Ligand-induced internalization of CD38 results in intracellular Ca\(^{2+}\) mobilization: role of NAD\(^+\) transport across cell membranes

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ABSTRACT CD38, a transmembrane glycoprotein widely expressed in vertebrate cells, is a bifunctional ectoenzyme catalyzing the synthesis and hydrolysis of cyclic ADP-ribose (cADPR). cADPR is a universal second messenger that releases calcium from intracellular stores. Since cADPR is generated by CD38 at the outer surface of many cells, where it acts intracellularly, increasing attention is paid to addressing this topological paradox. Recently, we demonstrated that CD38 is a catalytically active, unidirectional transmembrane transporter of cADPR, which then reaches its receptor-operated intracellular calcium stores. Moreover, CD38 was reported to undergo a remarkable and sustained increases of intracellular free calcium concentration in different cells exposed to either NAD\(^+\), or GSH, or N-acetylcysteine. This effect of CD38-internalizing ligands on intracellular calcium levels was found to involve a two-step mechanism: 1) influx of cytosolic NAD\(^+\) into the endocytotic vesicles, mediated by a hitherto unrecognized dinucleotide transport system that is saturable, bidirectional, inhibitable by 8-N3-NAD\(^+\), and characterized by poor dinucleotide specificity, low affinity, and high efficiency; 2) intravesicular CD38-catalyzed conversion of NAD\(^+\) to cADPR, followed by outpumping of the cyclic nucleotide into the cytosol and subsequent release of calcium from thapsigargin-sensitive stores. This unknown intracellular trafficking of NAD\(^+\) and cADPR based on two distinctive and specific transmembrane carriers for either nucleotide can affect the intracellular calcium homeostasis in CD38\(^+\) cells.—Zocchi, E., Usai, C., Guida, L., Franco, L., Bruzzone, S., Passalacqua, M., De Flora, A. Ligand-induced internalization of CD38 results in intracellular Ca\(^{2+}\) mobilization: role of NAD\(^+\) transport across cell membranes. FASEB J. 13, 273–283 (1999)

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CD38 is a bifunctional ectoenzyme present in a wide number of different mammalian cells (including muscle, neuronal, and endocrine tissues) but predominantly expressed on hemopoietic cells where its expression correlates with differentiation and proliferation processes (1–6). The two enzyme activities displayed by CD38 are an ADP-ribosyl cyclase and a cyclic ADP-ribose (cADPR)\(^2\) hydrolase that catalyze the synthesis from NAD\(^+\) and the hydrolysis of cADPR, respectively (7–11). Under specific conditions, i.e., at acidic pH values, CD38 can also catalyze the synthesis of nicotinic acid adenine dinucleotide (NAADP\(^+\)) via a base exchange reaction between NADP\(^+\) and nicotinic acid (12). Both cADPR and NAADP\(^+\) are powerful Ca\(^{2+}\) mobilizers in different cell types (12–14). Specifically, cADPR has recently emerged as a novel second messenger for many agonists from plant (15) to...
human cells, where it can modulate the intracellular calcium levels by releasing it from ryanodine-sensitive intracellular stores (5, 13, 16, 17). The well-established role of Ca\(^{2+}\) in the control of such fundamental processes of cell physiology as secretion, contraction, differentiation, proliferation, and apoptosis (18) suggests a possible involvement of the CD38/cADPR system in the regulation of these events. However, the topological problem of the ectocellular production of an intracellular Ca\(^{2+}\)-mobilizer defies an obvious interpretation of the function of the CD38/cADPR system in mammalian cells (5, 19, 20).

We recently described two different processes by which this topological paradox can be overcome: 1) the ligand (GSH or NAD\(^{1}\)) -induced, vesicle-mediated internalization of CD38 in B-lymphocytic, human Namalwa cells, which is followed by an increase of intracellular cyclase activity and of intracellular cADPR concentration ([cADPR]), (21); and 2) the cADPR-transporting function of membrane-bound CD38, which behaves as a catalytically active transporter responsible for generation and selective influx of cADPR across membranes (22). Both mechanisms could in fact cooperate to yield the same result: import of cADPR metabolism from the cell surface into the cytosol of CD38\(^{+}\) cells. Though it is still unknown whether the increased [cADPR], produced by CD38 internalization also induces an increased cytosolic calcium concentration ([Ca\(^{2+}\)]\(i\)), an unequivocal correlation between the two facts has been demonstrated during the opposite process of exocytotic transport of CD38 to the plasmamembrane (23). In this case, intracellularly produced cADPR was demonstrated to be cytosolic by the increase of basal [Ca\(^{2+}\)]\(i\), it elicits in CD38\(^{+}\)-transfected cells as compared to antisense-transfected controls.

