

NAD⁺-dependent internalization of the transmembrane glycoprotein CD38 in human Namalwa B cells

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Abstract CD38 is a transmembrane glycoprotein involved as an orphan receptor in many physiological processes of lymphocytes. It is also a bifunctional enzyme that catalyzes at its ectocellular domain the synthesis from NAD⁺ (cyclase) and the hydrolysis (hydrolase) of the calcium-mobilizing metabolite cyclic ADP-ribose (cADPR). A still unexplained paradox concerns the relationship between ectocellular localization of CD38 and intracellular calcium-releasing activity of its intermediate product cADPR. Incubation of CD38⁺ human Namalwa B cells with external NAD⁺ elicited extensive membrane down-regulation of CD38 and its internalization in non-clathrin-coated vesicles. Since the internalized CD38 was demonstrated to be enzymatically active, this NAD⁺-dependent process is a hitherto unrecognized means for shifting cADPR metabolism from the cell surface to the intracellular environment.

Key words: CD38; NAD⁺; GSH; ADP-ribosyl cyclase; Endocytosis; Ecto-enzyme

1. Introduction

CD38 is a type II transmembrane glycoprotein predominantly expressed in early and late stages of lymphocyte development [1,2]. Its involvement in many processes of lymphocyte proliferation and differentiation as a receptor for still unidentified ligand(s) is supported by a wide range of effects on lymphocyte physiology elicited by ligation of CD38 with specific monoclonal antibodies (MoAbs) [1,2].

CD38 is also a bifunctional ectoenzyme that catalyzes, at the outer surface of many cell types [3], the sequential synthesis and degradation of cyclic ADP-ribose (cADPR) [4–8]: the two enzyme activities involved are an ADP-ribosyl cyclase (responsible for the conversion of NAD⁺ to nicotinamide and cADPR) and a cADPR hydrolase that degrades cADPR to ADP-ribose (ADPR).

The potent calcium-releasing activity of cADPR on ryanodine-sensitive intracellular stores in several invertebrate and vertebrate cells [9–11] has raised expectations that this cyclic nucleotide may play a role in mediating CD38 receptor functions. An enhanced transmembrane calcium influx has been detected in murine B cells [12] following ligation with anti-CD38 antibodies, but there is no evidence that cADPR is involved.

A major, still unresolved question on the CD38-cADPR system concerns the apparent contradiction between the ectocellular site of CD38-catalyzed production of cADPR and its intracellular calcium-mobilizing activity [2,11,13,14]. Neither cADPR influx across the plasma membrane nor cADPR-initiated signal transduction in responsive cells has been demonstrated so far, although external cADPR has been reported to enhance the proliferation of activated murine B lymphocytes [4]. In addition, extracellular cADPR has been recently demonstrated to enhance the calcium response to depolarization in intact rat cerebellar granule cells [15].

An alternative mechanism for coupling the extracellular synthesis of cADPR with its intracellular functions could be the internalization of CD38. We have previously demonstrated that CD38, both purified from human erythrocyte ghosts and in situ in erythrocyte membranes, undergoes extensive self-aggregation in the presence of either thiol compounds or NAD⁺ [13,16,17]. Since ligand-induced receptor oligomerization is known to trigger subsequent internalization [18,19], we investigated the fate of membrane CD38 following exposure of intact CD38⁺ Namalwa cells (a continuous B-cell-derived line from Burkitt's lymphoma) to NAD⁺ or GSH. The data provided in this paper indicate that incubation of these cells with either reagent results in the cell surface depletion of CD38. Concomitantly, NAD⁺ proved to induce CD38 internalization in these cells. This process, coupling down-regulation of surface CD38 with increased intracellular cyclase activity, may provide a hitherto unrecognized mechanism underlying CD38 functions.

2. Materials and methods

2.1. Materials

Nitrocellulose membranes for ECL were purchased from Amersham, Milan, Italy. The IB4 MoAb [20] was a kind gift by Professor F. Malavasi. All chemicals were of the highest purity grade available from Sigma Chem Co., St. Louis, MO.

2.2. Cell culture

Namalwa cells (obtained from American Type Culture Collection (ATCC) Bethesda, MD) were grown in RPMI (Sigma) supplemented with 10% fetal calf serum (Sigma), penicillin (1 U/ml) and streptomycin (1 µg/ml) at 37°C in a humidified atmosphere with 5% CO₂. When incubated in the presence of NAD⁺, cells were seeded at 5 × 10⁵/ml and cultured for up to 18 h. Cell density never exceeded 1.5 × 10⁶/ml and cell viability was ≥90%. No apparent inhibition of cell growth was observed in the presence of NAD⁺.

