Dimeric and tetrameric forms of catalytically active transmembrane CD38 in transfected HeLa cells

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Abstract CD38, a type II transmembrane glycoprotein, behaves as a catalytically active transporter responsible for ectocellular generation of cyclic ADP-ribose (cADPR) from NAD^+ and for subsequent influx of cADPR across membranes [Franco, L., Guida, L., Bruzzone, S., Zocchi, E., Usai, C. and De Flora, A. (1998) FASEB J. in press]. cADPR regulates intracellular calcium homeostasis by releasing calcium from responsive stores. The cADPR-transporting function of CD38 requires channel-generating oligomeric forms of the protein rather than the 46 kDa monomers that have been described so far in $CD38^+$ cells. Here we demonstrate that CD38, both in reconstituted proteoliposomes and in CD38-transfected HeLa cells, is a mixture of catalytically active monomers, homodimers and homotetramers. A soluble recombinant form of CD38 corresponding to its ectocellular region proved to be monomeric. Thus, association of native CD38 with either artificial or natural membranes seems to result in a reversible juxtaposition of monomers suitable to cADPR-transporting activity.

Key words: CD38; Cyclic ADP-ribose; CD38 oligomer; Ectoenzyme; NAD^+

1. Introduction

CD38, a 46 kDa type II transmembrane glycoprotein widely expressed in mammalian cells, is a bifunctional ectoenzyme. Its two activities are ADP-ribosyl cyclase, responsible for the generation of nicotinamide and cyclic ADP-ribose (cADPR) from NAD^+, and cADPR-hydrolase, converting cADPR to ADP-ribose (ADPR) [1–4]. cADPR is a powerful calcium mobilizer from intracellular stores, active in plants, invertebrate and mammalian cells [5], where it is implicated as a second messenger for many agonists including NO, cholecystokinin and acetylcholine.

While CD38 is a membrane-bound ectoenzyme, the invertebrate ADP-ribosyl cyclase is a cytoplasmic, soluble enzyme [6]. The ectocellular localization of CD38 in vertebrates raises two topological problems [7]: (1) availability of NAD^+ to the catalytic domain of CD38, and (2) accessibility of ectocellularly produced cADPR to its intracellular receptors. Two recent findings from our laboratory seem to provide potential solutions to both problems. Presence of NAD^+ in plasma and interstitial fluids has been detected at nanomolar concentrations [8], thus enabling, in principle, the ectocellular production of cADPR by CD38. In addition, we demonstrated that CD38 is a catalytically active transporter of its product cADPR: CD38-reconstituted proteoliposomes and $CD38^+$ resealed erythrocyte membranes are active in concentrating NAD^+-derived cADPR, but not ADPR, inside the vesicle compartment [9].

The aim of this study was to provide a structural basis to the novel cADPR-transporting function of CD38 in intact cells [9]. This activity requires a channel-forming structure, rather then the monomeric form of CD38 described until now. Here we demonstrate the presence of dimeric and tetrameric forms of CD38, as enzymatically active bands on mildly denaturing SDS-PAGE gels, in CD38-transfected HeLa cells ($CD38^+$ HeLa cells) [10] and in CD38-reconstituted proteoliposomes as well. CD38 dimers and tetramers were also detected as radiolabeled bands on SDS-PAGE gels from lysates of [^{35}S]methionine/cysteine-labeled $CD38^+$ HeLa cells.

2. Materials and methods

2.1. Materials

BS^3 was purchased from Pierce, Rockford, IL. ADP-ribosyl cyclase from *Aplysia californica* and recombinant human CD38 [11] were kindly provided by Prof. H.C. Lee, Minneapolis, MN. This rCD38 is a soluble species corresponding to the ectocellular, C-terminal region of native CD38 and is completely deglycosylated [11]. All chemicals were of the highest purity grade available from Sigma (Milan, Italy). CD38 sense- and antisense-transfected HeLa cells were obtained and cultured as described in [10]. Anti-CD38 mAb IB4 [12] was a generous gift of Prof. F. Malavasi, Ancona, Italy. [^{35}S]Met/Cys (specific activity 1175 Ci/mmol) was purchased from ICN Pharmaceuticals, Inc., Irvine, CA.

