, at all developmental stages studied. The CD38-dependent activities were observed as early as embryonic day 15 (E15) and increased with the age of the animal. The developmentally regulated activities were correlated with the level of CD38 mRNA transcripts. In primary cultures of hippocampus cells, CD38 was shown to be enriched in neuronal perikarya. NAD^+-metabolizing activity was measured in intact hippocampal cells. The activity was enhanced in the same hippocampal cells when they were permeabilized, suggesting that a significant fraction of CD38 enzyme activity is localized inside the cell. This result correlates with the observation that increased CD38-dependent cyclase activity was found in cellular fractions enriched in intracellular membranes. The implications for the role of CD38 in regulating cADPR production and calcium signalling in neural tissue are discussed.

EXPERIMENTAL

Materials

ADP-ribosyl cyclase from *Aplysia californica*, nucleotide pyrophosphatase from *Croatus atrox* venom, NAD^+-glycohydrolase from *Neurospora crassa*, nicotinamide–guanine dinucleotide (NGD^+) and NAD^+ were purchased from Sigma. [^{14}C]NAD^+ (300 mCi/mmol) was purchased from Amersham Pharmacia Biotech Europe (Orsay, France).

Reverse transcription–PCR (RT-PCR) amplification

mRNA was prepared from developing and adult mice brains using the Dynabeads isolation kit (Dynal France S.A., Compiégne, France). Each RT-PCR reaction was performed in a total volume of 50 μl in the presence of 100 ng of mRNA using the Superscript™ One step™ RT-PCR system (Invitrogen SARL, Cergy Pontoise, France).

A 567 bp product from mouse CD38 cDNA (GenBank accession number L11332; [25]) was amplified using the primers 5′-GCAACATCACAAGAGAAGACTACGC (nucleotides 308–332) and 5′-ACACACTGAAGAAACCTGGCAGGCC (nucleotides 875–851). Reverse transcription was achieved within 30 min of incubation at 50 °C. Amplification was obtained after 40 cycles of 45 s at 94 °C, 30 s at 50 °C and 1 min at 72 °C.

As control for mRNA preparation and quantification, a 540 bp product from the mouse glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA was amplified using primers 5′-ACCACAGTCCATGCCATCACTGCC and 5′-CGAGTTGGGATAGGGCCTCTCTTGC. Reverse transcription was achieved within 30 min of incubation at 50 °C. Amplification was obtained after 35 cycles of 45 s at 94 °C, 30 s at 56 °C and 1 min at 72 °C.

Brain protein extracts

Protein extracts were prepared from developing [at E15 and postnatal day 1 (PN1)] and adult brains of C57Bl/6J WT and B6.192P2-(N12) *Cd38*$^{-/-}$ [26] mice. Adult brains were perfused with artificial cerebral spinal fluid as in [27] to which was added 10 units/ml heparin (Sigma), or with PBS/5 mM EDTA. After dissection, tissues were flash frozen in liquid nitrogen until use. Frozen tissues were first ground using a mortar and pestle, and then suspended in lysis buffer containing 20 mM Tris/HCl, pH 7.0, 10 % (w/w) glycerol, 10 mM $MgCl_2$, 300 units/ml DNase I (Roche Molecular Biochemicals, Meylan, France) and protease inhibitors (Complete™; Roche Molecular Biochemicals). The extract obtained at this step is referred to as the crude (or total) protein extract. After centrifugation of the total extract for 30 min at 1000 ***g*** to eliminate non-lysed cells, the supernatant was centrifuged for 30 min at 10000 ***g***. The pellet (referred to as the P10 fraction) was resuspended in 20 mM Tris/HCl, pH 7.0, 10 % (w/w) glycerol and protease inhibitors, and the supernatant was centrifuged again for 30 min at 540000 ***g***. The pellet obtained by high-speed centrifugation (referred to as P540) was resuspended in 20 mM Tris/HCl, pH 7.0, 10 % (w/w) glycerol and protease inhibitors, and the soluble extract was collected. In some experiments the crude extract was centrifuged directly for 30 min at 540000 ***g*** to obtain a pellet containing total membranes. For control purposes, total protein extracts from the spleens of WT mice were also prepared. Protein content was determined according to Bradford [28] using BSA as a standard.

Western blot analysis

The presence of CD38 in brain and spleen extracts was tested using an antibody raised against its C-terminus at a 1:100 dilution (Santa Cruz Biotechnology Inc., TEBU, Le-Perray-en-Yvelines, France). An antibody raised against plasma membrane Na^+/K^+-ATPase (Upstate Biotechnology, Lake Placid, NY, U.S.A.) was used at 0.1 μg/ml. Antibodies raised against mitochondrial oligomycin-sensitivity-conferring protein, lamin B1 and endoplasmic reticulum Ca^{2+}-ATPase (developed against the recombinant protein described in [29]) were used at a dilution of 1:2000, 1:1000 and 1:20000 respectively.

Proteins were separated on 10 or 12 % (w/v) polyacrylamide denaturing gels. They were then electrotransferred for 2 h at 500 mA to Immobilon P sheets (Millipore). After a 1 h incubation with 4 % (w/v) non-fat dry milk (Bio-Rad), the primary antibody was added and incubated overnight at 4 °C. The blots were then stained for 3 h at room temperature with horseradish peroxidase-conjugated anti-goat IgG (Pierce). After washing the blots, the reactive proteins were detected using the Renaissance chemilumiscent reaction (NEN Life Science Products) followed by exposure to X-ray films (Hyperfilm™ ECL™ or Hyperfilm™ βmax; Amersham Biosciences Europe).

Assays of GDP-ribosyl cyclase activity

The conversion of NGD^+ into cyclic GDP-ribose (cGDPR) [30] was followed fluorimetrically by monitoring the increase in fluorescence at 420 nm upon excitation at 300 nm. Assays were performed in a 1 cm × 1 cm fluorescence cuvette with continuous stirring using the MOS-200 optical system (Bio-Logic, Claix, France). The enzyme reaction was started by adding 20 μM NGD^+ to either *Aplysia* ADP-ribosyl cyclase (0.4 μg/ml) or protein extracts (200 μg/ml) that were incubated at 32 °C in 20 mM Tris/HCl, pH 7.0, in a final volume of 2 ml with continuous stirring.