2.3. Flow cytometry

This was performed as reported in the legend to Fig. 1, using a FACScan (Becton Dickinson, Milan, Italy). Five thousand events were analyzed per sample.

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Abbreviations: cADPR, cyclic ADP-ribose; ADPR, ADP-ribose; BSA, bovine serum albumin; NGD⁺, nicotinamide guanine dinucleotide; cGDPR, cyclic GDP-ribose; RIA, radioimmunoassay

2.4. Biotinylation of Namalwa cells and affinity purification of biotinylated CD38

Cells were washed in RPMI without serum and incubated at 4°C in PBS containing 5 mM glucose, 0.1 mM CaCl_2 , 1 mM MgCl_2 and 0.8 mM NHS-SS-biotin (Pierce, Milan, Italy) at a density of $5 \times 10^5/\text{ml}$ [21]. After 20 min cells were centrifuged and the incubation repeated with a new solution. Cells were then washed twice in RPMI containing 0.2% BSA (fatty acid free), resuspended in complete medium at a density of $5 \times 10^5/\text{ml}$ and cultured in the presence or absence (control) of 10 mM NAD^+ for 18 h. In a typical experiment, 1.5×10^7 cells were used for each incubation. Removal of surface biotin from control and NAD^+ -treated cells was obtained by incubating the cells at 37°C in RPMI with 50 mM GSH at a density of $10^6/\text{ml}$ for 5 min. This procedure was repeated twice. To remove unreacted GSH, cells were then washed twice in PBS containing 5 mg/ml iodoacetamide and once in RPMI. Finally, cells were solubilized at $1.5 \times 10^7/\text{ml}$ in lysis buffer (PBS containing 1% Triton X-100, 5 mM EDTA, 10 $\mu\text{g}/\text{ml}$ leupeptin, 50 $\mu\text{g}/\text{ml}$ aprotinin, 250 $\mu\text{g}/\text{ml}$ trypsin inhibitor) and centrifuged at $10000 \times g$ for 10 min. The clear supernatants were incubated with immobilized streptavidin (Pierce), at 300 μl packed resin/ml lysate, for 2 h at 4°C on a rotary shaker. The supernatants were recovered by centrifugation, the resin was washed in lysis buffer and the adsorbed (i.e. biotinylated) proteins were eluted with 0.1 M glycine, pH 2.0. The pH of the eluate was immediately neutralized with 1 M Tris-HCl, pH 8.3, and eluates and supernatants from control and NAD^+ -treated cells were dialyzed overnight at 4°C against 5 mM Tris-HCl, pH 6.5, containing 0.05% Triton X-100, and concentrated to 1/10 the original volume. Protein concentration was determined according to Bradford [22] with the appropriate detergent concentrations in the blank and standard reagents. Each sample was then analyzed by Western blot (following SDS-PAGE) and dot blot (see below).

2.5. Western blot and dot blot

SDS-PAGE was performed on 10% gels, according to [23], and proteins were blotted on nitrocellulose membranes as described in [13]. Saturation of the membranes and incubation with the first (IB4) and second Ab (anti-mouse IgG, Amersham, Milan, Italy) were performed following instructions of the Amersham ECL immunodetection kit.

2.6. Assays of cyclase and hydrolase activities

These were performed on intact cells as in [17], using 0.5 mM NGD^+ [24] and 0.5 mM cADPR, respectively, as substrates. Enzyme assays on solubilized cells (prepared either with 1% Triton X-100 or by freezing-thawing) were carried out as described in the legend to Table 1.

2.7. Immunofluorescence

Visible pellets of Namalwa cells, either untreated or treated for 2 or 6 h with 10 mM NAD^+ , were processed as described [25]. Briefly, cells were fixed in 3.7% paraformaldehyde in PBS, embedded at 4°C in ascending concentrations of sucrose in PBS, followed by OCT (Tissue-Tek, Miles Inc., Elkhart, IN) and frozen in liquid nitrogen. Samples were then sectioned in a cryostat, laid on gelatin-coated glass slides and air dried. The primary IB4 antibody was used at 5 $\mu\text{g}/\text{ml}$, the rabbit anti-clathrin antiserum (Sigma) was used at a 1:60 dilution. TRITC-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit IgG affinity purified antibodies (Jackson ImmunoResearch, West Grove, PA) were used as secondary reagents.