2.2. Purification of $CD38^+$-transfected HeLa cells

$CD38^+$-transfected HeLa cells (90×10^6) [10] were extensively washed in PBS and lysed in 4 ml of 300 mM sucrose containing 10 mM Tris-HCl, pH 6.5, 2 mM PMSF and 40 μg/ml each of leupeptin, aprotinin and trypsin inhibitor. Cells were sonicated in ice for 30 s (Heat System-Ultrasonics, W-380) and centrifuged for 10 min at $1000\times g$ at 4°C; the supernatant was again centrifuged for 15 min at $100\,000\times g$ at 4°C. The pellet was resuspended in 2 ml of lysis buffer and solubilized in 2% β-octylglucopyranoside (β-OG). Native CD38 was purified as described [3], except that β-OG replaced Triton X-100 throughout all purification steps (hydroxylapatite, immobilized-Cu^{2+} and immunoaffinity chromatography). Purified CD38 in resuspension buffer (5 mM Tris-HCl, pH 6.5, with 1% β-OG) displayed a

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Abbreviations: cADPR, cyclic ADP-ribose; NAD^+, nicotinamide adenine dinucleotide; ADPR, ADP-ribose; BS^3, bis(sulfosuccinimidyl)suberate; rCD38, recombinant soluble CD38; β-OG, β-octylglucopyranoside; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; NHD^+, nicotinamide hypoxanthine dinucleotide; ε-NAD^+, 1-N^6-etheno NAD^+; ε-ADPR, 1-N^6-etheno ADP-ribose; cIDPR, cyclic inosine diphosphoribose; IDPR, inosine diphosphoribose; M_r, molecular weight; mAb, monoclonal antibody; GSH, glutathione (reduced form)

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specific NADase activity of 120 nmol ADPR/min/ml, assayed as in [10]. Protein content was determined according to Bradford [13].

2.3. Chemical cross-linking with BS^3

The cross-linking activity of BS^3 was preliminarily tested in our experimental conditions (PBS, pH 7.4, at 25°C) on rabbit muscle aldolase [14]. Both purified *Aplysia* cyclase (0.3 μg) and recombinant human CD38 (5 μg) were incubated in the presence or absence of 1–5 mM BS^3 in 50 μl of PBS for 2 h at 25°C. Intact CD38 sense- and antisense-transfected HeLa cells (the latter cells were treated to provide a $CD38^-$ control for possible unrelated BS^3 effects) [10] were resuspended in PBS containing 10 mM glucose at 10^6 cells/ml. Aliquots of 0.5 ml were incubated with 1 or 5 mM BS^3 (dissolved in the PBS-glucose solution) under gentle stirring at 25°C for 2.5 h. In order to quench excess BS^3, 0.15 vol of 50 mM Tris-HCl, pH 7.5, were added to reaction mixtures and incubated at 25°C for 15 additional min. BS^3 solutions were prepared immediately prior to use.

2.4. Cell radiolabeling

CD38 sense- and antisense-transfected HeLa cells were incubated in cysteine/methionine-free RPMI 1640 medium, supplemented with 2 mM glutamine for 30 min and pulsed with [^{35}S]Met/Cys (30 μCi/ml) for 15 h. The metabolically labeled cells (2×10^7) were washed with PBS, lysed in 0.5 ml of a buffer containing 10 mM Tris-HCl, pH 6.5, 0.3 M sucrose, 2 mM PMSF and 2% Triton X-100, and sonicated for 30 s in ice. Native [^{35}S]Met/Cys-labeled CD38 was purified from cell lysates as described in [3] and analyzed by SDS-PAGE as described in Section 2.5. The gels were fixed in isopropyl alcohol:acetic acid:water (25:10:65), dried and radiolabeled protein bands were visualized by phosphorimager (Packard, Canberra, Australia).

2.5. SDS-PAGE and identification of enzymatic activities in gels

All samples subjected to SDS-PAGE analysis were diluted in a modified Laemmli sample buffer [15] lacking β-mercaptoethanol and EDTA and containing 1.5% SDS (instead of 8%). Samples were heated at 50°C for 3 min and electrophoresed on a refrigerated 7–10% acrylamide gradient SDS-PAGE gel. M_r standards were prepared as the samples and run in parallel.