After incubation with NGD^+, each sample containing brain extract was centrifuged at 4 °C for 30 min at 540000 ***g*** to remove membrane fractions. The supernatant was filtered through a Microsep 10K filter (Gelman Laboratory, Northborough, MA, U.S.A.) to remove the remaining proteins, and a fluorescence spectrum was recorded (excitation 300 nm; emission from 350 to 500 nm). The cGDPR content was determined by comparison of

the spectrum from the brain extract with a spectrum obtained using known concentrations of cGDPR. This latter was obtained by incubating 20 μM NGD^+ with 0.4 μg/ml *Aplysia* ADP-ribosyl cyclase for 15 min in 20 mM Tris/HCl, pH 7.0. Under these conditions, 100 % of NGD^+ was transformed into the cyclic compound, as verified by HPLC (results not shown).

Enzymic reactions with [^{14}C]NAD^+ and analysis by HPLC

Protein extracts were incubated in 20 mM Tris/HCl (pH 7.0) at 32 °C with 60 μM [^{14}C]NAD^+ (8 μCi/mmol) for various times (see Figure legends). Intact or permeabilized cells (60000 suspended cells per 100 μl of reaction medium; see below) were incubated in saline hippocampus medium (SH medium: 135 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 30 mM glucose and 10 mM Hepes, pH 7.5) at 37 °C with 200 μM [^{14}C]NAD^+ (4 μCi/mmol) for 2 h.

Reactions were stopped by adding 10 % (v/v) perchloric acid, followed by incubation for 10 min at 4 °C and then centrifugation of the samples (16000 ***g***, 10 min). After neutralization of the supernatant with K_2CO_3 and a short centrifugation to eliminate salt precipitates, the supernatant containing the reaction products was analysed by HPLC on a 300 mm × 3.0 mm μBondapack C18 column (Waters) equipped with a guard column (LiChroCART® 4-4 LiChrospher® 100 RP-18 5 μm; Merck Eurolab), as in [8]. Aliquots of 50–100 μl were injected and eluted isocratically with a medium containing 10 mM ammonium phosphate buffer, pH 5.5, and 1 % (v/v) acetonitrile. Radiodetection of the products was performed with an online radioactivity monitor (LB 506D, Berthold) after mixing eluting products with a scintillation liquid (Quickzint flow 302, Zinsser Analytic) using a pump (LP 5035, Berthold).

Standards were prepared by mixing (1:1:1, by vol.) the products formed when reacting NAD^+ with *Aplysia* ADP-ribosyl cyclase (60 μM NAD^+, 0.4 μg/ml enzyme; 15 min incubation at 32 °C), with nucleotide pyrophosphatase (60 μM NAD^+, 0.04 unit/ml enzyme; 15 min incubation at 32 °C) and with NAD^+-glycohydrolase (60 μM NAD^+, 0.04 unit/ml enzyme; 15 min incubation at 32 °C).

Products derived from NAD^+ (ADPR, cADPR, AMP) were identified on the chromatograms by separately injecting standards corresponding to the products of the above reactions. cADPR was further identified by its chemical conversion into ADPR after incubation for 15 min at 100 °C. ADPR was also characterized on the basis of its enzymic transformation into AMP catalysed by nucleotide pyrophosphatase.

Hippocampal cell cultures

Hippocampuses from neonatal mice (between days 1 and 2 post partum) were dissected and cleaned of meninges. They were incubated at 37 °C for 5 min in Hanks balanced salt solution (HBSS; In Vitrogen) containing 1 % (w/v) trypsin/EDTA and 1 mg/ml DNase I. After centrifugation to eliminate the supernatant, a 10-fold dilution of the tissue was resuspended in HBSS containing 0.4 mg/ml trypsin inhibitor. The tissue was gently triturated using a plastic pipette until a homogeneous suspension was obtained. After centrifugation, the cell pellet was resuspended in Neurobasal/B27 medium (In Vitrogen) containing 2 mM glutamate. Cell cultures were seeded at a density of 10^5 cells/cm^2 on culture dishes coated previously with 20 μg/ml poly(D-lysine) at 37 °C for 2 h. After 2 days, 3 μM cytosine arabinoside was added to the cultures to control the proliferation of non-neuronal cells, and 24 h later the medium was replaced. The cultures were then subsequently fed every other day.

For activity measurements, cells were dissociated by incubation for 5 min in HBSS containing 0.25 % trypsin. A 10-fold dilution in HBSS containing trypsin inhibitor was then performed. After two rinses with SH medium at 37 °C (see above), cells were resuspended in this medium. The same pool of suspended cells was divided in order to compare the activity of intact and permeabilized cells. Permeabilization was performed by adding 1 % Triton X-100 and 2 mM EDTA and homogenizing.

Immunofluorescence microscopy of neural cell cultures

Cultures of hippocampal cells isolated from neonatal WT mice were used at 8–12 days of culture. After three washes with SH medium (see above) at 37 °C, cells were fixed by incubation for 30 min at 0 °C in 70 % (v/v) ethanol. They were then rehydrated by incubation for 3 h at 4 °C in PBS. Cells were permeabilized by incubation for 45 min at room temperature in PBS containing 0.25 % Tween-20, 2.5 % (w/v) BSA (IgG-free; Jackson Immunoresearch) and 2.5 % (v/v) goat serum. They were then incubated at room temperature with the primary antibodies. Antibody raised against mouse CD38 {monoclonal rat anti-(mouse CD38) [31]} was used at 1:100 dilution (or at 1:2000 dilution for the biotinylated version). Prior to the addition of the biotinylated antibody, cells were treated with a blocking kit (Vector Laboratories). An antibody raised against neuronal tubulin [TUJ-1; monoclonal mouse anti-(rat βIII tubulin); Babco] was used at 1:500 dilution. After three washes with PBS containing 0.25 % Tween-20, preparations were incubated for 30 min at room temperature with the secondary antibodies. Anti-(rat IgG)–AlexaFluor 488 (Molecular Probes) was used at a dilution of 1:1000, and anti-(mouse Fc)–cyanin5 (Jackson Immunoresearch) was used at a dilution of 1:500. Cyanin3-conjugated streptavidin (Jackson ImmunoResearch) was used at 0.5 μg/ml. Cells were washed five times and then stored at 4 °C in anti-fading mounting medium (DAKO) until analysis.