These data prompted us to address the ‘subcellular’ topological inconsistency of the CD38/cADPR system: the endocytotic vesicles mediating ligand-induced CD38 internalization derive from invaginations of the plasmamembrane and therefore the active site of CD38 is intravesicular, i.e., apparently not accessible to cytosolic NAD\(^{+}\). The same asymmetric organization shields the catalytic region of CD38 inside exovesicles (5, 19); however, these vesicles in fact produce functionally active cADPR during de novo expression of CD38 (23). To ensure production of cytosolic cADPR by the intravesicular catalytic domain of CD38, two subsequent events are required: NAD\(^{+}\) influx from the cytoplasm into the intravesicular space, and cADPR efflux into the cytoplasm to reach its micosomal receptors and release calcium (24–27). Whereas transport of intravesicularly produced cADPR across the vesicle membrane is the result of the channel activity of oligomeric CD38 (22), NAD\(^{+}\) fluxes across the plasma membrane of intact cells have not yet been reported.

Thus, the aim of this study was twofold: to ascertain whether cytosolic calcium is actually increased after ligand-induced CD38 internalization, as it proved to be during CD38 export to the cell membrane (23); and, on the other hand, to elucidate the mechanisms overcoming these topological problems. Results obtained demonstrate that CD38 internalization in various cell types is causally linked to increased [Ca\(^{2+}\)]\(i\). The mechanism underlying this process (also accounting for enhanced [Ca\(^{2+}\)]\(i\), during CD38 exocytosis) was shown to involve influx of cytosolic NAD\(^{+}\) into the endocytotic CD38-containing vesicles, mediated by a hitherto unrecognized transmembrane pyridine dinucleotide transporter.

These data identify the occurrence of NAD\(^{+}\) and cADPR trafficking across cell membranes and demonstrate that internalization of CD38 ensures the intracellular production of functionally significant and topologically adequate (i.e., cytosolic) cADPR concentrations in CD38\(^{+}\) cells. Finally, the demonstration of NAD\(^{+}\) fluxes across the plasmamembrane of mammalian cells and of a functional effect of extracellular, micromolar NAD\(^{+}\) on [Ca\(^{2+}\)]\(i\), mediated by CD38 internalization and production of intracellular cADPR, imply a hitherto unrecognized extracellular, hormone-like function of NAD\(^{+}\) on CD38\(^{+}\) cells.

**MATERIALS AND METHODS**

N-Acetylcysteine (NAC), reagent grade, was kindly provided by Zambon Group S.p.A., Vicenza, Italy. Fura-2-AM and thapsigargin were purchased from Fluik, Milano, Italy. [carboxy-\(^{14}\)C]Nicotinamide (53 mCi/mmol) was obtained from Moravek (Brea, Calif.). Anti-CD38 monoclonal antibodies (mAb’s) were kindly provided by Prof. F. Malavasi, Torino, Italy (IB4 and IB6), or obtained from Sigma, Milan, Italy (OKT10). Silicon oil (OEL AR 200) was purchased from Wacker-Chemie GmbH, Munich, Germany. 8-N3-NAD\(^{+}\) was chemically synthesized from 8-N3-AMP and β-NMN (both purchased from Sigma), and was purified by high-pressure liquid chromatography (HPLC) as described (28, 29). All other chemicals were obtained from Sigma.

**Cell cultures**

Namalwa, Jurkat, and wild-type HeLa and NIH 3T3 cells (3T3) were purchased from ATCC (Rockville, Md.). CD38 sense- and antisense-transfected HeLa and 3T3 cells (CD38\(^{+}\), and CD38\(^{-}\), respectively) were obtained and cultured as described (23). Peripheral blood lymphocytes (PBL) were recovered after Ficoll gradient separation from fresh blood samples after informed consent from healthy donors. All cells were cultured in Dulbecco’s modified Eagle’s medium (without phenol red) (DME) supplemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), streptomycin (100 μg/ml), and glutamine (2 mM) in a humidified 5% CO\(_2\) atmosphere at 37°C.
Immunofluorescence microscopy