2.8. Cryoimmunoelectron microscopy

Visible pellets of Namalwa cells, either untreated or treated for 2 or 6 h with 10 mM NAD^+ , were processed as described [26]. All antibodies used were diluted in PBS, containing 0.02 M glycine. The primary IB4 antibody was used at 5 $\mu\text{g}/\text{ml}$. The second step reagent was a 10 nm gold-labeled goat anti-mouse IgG (ICN Biomedicals, Milan, Italy), diluted 1:50. The grids were observed and photographed with a Zeiss EM 10/C electron microscope.

3. Results

We used both flow cytometry and assays of ectocellular cyclase and cADPR hydrolase activities to monitor surface

CD38 upon incubation of intact Namalwa cells with either of the two CD38-clustering agents, NAD^+ or GSH [13,16,17]. In all experiments, both reagents were used at deliberately high concentrations to obviate any instability (mostly due to enzymatic degradation of NAD^+ and to oxidation of GSH) over the incubation times required to induce the changes under study. However, 10-fold lower NAD^+ or GSH gave the same effects on ectocellular CD38 enzyme activities at the shortest incubation times.

3.1. Cytofluorimetric analysis

Following 3 h incubation of Namalwa cells in the presence of 10 mM NAD^+ the intensity of CD38 immunofluorescence, as detected with the IB4 MoAb, was reduced to approximately 30% (Fig. 1). The same result was obtained using two different anti-CD38 MAbs, IB6 and OKT10, which recognize different epitopes of the glycoprotein [27]. The intensity of cell surface immunofluorescence with an anti-HLA I MoAb was unaffected by treatment with NAD^+ . A comparable extent of surface CD38 down-regulation was obtained upon exposure of the cells to 10 mM GSH (not shown).

3.2. Assay of ectocellular and total cyclase activities

Cyclase activity of Namalwa cells was assayed on NGD^+ , to avoid interference by the cADPR hydrolase [13,24]. After 2, 3 and 18 h incubation of the cells at 37°C with 10 mM NAD^+ (see Section 2.2), ectocellular cyclase activity decreased to $53 \pm 5\%$, $27 \pm 5\%$ and $17 \pm 3\%$ (mean \pm S.D. of 5 experiments), respectively, of the value of control cells (3.66 ± 0.20 nmol cGDP/ min/mg protein). A similar decay of the cyclase activity was observed upon culturing the cells with 10 mM GSH. With either reagent, disappearance of surface cyclase activity was closely paralleled by loss of the hydrolase. Removal of NAD^+ from the medium following 3 h incubation resulted in slow re-expression of membrane CD38, as shown by the increase of cyclase activity from $27 \pm 5\%$ to $48 \pm 8\%$ (mean \pm S.D. of 3 experiments) of control levels over 24 h at 37°C.

Cyclase activity was also assayed at the same times of in-

Table 1

Cyclase activity of intact and Triton X-100-solubilized Namalwa cells cultured with NAD^+

Incubation time (h)	Total ectocellular cyclase activity $\times 100$	
	Control	NAD^+ -treated
0	138	140
2	140	167
2+Sucrose (0.45 M)	141	168
2+Cycloheximide (100 μM)	142	165
18	139	215

Namalwa cells were cultured at 37°C and 5% CO_2 in complete medium (5×10^5 cells/ml) with 10 mM NAD^+ . At the end of the 2 h incubation with NAD^+ and cycloheximide, protein synthesis was 90% inhibited as determined by TCA-insoluble [^{35}S]Met incorporation. At the times indicated, cells were washed, resuspended in PBS with 10 mM glucose at 10^6 cells/ml and divided into two aliquots: one was solubilized by addition of 1% Triton X-100 (solubilized cells), while the other received an equal volume of PBS (intact cells). Cyclase activity was assayed in both solubilized (total) and intact cells (ectocellular) with 0.5 mM NGD^+ as substrate. After 15 and 30 min incubation with NGD^+ at 37°C aliquots were withdrawn, deproteinized with TCA (5% final concentration), centrifuged and the excess TCA removed with diethylether. HPLC analysis was as in [17]. Results are the mean of 5 experiments (S.D. $\leq 12\%$).