Cells were imaged using a fluorescent confocal microscope (Leica TCS-SP3). Control experiments in which primary antibodies were omitted did not display any detectable signal.

RESULTS

We studied the presence of CD38 transcripts and proteins in brain tissue isolated from developing and adult WT mice. We then analysed the contribution of CD38 to brain ADP-ribosyl cyclase and NAD^+-glycohydrolase activities by analysing results obtained with extracts isolated from the brain tissue of developing and adult WT and *Cd38*$^{-/-}$ mice. Finally, the location of CD38 and the possible occurrence of active intracellular CD38 protein in brain cells were examined.

CD38 transcripts and protein in developing and adult mouse brain

It is known that CD38 is expressed in a number of human tissues, including adult brain. However, it has not been determined whether CD38 is expressed in developing or adult mouse brain tissue. Therefore we analysed CD38 mRNA transcript levels by RT-PCR in these tissues. Figure 1(A) shows that a PCR product corresponding to a portion of the CD38 cDNA (confirmed by sequencing) was amplified from brain mRNA from E15, PN1 and adult mice. CD38 transcripts were present at equivalent levels in the brains of E15 embryos and PN1 neonates, and were greatly increased in abundance in adult brain tissue (see control reaction for G3PDH; Figure 1B).

To determine if mRNA transcript levels were correlated with protein expression levels, we analysed protein extracts isolated

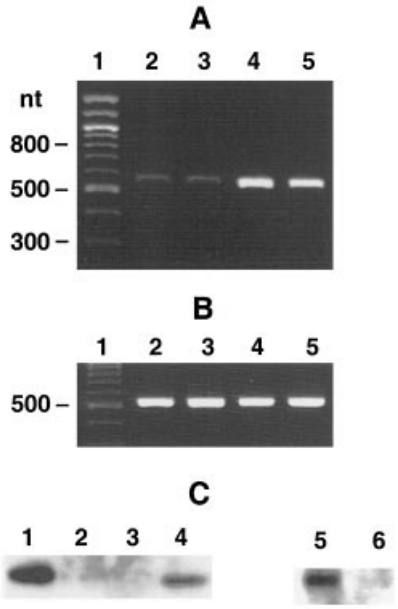

Figure 1 Analysis of CD38 transcripts and protein in brain extracts from WT mice

(**A**) RT-PCR analysis of CD38 mRNA transcripts was performed using RNA samples isolated from the brains of E15 (lane 2), PN1 (lane 3) and adult (lane 4) WT mice. Adult spleen RNA was used as a positive control (lane 5). (**B**) The quality and quantity of the transcripts in mRNA preparations were controlled by a G3PDH RT-PCR reaction using the same samples as in (**A**). (**C**) Western-blot analysis was performed to detect CD38 protein in total membrane protein extracts of brain from E15 (4 μg of protein; lane 2), PN1 (4 μg; lane 3) and adult (4 μg, lane 4; 8 μg, lane 5) WT mice. The protein was revealed with an antibody raised against the mouse CD38 C-terminus. A protein extract from spleen (8 μg; lane 1) served as positive control. A protein extract from adult *Cd38*$^{-/-}$ brain (8 μg; lane 6) served as a negative control.

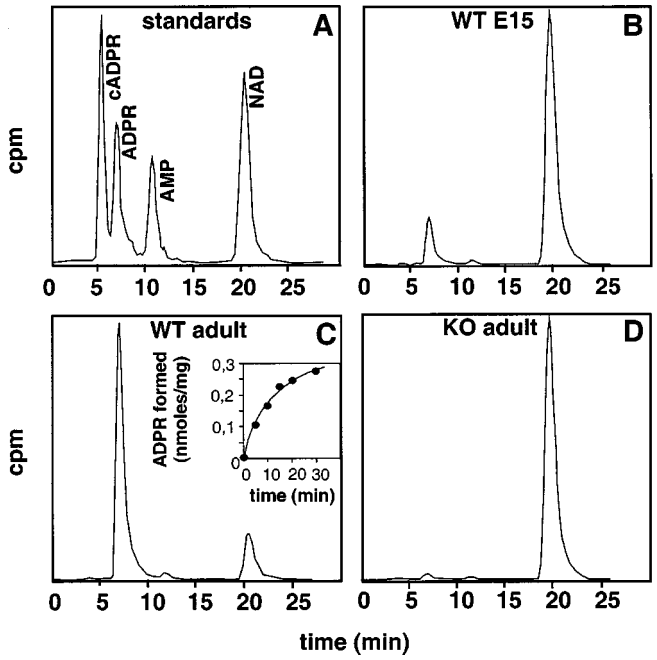

Figure 2 Analysis of NAD$^+$-metabolizing activities in brains of WT and *Cd38*$^{-/-}$ mice

Crude protein extracts (0.2 mg/ml) were incubated in 20 mM Tris/HCl at 32 °C and pH 7.0 with 60 μM [^{14}C]NAD$^+$ (8 μCi/mmol) in the presence of 2 mM EDTA (to inhibit nucleotide pyrophosphatases). Reactions were stopped by adding perchloric acid. Analysis of product formation by the protein extracts was performed by HPLC using an on-line radioactivity detector, as described in the Experimental section. (**A**) Standard nucleotides (see the Experimental section): cADPR appears at 5.2 min, ADPR at 6.5 min, AMP at 11 min, and NAD$^+$ at 20 min. (**B**) Products obtained with an extract isolated from the brains of embryonic (E15) WT mice after a 30 min reaction. (**C**) Products obtained with an extract isolated from the brains of adult WT mice after a 15 min reaction. (**D**) Products obtained with an extract isolated from the brains of adult *Cd38*$^{-/-}$ (KO) mice after a 15 min reaction.