After SDS-PAGE of both *Aplysia* ADP-ribosyl cyclase and recombinant human CD38, gels were washed for 60 min in 500 ml of 20 mM Tris-HCl, pH 7.5. To identify *Aplysia* cyclase, the gels were incubated for 20 min at 37°C in 20 mM Tris-HCl, pH 7.5, containing 0.2 mM NHD^+ [16]. To visualize the NADase activity of recombinant human CD38, the gels were incubated for 20 min at 37°C in a buffer containing 20 mM Tris-HCl, pH 7.5, and 0.2 mM ε-NAD^+ [17]. After incubation, gels were placed on an UV-transilluminator to detect formation of cIDPR and of ε-ADPR, respectively. Photographs were taken using a 550 nm interference filter.

After SDS-PAGE of cell lysates (50 μg protein), each longitudinal lane was cut into transversal slices of 1 cm. Each gel slice was put into a separate dialysis tubing containing 0.75 ml of 10 mM Tris-HCl, pH 6.5, and 0.1% Triton X-100. Slices were dialyzed against 4 l of the same buffer for 16 h at 4°C. Thereafter, the content of each tubing was incubated with 0.04 mM ε-NAD^- at 37°C for 4 h. The production of ε-ADPR was detected using a LS50B Fluorimeter (Perkin Elmer) set at 300 nm excitation and 410 nm emission. Recovery of NADase activity was about 7% of the loaded activity. Control samples were obtained from gel slices cut from a lane loaded with antisense-transfected HeLa cells [10].

2.6. Reconstitution of purified CD38 in proteoliposomes

Preparation and characterization of the CD38-reconstituted unilamellar proteoliposomes are reported in [9]. A typical proteoliposome preparation displayed an extravesicular NADase activity of 3.3 ± 0.3 nmol ADPR/min/ml and a lipid concentration of 2.5 ± 0.1 mg/ml. CD38 proteoliposomes (2 ml) were incubated in the presence or absence of 2.5 mM BS^3 at 25°C under gentle stirring. After 2 h incubation, 350 μl of 50 mM Tris-HCl, pH 7.5, were added to quench excess BS^3. Samples were then centrifuged for 15 min at $100\,000\times g$ and pellets were resuspended in 300 μl of 0.15 M NaCl. Samples were solubilized with 0.5% Triton X-100, diluted in modified Laemmli buffer and analyzed by SDS-PAGE (see Section 2.5). The gel lanes were cut and NADase activity on the gel slices was analyzed as described in Section 2.5.

3. Results

Usually, structural studies on transmembrane proteins require detergent solubilization; however, this treatment can result in loss of native oligomeric structures. Thus, BS^3, a homobifunctional reagent widely used to cross-link protein subunits [14], was used in order to stabilize transmembrane CD38 molecules before detergent solubilization. Moreover, SDS-PAGE samples were prepared according to a more conservative procedure: SDS concentration in the sample buffer was reduced from 8% to 1.5%, β-mercaptoethanol and EDTA were omitted and samples were heated at 50°C only (see Section 2.5).

These experimental conditions were first checked on the monofunctional ADP-ribosyl cyclase from *Aplysia californica*, which has been recently demonstrated by X-ray crystallography to be a homodimer [6]. Cyclase activity was visualized directly on the gel, following incubation with NHD^+, by detecting the fluorescent product cIDPR on a transilluminator [16]. As shown in Fig. 1A, the control sample displayed a single active band at 30 kDa (lane a), while in the BS^3-preincubated sample a 60 kDa band was visible without any monomer left (lane b). Thus, both the monomeric and the dimeric forms proved to retain cyclase activity. Accordingly, the homodimeric structure of native *Aplysia* cyclase [6] was confirmed with this experimental procedure and BS^3, strictly required to prevent its dissociation that occurs even in our mildly denaturing conditions (lane a), proved not to inactivate the cyclase activity.