CD38<sup>+</sup>, HeLa cells were grown overnight on glass coverslips. Incubations with the various internalizing agents at millimolar concentrations were performed at 37°C in a petri dish with a single addition at zero time, while micromolar NAD<sup>+</sup> or 8-N<sub>2</sub>-NAD<sup>+</sup> were continuously infused for 30 min at 25°C through a 200 µl chamber at a flow rate of 50 µl/s. All solutions were in DME without FCS. Cells were fixed with the Triton/parafomaldehyde method (30). Nonspecific protein binding to cells was blocked by a 30 min incubation with 5% FCS. Cells were then incubated with anti-CD38 or anti-HLA (histocompatibility leukocyte antigen) class I (Sigma) mAb’s (10 µg/ml). A FITC-conjugated anti-mouse immunoglobulin G (IgG) mAb (Jackson Immunoresearch Laboratories Inc., West Grove, Pa.) was used as secondary antibody, at a concentration of 2.5 µg/ml.

Images of the samples were collected by confocal microscopy with a BioRad MRC 1024 Instrument (krypton/argon laser) on a Nikon DIAPHOT 200, ×60 oil plan apo objective with NA1.4.

Cyttofluorimetric analyses

CD38<sup>+</sup>, HeLa cells, either untreated or treated with internalizing agents, were detached with trypsin and incubated with the primary antibody against HLA class I (10 µg/ml) in complete medium for 30 min at 4°C. Thereafter, cells were washed and incubated with 10 µg/ml FITC-conjugated antimouse IgG antibody in complete medium for 30 min at 4°C. Cells were then washed and fluorescence intensity was quantitated by flow cytometry (21). Control samples were incubated with the secondary antibody only.

Fluorimetric determination of the [Ca<sup>2+</sup>]<sub>i</sub>

The experimental setting used for determination of [Ca<sup>2+</sup>]<sub>i</sub> on Fura2-loaded, adherent cells has been described in detail elsewhere (23). Briefly, cells were seeded on glass coverslips 18 h before the experiment. After Fura2 loading of the cells, the glass coverslip was mounted in a 200 µl recording chamber, mounted on the stage of an inverted microscope (Zeiss IM35, Stuttgart, Germany), continuously perfused at 25°C with solutions fed by gravity through solenoid microvalves, and removed by a hydraulic vacuum pump. Calcium measurements were performed on fields containing 2–10 cell bodies. At the beginning of each experiment, cells were washed in saline zero calcium solution (155 mM NaCl, 5.4 mM KCl, 1 mM MgCl<sub>2</sub>, 10 mM glucose, and 5 mM Hepes, pH 7.4). Intact cells were either exposed to millimolar concentrations of internalizing agents under conditions of stopped flow or to micromolar concentrations under conditions of continuous perfusion in the zero calcium solution. Calcium variations were recorded as described (23). Nonadherent cells (Namalwa, Jurkat, and PBL) were treated similarly except that Fura2 loading and calcium measurements were performed in suspension in a 2 ml cuvette (0.5×10<sup>6</sup> cells/ml).

Enzymatic assays

Ectocellular cyclase activity on NGD<sup>+</sup> (23) and protein content (31) were determined on the same samples used for calcium measurements. Glucose 6-P dehydrogenase (G6PD) and hexokinase activities were determined on sonicated cells and on centrifuged supernatants as described (32). The percentage of cell lysis was calculated as the percentage of total intracellular enzyme activity released into the supernatant.

Determination of pyridine dinucleotide concentrations

The pyridine dinucleotide content of alkaline cell extracts and of alkalized, centrifuged cell supernatants was determined by a sensitive enzymatic cycling assay procedure (33), as described (29). Briefly, this assay enables the sensitive (±10 pmol per assay) and specific determination of the amount of each pyridine dinucleotide species (NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, and NADPH). A different assay mixture was used to detect NAD<sup>+</sup>/NADH or NADP<sup>+</sup>/NADPH, the appropriate substrate and enzyme being ethanol and alcohol dehydrogenase or glucose 6-phosphate and G6PD, respectively. An aliquot of each sample was heated to 60°C for 30 min to destroy the oxidized forms. The amount of the oxidized forms was calculated from the difference between the unheated (oxidized + reduced dinucleotides) and the heated (reduced form only) samples (33). Assays were done in duplicate; in controls (run in parallel), the enzyme was omitted from the assay mixture. Alternatively, aliquots of each sample were incubated 60 min at 37°C with 1 U/ml of nucleotide pyrophosphatase (Sigma); both controls gave comparable results and were used alternatively. All assays were performed on freshly obtained (not frozen) samples. Protein determination was obtained on aliquots of cell extracts (31).