from WT and *Cd38*$^{-/-}$ mouse brain tissue by Western blotting using an antibody developed against the C-terminus of mouse CD38. This analysis clearly revealed the presence of a band at 46 kDa in the total membrane extract from WT adult mouse brain (Figure 1C, lanes 4 and 5). This band was not present in brain extracts from adult *Cd38*$^{-/-}$ mice (Figure 1C, lane 6), indicating that the band observed in WT brain is CD38 specific. Additionally, CD38 protein in the extract of WT adult brain appeared to be due to expression of CD38 on neural or epithelial brain cells, but not on blood cells, as similar results (not shown) were obtained with extracts from perfused and non-perfused brains. Finally, CD38 protein could not be detected by Western blot in extracts from developing brain (Figure 1C, lanes 2 and 3), even when loading 5-fold higher quantities of membrane protein (i.e. 20 μg per well; results not shown).

Together, these results indicate that CD38 is expressed at both the mRNA and protein levels in adult mouse brain tissue, and that CD38 protein must either be absent or be expressed at significantly lower levels in embryonic and neonatal brain tissue.

CD38-dependent NAD$^+$-metabolizing activities in extracts from adult and developing brain

We wished to determine whether CD38 is enzymically active in developing and adult mouse brain tissue. Therefore we studied the transformation of [^{14}C]NAD$^+$ and analysed the reaction products by HPLC using an on-line radioactivity detector. Total extracts from the brains of WT and *Cd38*$^{-/-}$ mice were tested for their ability to transform the labelled substrate (60 μM) into cADPR and ADPR after incubation for between 5 min and 1 h at 32 °C and pH 7.0. To allow a better analysis of activities, 2 mM EDTA was added to the incubation medium in order to inhibit nucleotide pyrophosphatase activities [32,33] present in the brain extracts (results not shown) that hydrolytically cleave the pyrophosphate bond of NAD$^+$ and the formed ADPR. A chromatogram of standards (prepared as described in the Experimental section) is presented in Figure 2(A): under our conditions, cADPR was eluted at 5.2 min, ADPR at 6.5 min, AMP at 11 min and NAD$^+$ at 20 min (peaks were identified unambiguously as described in the Experimental section).

Figures 2(B)–2(D) show representative ^{14}C HPLC profiles obtained after transformation of NAD$^+$ in the presence of a total protein extract isolated from brains of WT mice at E15 (0.2 mg/ml; 30 min reaction) and from brains of both WT and *Cd38*$^{-/-}$ adult mice (0.2 mg/ml; 15 min reaction). The formation of cADPR (peak at 5.2 min) by WT or *Cd38*$^{-/-}$ brain extracts could not be detected by this HPLC method; because of the sensitivity of the analytical method used, this indicates that the production of cADPR (if any) must represent less than 0.5 % of the total reaction products. In contrast, peaks corresponding to ADPR (at 6.5 min) were observed in reactions performed with extracts isolated from WT brain. Approx. 12 % of the substrate, NAD$^+$, was converted into ADPR in the reactions containing the brain extract from WT E15 mice (Figure 2B), and 76 % of the substrate was converted into ADPR in the reaction containing

the adult WT brain extracts (Figure 2C). Kinetic analysis of the enzyme reaction with the adult WT brain extract (inset of Figure 2C) gave an initial reaction rate of 25 nmol of ADPR formed/min per mg of protein. Reactions with extracts prepared from E15 (results not shown) and adult (Figure 2D) *Cd38*$^{-/-}$ brains did not show any ADPR production under the same experimental conditions, indicating that CD38 is the primary NAD^+-glycohydrolase expressed in the brain. The small peak observed at 6.5 min (2 % of total radioactivity) in Figure 2(D) corresponds to contaminating ADPR present in the sample of [^{14}C]NAD^+ used. A very small peak (1 % of total radioactivity) was also observed in all samples (Figures 2B–2D). This peak co-eluted with AMP, and is probably the result of a residual nucleotide pyrophosphatase activity that was not completely inhibited by EDTA.

Together, these results demonstrate that CD38 is the major constitutively active NAD^+-glycohydrolase present in developing and adult mouse brain. The higher activity in the adult brain when compared with embryonic tissue is most probably due to the significantly higher level of expression of the protein (see Figure 1).

CD38-dependent GDP-ribosyl cyclase activity in extracts from adult and developing brain

Since we could not measure cADPR production in brain tissue by the HPLC method, we next used NGD^+ as a surrogate substrate of ADP-ribosyl cyclase [30] and specifically measured GDP-ribosyl cyclase activity in the protein extracts. This method is more sensitive, as NGD^+ is converted, in excellent yield, by all known ADP-ribosyl cyclases (including CD38) into a cyclic derivative, cGDPR, which is fluorescent and resistant to hydrolysis. Thus time-dependent formation of the fluorescent product cGDPR can be measured and quantified.

We have compared the formation of cGDPR catalysed by total protein extracts (200 μg/ml) obtained from the brains of embryonic (E15), neonatal (PN1) and adult WT and *Cd38*$^{-/-}$ mice in the presence of 20 μM NGD^+ at 32 °C and pH 7.0. In extracts of brains from WT mice, GDP-ribosyl cyclase activity was observed as early as E15 (Figures 3A and 3B). Analysis of the reactions gave initial rates of 0.6, 0.6 and 9.3 nmol of cGDPR formed/min per mg for brain extracts from E15, PN1 and adult mice respectively. Under these experimental conditions, no cyclase activity could be detected in extracts of brains isolated from developing or adult *Cd38*$^{-/-}$ mice (Figures 3A and 3B). The same reactions were then carried out using higher concentrations of NGD^+ (100 μM and 1 mM), and again no GDP-ribosyl cyclase activity was detected in brain extracts from *Cd38*$^{-/-}$ mice, even though this method allows detection of cyclase activity that is only 0.2–0.5 % of the adult WT activity. As expected, under the same experimental conditions, *Aplysia* ADP-ribosyl cyclase converted 100 % (40 nmol) of NGD^+ into cGDPR, whereas brain extracts from both developing and adult WT mice transformed only 70–85 % of NGD^+ into the cyclic compound (the remaining reaction product was GDPR, the hydrolytic product).