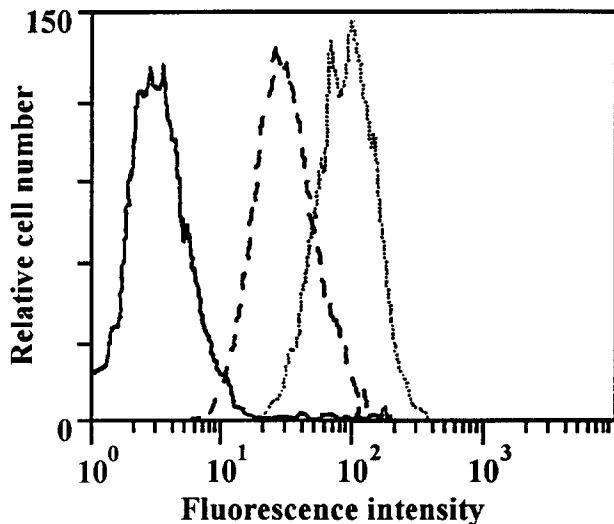


Fig. 1. CD38 immunofluorescence of Namalwa cells incubated with NAD^+ . Namalwa cells (5×10^5 cells/ml) were cultured at 37°C and $5\% \text{CO}_2$, as described in Section 2.2, without (control) or with 10 mM NAD^+ . After 5 h cells were washed and incubated in $50 \mu\text{g/ml}$ IB4 in complete medium for 30 min at 0°C . Thereafter, cells were washed and incubated with $50 \mu\text{g/ml}$ FITC-conjugated anti-mouse IgG in complete medium for 30 min at 0°C , then washed again and fluorescence intensity was quantitated by flow cytometry. No fluorescence was detectable on cells incubated with the second antibody only (continuous line). Dotted line, control cells. Dashed line, NAD^+ -treated cells. Results of a typical experiment are shown.

cubation in the corresponding solubilized cells (total activity) obtained with freezing-thawing or Triton X-100. The latter procedure proved to be more disruptive: thus, the ratios between total and ectocellular cyclase activities in control cells were 1.15 ± 0.04 ($n=4$) and 1.40 ± 0.06 ($n=6$) for the frozen-thawed and the Triton X-100-treated cells, respectively. The ratio of total to ectocellular cyclase activity was consistently higher in the NAD^+ -treated than in the control cells and increased with the time of exposure to NAD^+ (Table 1). An actually higher intracellular cyclase activity in the cells exposed to NAD^+ , as compared to controls, is demonstrated by an increased cADPR content, estimated by a sensitive and specific RIA procedure [28]. The relevant levels of cADPR were 3.13 ± 0.45 and $1.63 \pm 0.24 \text{ pmol/mg protein}$ in the perchloric acid extracts from NAD^+ -treated cells (2 h) and control cells, respectively, following extensive washing of the relevant cell populations.

The presence of cycloheximide ($100 \mu\text{M}$) or hyperosmolar sucrose (0.45 M), an inhibitor of clathrin-dependent endocytosis [29], during incubation of the cells with NAD^+ did not exert any significant effect on the changes in the ratio of total to ectocellular cyclase activity (Table 1).

No appreciable cyclase activity was detectable in the supernatants of NAD^+ - or GSH-treated cells throughout the incubation time. This rules out any shedding of CD38 under these conditions [30].

3.3. Internalization of CD38

To further confirm internalization of CD38 in NAD^+ -treated Namalwa cells, cell surface biotinylation experiments were carried out. Thus, after cell biotinylation and subsequent 18 h culture in the absence (control) or presence of 10 mM NAD^+ , biotin was removed from surface proteins, the cells were sol-

ubilized and the cell lysates were applied onto immobilized streptavidin (see Section 2.4). Supernatants and eluates from the affinity purification step, corresponding to ectocellular and to internalized, biotinylated proteins, respectively, were analyzed both by dot blot and by SDS-PAGE followed by Western blot. Results are shown in Fig. 2.