Influx of pyridine dinucleotides into cells

Two different experimental approaches were followed in the studies investigating pyridine dinucleotide influx into cells. As the transport proved to be bidirectional, a significant amount of the internalized nucleotides could be lost from the cells during the extensive washings necessary to remove the excess of extracellular coenzymes. In one type of experiment, adherent HeLa or 3T3 cells (approximately 10<sup>6</sup> cells per sample, seeded 24–36 h before the experiment) were rapidly washed once in DME to remove the FCS (which could be a source of dinucleotide pyrophosphatase; L. Guida, unpublished data). Cells were incubated with the various pyridine dinucleotides in DME at 37°C and subsequently washed at 0°C. A control flask, without cells, was incubated and washed in parallel with each sample. The number of washings was calibrated so as to reduce the concentration of extracellular dinucleotide in the last washing of the control flask to HPLC (34) undetectable concentrations, i.e., <50 nM: under these conditions, the total amount of extracellular dinucleotide remaining after removal of the last washing accounted for 1% to 10% (depending on the nucleotide type) of the intracellular amount of dinucleotide in untreated cells. In a parallel experiment, cells were incubated with each dinucleotide in suspension (approximately 10<sup>6</sup> cells per sample) and the extracellular nucleotides were alternatively removed by centrifugation of the cells through 3 vol of silicon oil (d=1.03–1.05 g/ml) for 15 s at 13,000 × g at 25°C. Pellets were centrifuged again through 0.5 ml of silicon oil, and cells were lysed in 200 µl of water and sonicated for 30 s (Heat Systems-Ultrasonics, Inc. W-380) in ice. The intracellular dinucleotide content was determined on alkaline cell extracts with the cycling assay (33) or, in the experiments designed to determine Κ<sub>m</sub> values, on TCA extracts by HPLC (34).

In the kinetic experiments, which were performed on both CD38<sup>-</sup> and CD38<sup>+</sup> cells, the ectocellular NADase activity of the latter cell types could interfere with the results by producing membrane-permeable nicotinamide and ADPR (22), from which intracellular NAD<sup>+</sup> synthesis might occur. Thus,
the incubation times were brief (from 30 s to 2 min) and control incubations were performed using nicotinamide and ADPR at the same concentrations as those actually produced during incubation of the CD38 \(^+\) cells with NAD\(^+\). This treatment proved not to modify the [NAD\(^+\)].

**RESULTS**

GSH- and NAD\(^+\)-induced internalization of CD38 in CD38\(^+\) cells

When HeLa cells transfected with human full-length CD38 (CD38\(^+\), HeLa) were incubated in the presence of 10 mM GSH, a time-dependent disappearance of ectocellular ADP-ribosyl cyclase activity was observed, with 50 ± 3, 20 ± 4, and 8 ± 4% of initial activity remaining after 1, 12, and 24 h incubation, respectively. The decrease of surface cyclase activity was paralleled by disappearance of membrane immunoreactivity tested at the confocal microscope with several anti-CD38 monoclonal antibodies and by the appearance of cytosolic dot-like staining (Fig. 1), consistent with vesicle-mediated internalization (21). Closely comparable results were obtained when either 10 μM NAD\(^+\) (Fig. 1) or 8-N\(_3\)-NAD\(^+\) (not shown) was used as internalizing agent. Ligand-induced endocytosis of CD38 proved to be specific; no internalization of HLA-class I antigens occurred in control, CD38 antisense-transfected HeLa cells incubated with either NAD\(^+\) or GSH, as detected by both confocal immunofluorescence and FACS analysis (not shown).

Similar results were obtained on CD38\(^+\), 3T3 cells, thus confirming and extending our previous observation of GSH-and NAD\(^+\)-induced, vesicle-mediated CD38 internalization in human B-lymphocytic Namalwa cells (21). In these cells, CD38 internalization was found to be followed by an increase of the intracellular cADPR concentration (21). This prompted us to investigate the intracellular free calcium concentration (\([\text{Ca}^{2+}]_i\)) in CD38\(^+\) cells undergoing CD38 internalization.