Together, these data demonstrate that NAD^+-glycohydrolase and ADP-ribosyl cyclase activities are present in mouse embryonic brain tissue. Furthermore, they show that these activities are dependent on CD38 expression in both developing and adult brain.

Distribution of CD38-dependent cyclase activity analysed by differential centrifugation

Since CD38 is expressed and enzymically active in the brain, it was important to determine whether it is present only as a membrane-bound form or whether it also has a soluble form. Therefore crude protein extracts of brains from adult WT and *Cd38*$^{-/-}$ mice were separated into three fractions: a soluble protein extract and two extracts of membrane proteins obtained by low-speed (P10) and high-speed (P540) centrifugation (see the Experimental section). These extracts were then analysed for their GDP-ribosyl cyclase activity under the same conditions as described above.

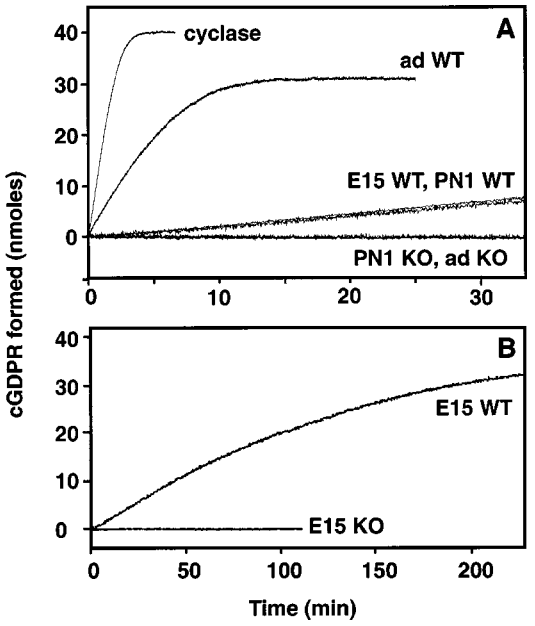

Figure 3 Analysis of ADP-ribosyl cyclase activity in brains of WT and *Cd38*$^{-/-}$ mice using NGD^+

Reactions were achieved by adding 20 μM NGD^+ to 0.4 μg/ml *Aplysia* ADP-ribosyl cyclase (positive control) or to crude protein extracts (200 μg/ml) incubated at 32 °C in 20 mM Tris/HCl at pH 7.0. Formation of cGDPR was detected by measuring fluorescence at 420 nm upon excitation at 300 nm. Estimation of the amount of cGDPR produced in the various extracts was determined by fluorescence spectrum measurements of the samples after eliminating protein at the end of the reaction and comparison with spectra of calibrated cGDPR standards (see Experimental). (**A**) Kinetics of cGDPR formation obtained with *Aplysia* cyclase and with crude protein extracts isolated from the brains of embryonic (E15), postnatal (PN1) and adult (ad) WT and *Cd38*$^{-/-}$ (KO) mice. (**B**) Kinetics of cGDPR formation obtained with crude protein extracts from E15 WT brain.

As expected, no activity could be detected in the three extracts isolated from the brains of *Cd38*$^{-/-}$ mice (results not shown). In contrast, extracts from WT mice possessed CD38-dependent cyclase activity in both the P10 and P540 membrane fractions (Figure 4A), but no activity was detected in the soluble fraction (Figure 4A). Analysis of reactions with the P10 and P540 membrane fractions gave initial rates of 17 and 3.7 nmol of cGDPR formed/min per mg of protein respectively. Similar to what we observed when analysing the total extract (Figure 3), both the P10 and P540 membrane fractions transformed only approx. 85 % of the NGD^+ into the cyclic compound.

Since ADP-ribosyl cyclase activity appeared to be located only within membranes, and was enriched in the P10 fraction, we next determined which subcellular membranes were present in the P10 and P540 fractions. The presence of proteins that are expressed specifically in mitochondrial, nuclear, plasma and endoplasmic reticulum membranes were analysed by Western blotting of these brain fractions (Figure 4B). Oligomycin sensitivity conferring protein was used to identify mitochondrial membranes, lamin B1 to identify nuclear membranes, Na^+/K^+-ATPase to identify