The same method was followed with a recombinant, soluble form of human CD38 (rCD38), which lacks both the transmembrane and intracellular domains and also the glycosidic moieties, present in the native protein [11]. Since the most abundant product of the CD38 enzyme activities is ADPR and not the cyclic nucleotide (cADPR), NHD^+ was not suitable as substrate because cIDPR is formed in too low amounts to allow detection of ADP-ribosyl cyclase on the gels and the NADase product IDPR is not fluorescent [16]. Thus, ε-NAD^+ was used as substrate of NADase activity, instead of NHD^+, to detect fluorescent ε-ADPR directly on the gel following SDS-PAGE (Fig. 1B). A single 30 kDa band

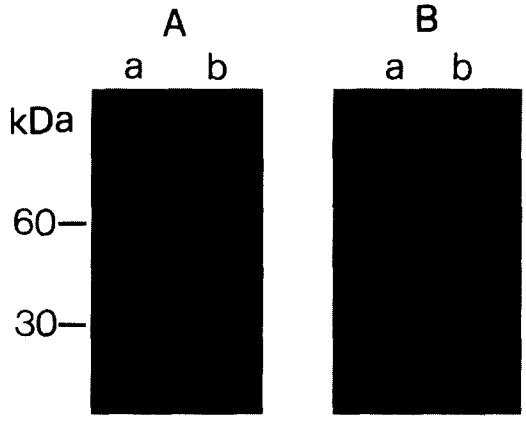

Fig. 1. Detection of catalytically active forms of *Aplysia* ADP-ribosyl cyclase and recombinant human CD38. *Aplysia* cyclase (A) and recombinant human CD38 (B) were incubated without (lane a) and with (lane b) BS^3, as described in Section 2.3. The samples were subjectted to SDS-PAGE and enzymatic activities were identified under UV light, as described in Section 2.5.

was observed with (b) or without (a) prior incubation of rCD38 with BS^3. Therefore, unlike the *Aplysia* cyclase, human rCD38 is a monomer and BS^3 does not induce any formation of oligomeric forms per se.

In order to investigate the putative oligomeric structure of transmembrane, native CD38 in situ, similar cross-linking experiments with BS^3 were performed on CD38 sense-transfected ($CD38^+$) HeLa cells [10]. However, the low activity levels present in these samples prompted us to use a more sensitive procedure in order to monitor production of ε-ADPR from ε-NAD^+, as described in Section 2.5. As shown in Fig. 2A, the NADase activity profile of the gel slices obtained after SDS-PAGE of solubilized lysates from $CD38^+$ HeLa cells showed three peaks corresponding to 46, 90 and 190 kDa, respectively. In the samples from cells incubated without BS^3 the two latter forms, apparently corresponding to dimers and tetramers, were quantitatively less represented than the monomers. Conversely, pre-treatment of HeLa cells with BS^3 resulted in a remarkable increase of dimers and especially of tetramers at the expense of monomers (Fig. 2A).