CD38 internalization and increased \([\text{Ca}^{2+}]_i\)

After 3 h incubation of all cell types tested (CD38\(^+\), HeLa and 3T3 cells and Namalwa B lymphoid cells) with either NAD\(^+\) (2 mM) or GSH (10 mM), the surface cyclase activity was reduced to approximately 35% of the initial value, while the \([\text{Ca}^{2+}]_i\) was markedly increased up to approximately twice the basal concentration (Table 1). No further calcium increase was observed upon incubation of the cells with either NAD\(^+\) or GSH for as long as 24 h. No significant differences between the various cell lines were apparent. Comparable results were obtained also on peripheral blood lymphocytes (Table 1).

Neither NAD\(^+\) nor GSH produced any increase in the \([\text{Ca}^{2+}]_i\) in CD38\(^-\), HeLa and 3T3 cells (23), thus ruling out any direct effect of either CD38-internalizing agent on the intracellular calcium levels. The time course of the calcium increase after addition of the CD38-internalizing agents to CD38\(^+\) HeLa cells was investigated. Upon addition of NAD\(^+\) (2 mM) or GSH (10 mM) to cells the \([\text{Ca}^{2+}]_i\) increased to reach maximal values (approximately twice the initial concentration) within 40–45 min (Fig. 2). NAC, a stable thiol-containing molecule, elicited the same extent of calcium increase as GSH and NAD\(^+\) (Fig. 2). After removal of any internalizing compound, the \([\text{Ca}^{2+}]_i\) decreased progressively and almost linearly, with 190, 165, and 105% of the basal \([\text{Ca}^{2+}]_i\) being recorded after 2, 6, and 24 h, respectively (mean of four experiments). The same kinetics and extent of \([\text{Ca}^{2+}]_i\) increase were also observed on CD38\(^+\), 3T3 cells and on constitutively
TABLE 1. [Ca\textsuperscript{2+}], in CD38\textsuperscript{+} cells incubated with NAD\textsuperscript{+} or GSH

<table>
<thead>
<tr>
<th>Cell type*</th>
<th>Addition</th>
<th>Cyclase activity\textsuperscript{a} (% of control)</th>
<th>[Ca\textsuperscript{2+}]\textsubscript{i} (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HeLa, CD38\textsuperscript{+}</td>
<td>NAD\textsuperscript{+}</td>
<td>38 ± 3</td>
<td>185 ± 15</td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>36 ± 3</td>
<td>195 ± 16</td>
</tr>
<tr>
<td>3T3, CD38\textsuperscript{+}</td>
<td>NAD\textsuperscript{+}</td>
<td>35 ± 3</td>
<td>198 ± 16</td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>32 ± 3</td>
<td>188 ± 15</td>
</tr>
<tr>
<td>Namalwa</td>
<td>NAD\textsuperscript{+}</td>
<td>32 ± 3</td>
<td>187 ± 15</td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>36 ± 3</td>
<td>170 ± 13</td>
</tr>
<tr>
<td>PBL</td>
<td>NAD\textsuperscript{+}</td>
<td>52 ± 5</td>
<td>200 ± 18</td>
</tr>
<tr>
<td></td>
<td>GSH</td>
<td>45 ± 4</td>
<td>230 ± 20</td>
</tr>
</tbody>
</table>

*Cells were incubated with 2 mM NAD\textsuperscript{+} or 10 mM GSH for 3 h at 37°C; ectocellular cyclase activity and [Ca\textsuperscript{2+}]\textsubscript{i} were determined as described in Materials and Methods. \textsuperscript{a}Results are expressed as percentage of control values obtained from the corresponding cell types incubated without NAD\textsuperscript{+} or GSH. Neither treatment determined any [Ca\textsuperscript{2+}]\textsubscript{i} modification in CD38\textsuperscript{+}, HeLa or 3T3 cells as compared to untreated CD38\textsuperscript{+}, controls. PBL, peripheral blood lymphocytes. For absolute values of cyclase activity and [Ca\textsuperscript{2+}]\textsubscript{i} in the cell types listed in the Table, see ref 23.

CD38\textsuperscript{+} Namalwa cells upon addition of each of the three internalizing agents. This calcium increase was not due to influx of Ca\textsuperscript{2+} from the extracellular medium because all experiments were performed in zero calcium saline solution (see Materials and Methods). Moreover, no increase in [Ca\textsuperscript{2+}]\textsubscript{i} was detectable upon addition of either NAD\textsuperscript{+} or GSH to thapsigargin-pretreated CD38\textsuperscript{+}, cells (not shown).