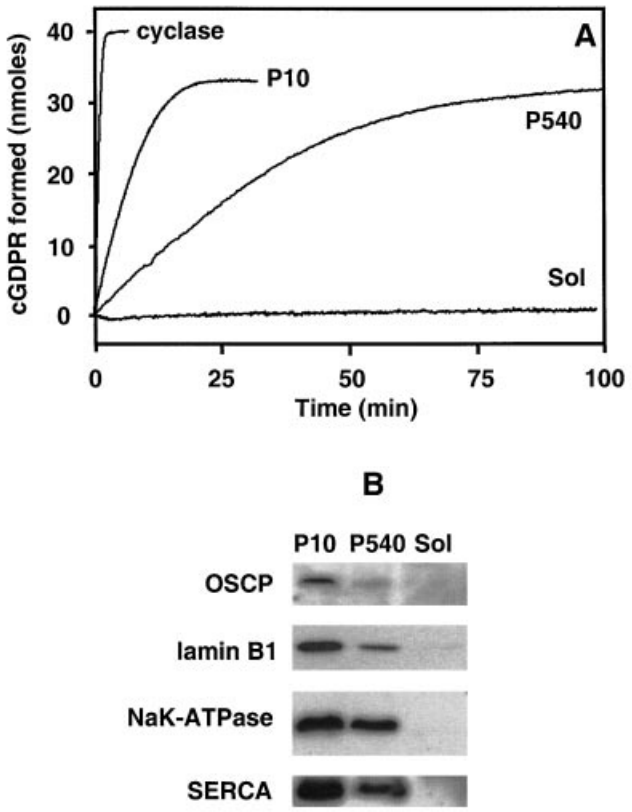

Figure 4 Distribution of CD38 cyclase activity in soluble and membrane extracts

(**A**) Comparison of the GDP^+-ribosyl cyclase activities of three protein extracts isolated from the brains of adult WT mice: a soluble (Sol) fraction and two membrane fractions obtained by low-speed (P10) and high-speed (P540) centrifugation (see the Experimental section). The reaction was performed by adding 20 μM NGD^+ to 0.4 μg/ml *Aplysia* cyclase (control) or to brain protein extracts (200 μg/ml) incubated at 32 °C in 20 mM Tris/HCl at pH 7.0. Formation of cGDPR was detected by fluorescence at 420 nm upon excitation at 300 nm. (**B**) Western-blot analysis to detect the distribution of mitochondrial, nuclear, endoplasmic reticulum and plasma membranes in the P10, P540 and soluble (Sol) extracts. Antibodies developed against oligomycin sensitivity conferring protein (OSCP), lamin B1, Na^+/K^+-ATPase and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) were used to identify proteins specific for mitochondrial, nuclear, plasma and endoplasmic reticulum membranes respectively.

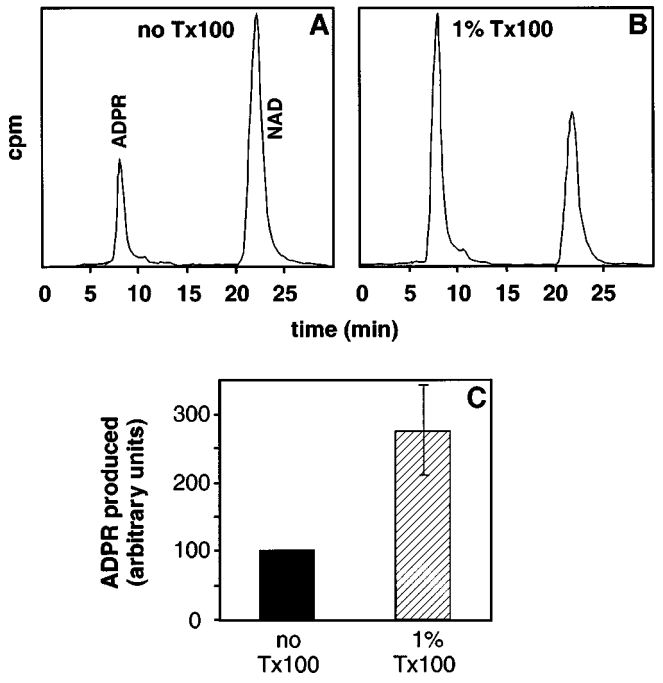

Figure 5 Comparison of NAD^+-metabolizing activities in intact (A) and permeabilized (B) hippocampal cells

Suspensions of 13–15-day-old cultures of cells obtained from the hippocampus of neonatal WT mice were separated into two pools. One pool was maintained in SH medium and the other was permeabilized in the presence of 4 mM EDTA and 1% Triton X-100 (Tx100). Intact and permeabilized cells (60000 per assay) were then incubated for 2 h at 37 °C with 200 μM [^{14}C]NAD^+ (4 μCi/mmol). Reactions were stopped by addition of perchloric acid. Analysis of the reaction products was performed by HPLC using an on-line radioactivity detector, as described in the Experimental section. (**A**) Products obtained with intact cells. (**B**) Products obtained with permeabilized cells from the same culture as in (**A**). (**C**) Bars indicate means $\pm$ S.E.M. ($n = 3$ primary cultures obtained from different animals), given in arbitrary units (100 units is the activity of the intact-cell control).

plasma membranes, and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase to identify endoplasmic reticulum. Analysis of the P10 fraction revealed that this fraction is enriched in mitochondrial, nuclear and endoplasmic reticulum membranes compared with the P540 fraction. Plasma membranes are relatively more abundant in the P540 fraction. CD38-dependent ADP-ribosyl cyclase activity is at least 4-fold greater in the P10 fraction. This suggests that CD38 is probably not localized exclusively on the plasma membrane in mouse brain tissue, but might also be present on intracellular membranes.

NAD^+-metabolizing activities in intact and permeabilized neural cells

In order to provide additional data supporting the possible intracellular location of the CD38 protein, we measured NAD^+-metabolizing activities in intact and permeabilized hippocampal cells isolated from the brains of neonatal WT mice. The hippocampus, isolated from the rest of the brain, was shown by Western blot analysis to contain CD38 protein (results not shown). Primary cell cultures (13–15 days old), composed of both neuronal and glial cells, were used. After resuspending the cells (6×10^5 per assay), they were tested for their ability to transform NAD^+ into cADPR and ADPR on incubation with [^{14}C]NAD^+ (200 μM) for 2 h at 37 °C and pH 7.0. To improve the analysis of activities, 4 mM EDTA was added to the incubation medium for permeabilized cells for which nucleotide pyrophosphatase activity was observed (results not shown).