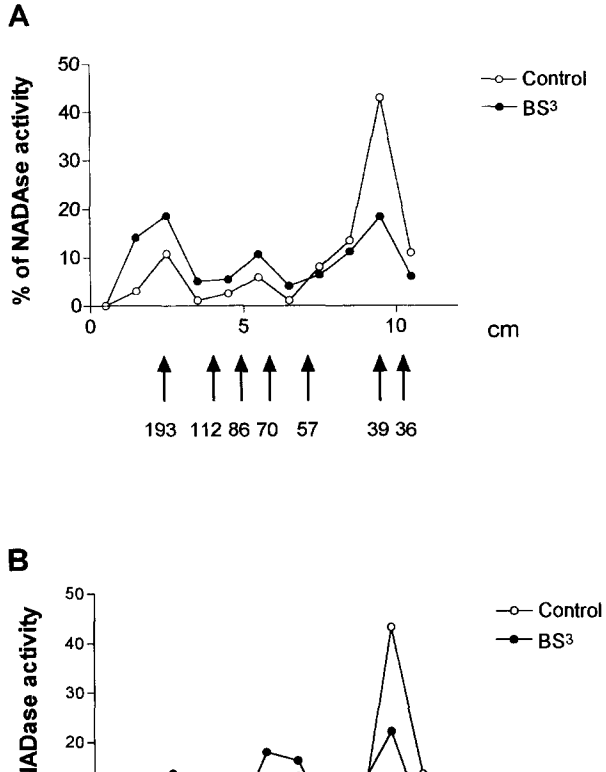

Fig. 2. Active oligomeric forms of CD38 in $CD38^+$ HeLa cells and in CD38-reconstituted liposomes. $CD38^+$ HeLa cell lysates (A) and CD38-reconstituted liposomes (B) were incubated either in the presence (●) or in the absence (○) of BS^3 and run on a 7–10% SDS-PAGE as described in Sections 2.3 and 2.5. Each gel lane was then cut into slices and NADase activity of each sample was determined by recording ε-ADPR fluorescence (Section 2.5). The abscissa indicates the distance in centimeters and arrows indicate the M_r markers. The ordinate indicates the percentage of the total NADase activity recovered from all samples. Data shown are the mean ± S.D. of five experiments.

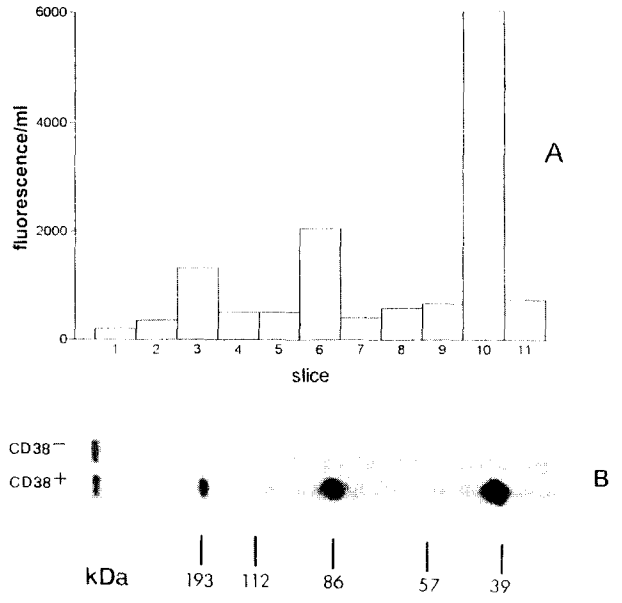

Fig. 3. NADase activity profile and autoradiogram of metabolically labeled CD38. $CD38^{+/-}$ HeLa cells were cultured with [^{35}S]Met/Cys and the procedure for the purification of CD38 (see Section 2.2) was applied to both cell lysates. The eluates from the immunoaffinity chromatography step were subjected to SDS-PAGE under mildly denaturing conditions (see Section 2.5). Lanes loaded with the radiolabeled samples, in duplicate, were either cut into slices and the NADase activity was analyzed fluorometrically with ε-NAD^+ as substrate (A), or visualized by a phosphorimager (B). Samples from $CD38^-$ cells did not reveal any NADase activity (not shown) nor any labeled bands (B).

In an attempt to demonstrate that these high M_r bands correspond to CD38 homooligomers and not to CD38 complexes with other membrane proteins, similar experiments were carried out on proteoliposomes reconstituted with human CD38 purified to homogeneity [3] from either $CD38^+$ HeLa cells or erythrocyte membranes. The distribution of monomeric, dimeric and tetrameric forms of catalytically active CD38 in the proteoliposomes was similar to that observed on the $CD38^+$ HeLa cells (Fig. 2B). BS^3 proved to enhance the peak of dimers and, to a lower extent, of tetramers over that of monomers. The source of purified CD38 proved to be irrelevant. Thus, Fig. 2B shows results obtained on proteoliposomes prepared with CD38 purified from HeLa cells, but a closely comparable activity profile was observed with proteoliposomes reconstituted with CD38 purified from erythrocyte membranes.