No CD38 was apparent in the streptavidin eluates from the control cell lysates, neither by dot blot (Fig. 2A, dot 2) nor by SDS-PAGE and Western blot (Fig. 2B, lane 2), while CD38 could be clearly visualized in the streptavidin supernatants by dot blot (Fig. 2A, dot 1) and as a 46 kDa band by SDS-PAGE and Western blot (Fig. 2B, lane 1). These results identify CD38 in control cell lysates as the unbiotinylated (i.e. membrane-bound) monomeric form only. Conversely, the streptavidin eluate from NAD^+ -treated cell lysates showed a strong immunoreactivity with the IB4 MoAb on dot blot analysis (Fig. 2A, dot 4), indicating the presence of biotinylated (i.e. internalized) CD38 in the lysates. The absence of a 46 kDa band on SDS-PAGE and Western blot lane from the same sample (Fig. 2B, lane 4) indicates that the internalized CD38 was in an aggregated form, unable to enter the polyacrylamide gel [13,16]. Some residual, unbiotinylated (i.e. ectocellular) and monomeric CD38 was detectable in the lysates of NAD^+ -treated cells both by dot blot (Fig. 2A, dot 3) and by SDS-PAGE (Fig. 2B, lane 3), although the amount of CD38 was apparently lower than in lysates from control cells.

Search of CD38 aggregates in the lysates from NAD^+ -treated cells by means of density gradient centrifugation (both on

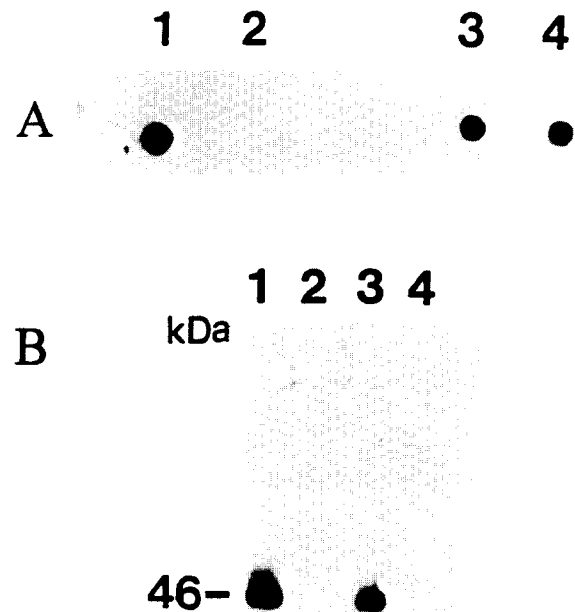


Fig. 2. Aggregation and internalization of biotinylated CD38. Namalwa cells were surface biotinylated as described in Section 2.4 and cultured for 18 h in the absence (control) or presence of 10 mM NAD^+ . After removal of surface biotin with a pulse of GSH, cells were washed and solubilized. Lysates from control and NAD^+ -treated cells were applied to immobilized streptavidin. Resin supernatants and eluates were concentrated and each sample was subjected to dot blot (A) and SDS-PAGE and Western blot (B). Immunodetection of CD38 was obtained with the IB4 MoAb and ECL. Results of a representative experiment are shown. 1, control supernatant; 2, control eluate; 3, supernatant from NAD^+ -treated cells; 4, eluate from NAD^+ -treated cells.