Figure 5 shows representative ^{14}C HPLC profiles obtained after reacting the labelled substrate with intact (Figure 5A) and Triton X-100-treated (Figure 5B) suspended cells from the same preparation. As observed with the brain extracts, cADPR formation could not be detected in either intact or permeabilized cells. Again this result indicates that cADPR represented less than 0.5% of the reaction products. In contrast, ADPR peaks were observed with both intact and Triton X-100-treated cells. The experiment was repeated with three different cultures, giving the mean results presented in Figure 5(C). Under our experimental conditions, in the reaction containing intact cells, between 10% and 20% of the substrate NAD^+ was converted into ADPR (approx. 0.5 nmol of ADPR formed/min per mg of protein). Under the same experimental conditions, the presence of Triton X-100 led to an average 2.7-fold increase in the production of ADPR. The large increase in activity in the Triton X-100-

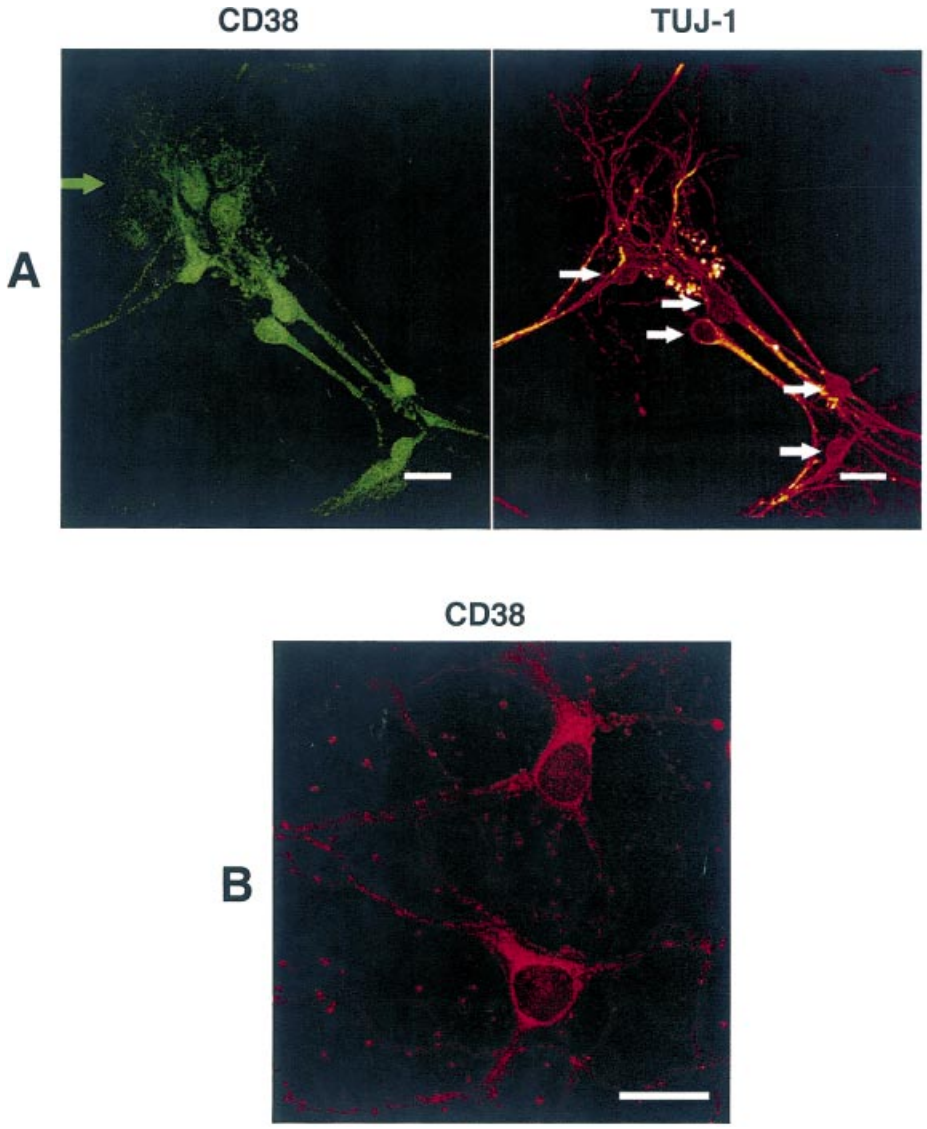

Figure 6 Localization of CD38 within mouse hippocampal cells

Hippocampal cells isolated from neonatal WT mice were cultured for 8–12 days, fixed using ethanol and then subjected to staining (see the Experimental section for details). (**A**) Left: anti-CD38 and anti-(rat IgG)–AlexaFluor 488; right: anti-(neuronal tubulin) (TUJ-1) and anti-(mouse Fc)–cyanin5. The green arrow indicates the location of glial cells. The white arrows indicate the neuronal bodies. (**B**) Biotinylated anti-CD38 and cyanin5-conjugated streptavidin. Scale bar = 20 μm.

permeabilized cells is probably due to intracellular activity, which is likely to be CD38-dependent, since no such NAD^+-glycohydrolase activity could be detected in the extracts of *Cd38*$^{-/-}$ mouse brain (Figure 2).

Together with the above result showing higher cyclase activity in brain extracts that were enriched in intracellular membranes, these data strongly suggest that active CD38 proteins are present intracellularly in neural cells.

Distribution of CD38 protein in hippocampal cells analysed by confocal microscopy

Since our activity measurements led us to consider the presence of intracellular CD38 in neural cells, we next used immunofluorescence microscopy and confocal imaging to examine the location of CD38 in neural cells. Experiments were performed on hippocampal cells isolated from neonatal WT mice and cultured for 8–12 days. Figure 6(A) shows a representative image in which neural cells were exposed to the TUJ-1 antibody (which stains the neurons) and to an anti-CD38 antibody. Figure 6(B) shows a simple staining obtained with the biotinylated anti-CD38 antibody at higher magnification.

Both TUJ-1-positive and -negative hippocampus cells were stained by the anti-CD38 antibody. In neuronal cells, the main location of the CD38 protein was in the cell body (Figure 6A); however, CD38 was also expressed on the processes. In glial cells (TUJ-1-negative; upper left of Figure 6A), the CD38 staining was more diffuse. Part of the CD38 staining observed certainly corresponds to staining of the ectocellular enzyme. However, a clear intracellular perikaryotic location of the protein was also observed in intensely stained neural cells in experiments

performed with the biotinylated anti-CD38 antibody (Figure 6B).

Our results with hippocampus cells are in agreement with those obtained by Yamada et al. [3], who demonstrated intracellular CD38 expression in rat cerebral and cerebellar cortices. Using immunoelectron microscopy, they showed that CD38 is expressed within the cells and is localized in several intracellular membranes, including mitochondrial membranes.

Together with the earlier results, these microscopy results suggest that CD38, in addition to being ectocellular, may also be localized to intracellular membranes in neural cells.