Finally, in order to unequivocally correlate oligomeric forms of membrane-bound CD38 with NADase activity, $CD38^{+/-}$ HeLa cells were metabolically labeled with [^{35}S]Met/Cys and CD38 protein was purified to homogeneity as described earlier [3]. Radiolabeled samples were loaded in duplicate on SDS-PAGE: one lane was cut into slices and the NADase activity was determined, while the other lane was fixed, dried and exposed to phosphorimager for detection of radiolabeled protein bands. Fig. 3A shows three major activity peaks, as recorded by ε-ADPR fluorescence, in the sample purified from $CD38^+$ HeLa cells. Phosphorimager record of the same sample (Fig. 3B) shows three radiolabeled bands corresponding to the peaks of enzymatic activity at 46, 90,

190 kDa respectively. These M_r values are consistent for monomeric, dimeric and tetrameric CD38 structures. No NADase activity was detectable in samples purified from $CD38^-$ cells (not shown) nor could any labeled bands be immunopurified with the anti-CD38 mAb IB4 from these $CD38^-$ cells (Fig. 3B).

4. Discussion

The present findings provide the first evidence for a discrete and apparently reversible oligomeric structure of native, catalytically active, transmembrane CD38 in cells. The only available report on high molecular weight forms of CD38 concerns a transglutaminase-cross-linked 190 kDa species of CD38 that has been immunopurified from retinoic acid-stimulated HL60 cells [18]. The dimeric and tetrameric forms of CD38 demonstrated in this study are not covalently cross-linked and can dissociate following SDS-PAGE: this was clearly indicated by the partially stabilizing effect of BS^3 on both $CD38^+$ HeLa cells and on CD38-reconstituted proteoliposomes (Fig. 2). Moreover, the trend to form both dimers and tetramers appears to be an intrinsic property of the CD38 protein, as indicated by the demonstration of the same oligomeric forms, in the same relative abundance, in both $CD38^+$ HeLa membranes and in proteoliposomes reconstituted with CD38 purified to homogeneity (Fig. 2).

The dimeric structure of the monofunctional *Aplysia* cyclase, which has been recently solved by X-ray crystallography [6], was confirmed, thus validating the experimental setting used in this study (Fig. 1A). Quite unexpectedly, we consistently failed to observe any oligomeric forms of soluble, truncated, rCD38 (Fig. 1B). Therefore, a discrepancy exists between soluble CD38 and the native protein, that may reflect a critical role of the transmembrane domain of native CD38 in promoting or stabilizing monomer association. Such reversible juxtaposition of interacting monomers is expected to create a solvent-filled channel allowing the in situ generated cADPR to cross CD38 dimers and/or tetramers. This model provides the structural counterpart to the recently demonstrated cADPR-transporting activity of native CD38 [9]. Monomers per se, while competent to display catalytic activity, as demonstrated in this study (Figs. 2 and 3A), are not structurally suitable to mediate any vectorial transport of cADPR.

We could not detect the two oligomeric forms of CD38 by immunoblot analysis after SDS-PAGE under mildly denaturing conditions, although the procedure was modified to enhance transfer of high molecular weight proteins (presence of SDS in the blotting buffer, high voltage transfer and low acrylamide concentration in the gel, see [19]). These negative results could be due either to inefficient transfer of the oligomeric forms of CD38 to the nitrocellulose or to failure of the anti-CD38 mAbs (IB4, IB6, OKT10) to recognize the denatured, immobilized oligomers.

The relative proportion of the oligomeric and the monomeric forms of native CD38 in the cell membranes remains to be determined. The low final recovery of enzymatic activity after dialysis of the SDS-PAGE protein bands (approximately 7%) and the fact that monomers and oligomers might renature differently do not allow to extrapolate the activity profiles shown in Figs. 2 and 3A to the in vivo situation. Specifically, CD38 monomers might represent an artefact or at least be overestimated (with dimers and tetramers being underestimated accordingly), due to the unavoidable membrane solubilization and subsequent electrophoretic separation under dissociating conditions. Therefore, these results prove unequivocally that transmembrane CD38 in cells has a structure suitable to catalyze cADPR formation and also its selective and unidirectional transport, as recently demonstrated [9]. Reversibility of monomer association might suggest modulation of this functional activity, which can elicit increased $[Ca^{2+}]_i$ levels in $CD38^+$, but not in $CD38^-$ HeLa cells [9].

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