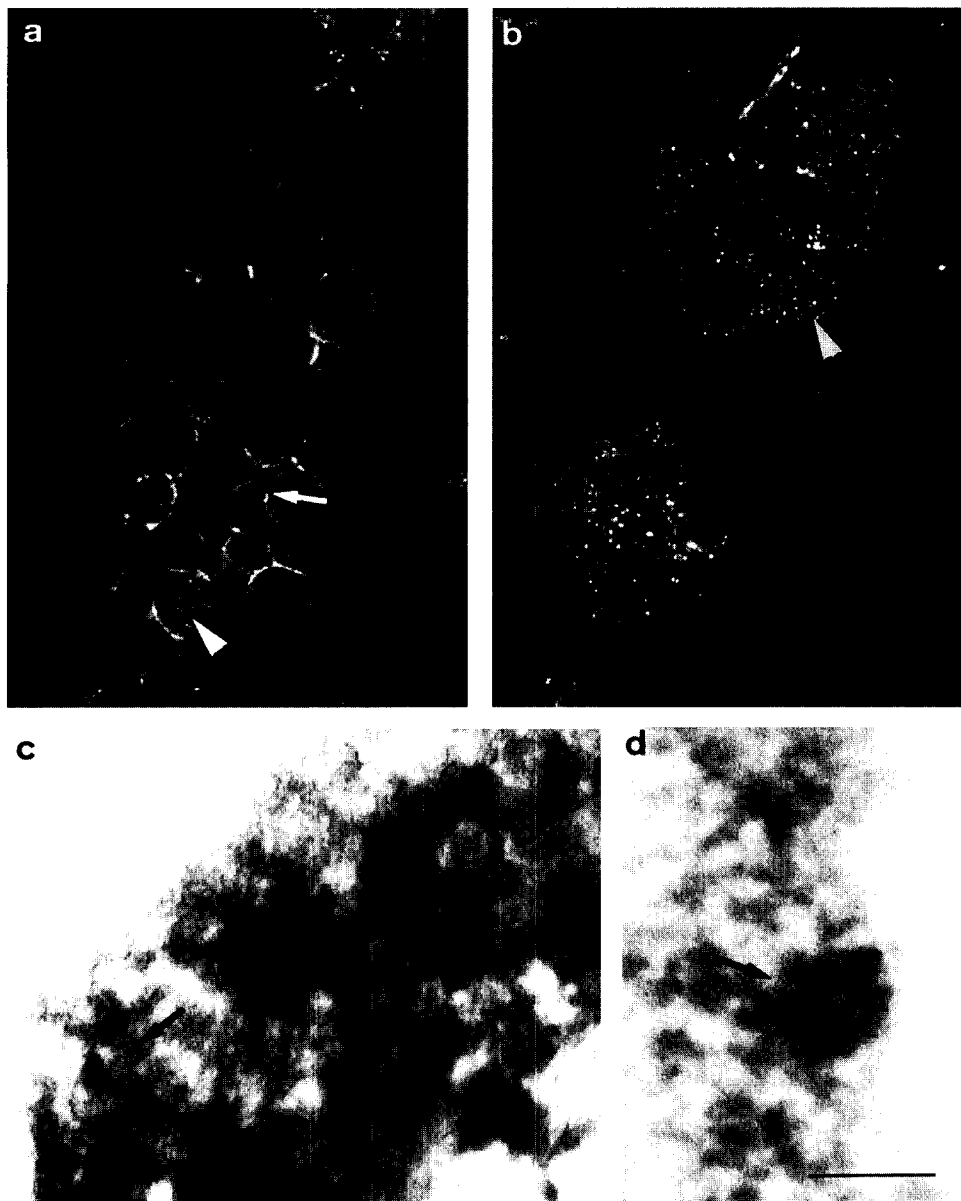


Fig. 3. Internalization of CD38 via smooth vesicles upon incubation of Namalwa cells with NAD^+ . a, b, Immunofluorescence staining for CD38 of Namalwa cells either untreated (a) or treated for 2 h with 10 mM NAD^+ (b). a: In most control cells CD38 was localized on the outer plasma membrane (arrow), but in a few cells a dot-like intracellular pattern of staining (arrowhead) was also evident. b: After NAD^+ treatment the plasma membrane localization disappeared, while all cells showed an intense dot-like intracellular staining (arrowhead). c, d: Ultrastructural immunogold localization of CD38 on ultrathin cryosections of Namalwa cells treated for 2 h with 10 mM NAD^+ revealed the presence of smooth vesicles containing gold particles (arrows). Bar: a, 30 μm ; b, 24 μm ; c, 166 nm; d, 110 nm.

sucrose and on glycerol) and of gel permeation experiments followed by dot blot assays on the individual fractions, was unsuccessful. The main reasons for these negative results were: (a) interference of sucrose and glycerol on the dot blot assays, even following dialysis of the fractions; (b) irreversible adhesion of polymeric CD38 to the gel matrices used for gel permeation analyses; (c) the apparent polydispersity of CD38 aggregates, in agreement with the lack of discrete oligomerization forms of the purified self-aggregated glycoprotein [16].

In order to define the pathway of CD38 internalization upon NAD^+ treatment, we performed immunofluorescence and cryoimmunoelectron microscopy studies. Fig. 3a shows

the peripheral membrane localization of CD38 in untreated control cells. After 2 h (Fig. 3b) and 6 h (not shown) of exposure to NAD^+ , most CD38 disappeared from the cell membrane and was found in a dotted pattern of staining within the cytoplasm, indicating a possible vesicular localization. However, when cells were double immunostained for CD38 and clathrin, the latter showed a more diffused and finely dispersed vesicular pattern of staining (not shown). After 2 h exposure of the cells to NAD^+ , and more evidently after 6 h, immunolocalization at the ultrastructural level identified several CD38-containing cytoplasmic smooth vesicles (Fig. 3c,d), with an average diameter of about 120–150 nm. These vesicles always contained more than one gold particle, indicat-

ing a possible clustering mechanism. In contrast, smooth vesicles containing gold particles were only exceptionally observed in control Namalwa cells (not shown).