DISCUSSION

In the present work, we have studied CD38-related ADP-ribosyl cyclase activity in developing and adult brain by comparing the activity in brain tissue isolated from WT and *Cd38*$^{-/-}$ mice. ADP-ribosyl cyclase activity was observed in a crude protein extract isolated from WT mouse embryonic brain as early as stage E15 by the use of the fluorescent analogue of NAD^+, NGD^+. In the extract from *Cd38*$^{-/-}$ mouse brain at the same embryonic stage, no GDP-ribosyl cyclase activity was detected, demonstrating that the activity measured in embryonic brain tissue is due to the CD38 enzyme. The result obtained with the WT embryonic brain tissue is consistent with the presence of CD38 mRNA transcripts detected by RT-PCR analysis of embryonic brain mRNA. However, despite detectable levels of CD38-dependent activity and CD38 transcripts in embryonic brain, CD38 protein could not be detected by Western blot using an antibody developed against its C-terminus. This is probably due to the fact that CD38 is expressed at low levels in embryonic brain and cannot be detected by less sensitive methods such as Western blotting. This could also explain why CD38 protein was not observed previously in human embryonic tissues [1]. The level of CD38-dependent ADP-ribosyl cyclase activity was almost identical in brain extracts prepared from PN1 neonates and E15 embryos. It then increased in adult brain tissue. Again, these results correlate with the presence of low, but equivalent, amounts of CD38 mRNA transcripts in brain tissue from mice at E15 and PN1, which were greatly increased in adult brain.

Moreover, the GDP-ribosyl cyclase activity in adult WT mouse brain was shown to be associated exclusively with membranes. This result is somewhat at odds with the results obtained by Matsumara et al. [34] with bovine brain suggesting the existence of a soluble enzyme in that tissue. Some differences between the protocols used to prepare the brain extracts may, however, be of importance. In particular, in the present study, a more powerful cocktail of protease inhibitors was employed during the initial steps of brain cell fractionation, ruling out the possibility that a hydrosoluble domain of CD38 might be released from the membranes by endogenous proteases. However, since the purified bovine soluble protein was not identified by Matsumara et al. [34], we cannot exclude the possibility of a difference between the two species and of the occurrence, in some bovine tissues, of an enzyme other than the membrane-bound CD38 that was purified from spleen and sequenced by Augustin et al. [8].

Under the same experimental conditions as those used to analyse brain extracts from WT mice, no ADP-ribosyl cyclase activity was observed in *Cd38*$^{-/-}$ mouse brain, either in developing or in adult brain tissue. These results are consistent with the observation that the other known mammalian ADP-ribosyl cyclase, BST-1, has not been shown to be expressed in adult brain by either Northern or Western blotting [35,36]. Our results are also consistent with the loss of brain-associated NAD^+-glycohydrolase activity in the brains of *Cd38*$^{-/-}$ adult mice [26], if we assume that both NAD^+-glycohydrolase and ADP-ribosyl cyclase activities are due to the same enzyme. However, our conclusion that ADP-ribosyl cyclase activity in the brain is entirely dependent on CD38 has to be reconciled with the observation that the cADPR content in homogenates prepared from *Cd38*$^{-/-}$ mouse brain tissue is only marginally reduced when compared with that in WT brain homogenate [16]. Thus, based on those results, we expected to observe ADP-ribosyl cyclase activity in the *Cd38*$^{-/-}$ mouse brain. Two explanations, at least, may reconcile these disparate results. First, it is possible that CD38 is the sole ADP-ribosyl cyclase present in brain tissue and that the cADPR found in *Cd38*$^{-/-}$ mouse brain is due to uptake of cADPR produced by another cyclase (such as BST-1) at a distant site. This explanation seems unlikely, as the cADPR would have to be very stable, to diffuse into the central nervous system, and then to be taken up by neural or epithelial cells. Alternatively, another non-CD38 and inducible ADP-ribosyl cyclase may be present in brain. This putative enzyme would be predicted to have a very low level of basal activity, and may be induced to produce cADPR *in vivo* (by unknown cellular agents or regulatory proteins), but not easily *in vitro*. This mechanism of inducible enzyme activity is used by signalling enzymes, such as adenylate cyclase, for their second-messenger production. With the analytical tools used in the present work *in vitro*, the cyclase activity of the non-CD38 enzyme would not be detectable; however, cADPR produced *in vivo* by this unidentified cyclase could still be measured, as in [16]. Moreover, NGD^+ used as a substrate in the sensitive fluorescent cyclase assay might be a poor substrate or even fail to be a substrate for the putative enzyme. A 'low basal activity' cyclase is reminiscent of the putative 'intracellular signalling ADP-ribosyl cyclase' postulated by several authors to exist in mammalian cells [5,23]. In agreement with this possibility, Guse et al. [23] identified an intracellular cyclase in T cells that seems unable to use NGD as a substrate. The next step to address this hypothesis will be to verify the possible occurrence of very low and inducible ADP-ribosyl cyclase activity in brain extracts from *Cd38*$^{-/-}$ mouse.

Finally, we demonstrated by confocal immunofluorescence microscopy on primary cultures of mice hippocampus cells that CD38 is expressed by both neurons and glial cells, and seems not to be localized exclusively on the plasma membrane of these cells. The protein may also be localized to intracellular membranes. Our results are consistent with the detection of the CD38 protein by immunohistochemistry on intracellular membranes in neural cells of rat and human [2,3]. Our activity measurements on both brain extracts and hippocampal cells cultures strongly suggest that the intracellular enzymes are active.

In summary, we have demonstrated that CD38 is expressed and is constitutively enzymically active in brain tissue from both developing and adult mice. CD38 expression increases during ontogeny and reaches its highest levels in the adult brain. CD38 may be distributed on multiple membranes of brain cells. Given the subcellular localization of CD38 in the brain and the known function of cADPR to regulate calcium signalling in neural cells [17–19], it is tempting to speculate that CD38 may regulate intracellular calcium signalling in neural cells via its production of cADPR.

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