4. Discussion

CD38 undergoes extensive aggregation upon incubation with either NAD^+ or thiol compounds [13,16,17]. The process is fast (less than 1 min) with the purified glycoprotein in solution, producing large aggregates with no apparent geometry [13,16], while it is slower in membranes (10–60 min), probably due to impairment of lateral mobility. CD38 aggregation in membranes is accompanied by inability to enter polyacrylamide gels. It takes place in human erythrocytes [17], human Molt-4 (a T cell line) and Swiss 3T3 murine fibroblasts transfected with human CD38 (E. Zocchi, unpublished). Therefore, NAD^+ -induced aggregation of CD38 seems to be related to the structure of the ectocellular region of the human glycoprotein [17] rather than to its membrane environment.

In this study we obtained biochemical and morphological evidence for CD38 internalization in Namalwa B cells incubated with NAD^+ . The NAD^+ -induced endocytosis, which follows aggregation of membrane CD38 (Fig. 2), involves formation of membrane vesicles (Fig. 3). It does not take place through clathrin-coated pits, as demonstrated by the different patterns of staining of CD38 and clathrin, by failure of CD38 to co-immunolocalize with clathrin by double immunofluorescence staining and by lack of inhibition by hyperosmolar sucrose. It does not involve any formation of caveolae, because these structures are absent in lymphoid cells [31] and, additionally, they are insoluble in Triton X-100 [32], which conversely disrupts CD38-containing intracellular membrane vesicles quite efficiently (Table 1). Therefore, it is not yet possible to assess the nature of the endocytic vesicles involved in the process of NAD^+ -dependent CD38 internalization.

It is worth noting that internalization of CD38 results in the import of ADP-ribosyl cyclase activity from the cell surface to the cytosol. This is demonstrated by the corresponding increase in cADPR levels in the NAD^+ -treated over the control cells. These findings do not necessarily contradict the large extent of enzyme inactivation by NAD^+ and by thiol compounds that occurs both with purified and with membrane-bound CD38 [13]. Indeed, the complexity of a whole cell with respect to purified or reconstructed systems does not allow an obvious comparison between *in vitro* and *in vivo* conditions. Specifically, in intact cells additional factors can limit enzyme inactivation, e.g. smaller aggregates, susceptibility to modulators, accelerated membrane-cytosol turnover of polymerized CD38. In any case, all the available data indicate that internalized CD38 is responsible for the enhanced intracellular cyclase activity over that of control cells. The brief time of exposure to NAD^+ (2 h) sufficient to elicit the changes in subcellular localization, the failure of cycloheximide to inhibit this effect under conditions that effectively block protein synthesis (Table 1) and the slow turnover of membrane CD38 upon removal of NAD^+ , are evidence supporting internalization rather than *de novo* synthesis of intracellular CD38.

The present results indicate that extracellular NAD^+ could represent a hitherto unrecognized means for shifting cADPR metabolism from the cell surface to the intracellular compartment. This possibility would make it possible to reconcile the

topological paradox of ectocellular localization of CD38 and intracellular site of action of its enzymatic product cADPR as a calcium mobilizer. Although, as pointed out in [2], endocytosis is expected to position the internalized CD38 within a membrane-bound intracellular vesicle (Fig. 3c,d), this seems to be accessible to cytosolic NAD^+ , as shown by the increase in cADPR levels. It has been speculated that extracellular NAD^+ might become available to CD38⁺ lymphoid cells, even at transiently elevated concentrations, following apoptosis of neighboring cells [1,2]. Recently, NAD^+ has been detected for the first time in the interstitial fluid of rat cerebellum [15], thus adding to ATP [33] as a previously unidentified extracellular signal metabolite. GSH, which shows additive effects with NAD^+ in eliciting CD38 self-aggregation, might also be an extracellular ligand resulting from cell lysis and cooperating with NAD^+ in triggering CD38 internalization in responsive cells.

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