To demonstrate that the increase in [Ca\textsuperscript{2+}]\textsubscript{i} is specifically dependent on CD38 internalization, GSH was externally supplemented to cells where CD38 internalization had been prevented by prior covalent cross-linking of surface membrane proteins. In the absence of glutaraldehyde, exposure of CD38\textsuperscript{+} HeLa cells to 10 mM GSH (40 min) resulted in an approximately 45% decrease of the ectocellular cyclase activity due to CD38 internalization (inset to Fig. 3) and produced a marked (80%) and time-dependent increase in [Ca\textsuperscript{2+}]\textsubscript{i} (Fig. 3). After glutaraldehyde pretreatment of the cells, however, the surface cyclase activity remained constant upon addition of GSH (inset to Fig. 3). Thus, exposure of cells to glutaraldehyde effectively prevented CD38 internalization without interfering with its enzymatic activity. Under these conditions, no [Ca\textsuperscript{2+}]\textsubscript{i} increase was observed in glutaraldehyde pretreated cells after addition of GSH (Fig. 3).

**NAD\textsuperscript{+} efflux from intact CD38\textsuperscript{-} cells**

These results clearly demonstrated that the calcium increase is dependent on ligand-induced transfer of...
CD38 into the cytoplasm. After internalization, however, the catalytic domain of CD38 is secluded from cytosolic NAD\(^+\) by the endocytotic vesicle membrane (21). When internalization is triggered by exposure of cells to NAD\(^+\), availability of NAD\(^+\) to the cyclase activity of CD38 is ensured by endocytosed NAD\(^+\) inside the vesicles. However, GSH and NAC induce the same extent and kinetics of [Ca\(^{2+}\)]\(_i\) increase as NAD\(^+\) (Table 1 and Fig. 2) on all cell types tested. This is consistent for the occurrence of a transport mechanism for cytosolic NAD\(^+\) in the membrane of the CD38 internalization vesicle. Subsequent efflux of catalytically generated cADPR from the vesicle to reach its microsomal receptors is made possible by the recently discovered cADPR-transporting activity of CD38 itself (22).

The presence of a transport mechanism responsible for NAD\(^+\) influx into the endocytotic vesicles would also allow NAD\(^+\) to be released from the cell. Thus, we investigated NAD\(^+\) efflux from wild-type HeLa and 3T3 cells. Absence of any NAD\(^+\)-hydrolyzing activity on the membrane of these cells was preliminarily checked as a necessary prerequisite for the detection of NAD\(^+\) efflux (S. Bruzzone, unpublished data). As illustrated in Fig. 4, repeated washings of adherent HeLa cells at 37°C in DME resulted in the progressive decrease of intracellular NAD\(^+\) and NADH content ([NAD\(^+\)+NADH])\(_i\), which was more extensive during the first three medium changes (Fig. 4). The sum of the NAD\(^+\)+NADH released into the supernatants of the sequential washings closely paralleled the amount of pyridine dinucleotide lost from the cells (Fig. 4). NAD\(^+\) was the most abundant species both in the cell extracts and in the supernatants, accounting for 90% of the total NAD\(^+\) + NADH pool.

Approximately 25% of the intracellular NAD\(^+\) + NADH pool was consistently released from the cells into the supernatant. This NAD\(^+\) efflux was not due to cell lysis, the extent of which in each supernatant was below 0.5% (Fig. 4). Comparable results were also obtained with wild-type 3T3 cells and with the CD38\(^-\) murine fibroblast cell line M210B4 (not shown).

Susceptibility to inhibition is one of the key features of protein-mediated transport mechanisms. In preliminary experiments we attempted to inhibit efflux of metabolically labeled [\(^{14}\)C-nicotinamide]-NAD\(^+\) from HeLa cells with extracellular unlabeled NAD\(^+\). We tested extracellular NAD\(^+\) concentrations of up to 0.25 mM without observing significant inhibitory effects. Higher extracellular concentrations of NAD\(^+\) were found to interfere with the HPLC separation of the excess [\(^{14}\)C]-nicotinamide present in the cells from the NAD\(^+\) peak (34). The remarkable [NAD\(^+\)], in these cells (7.04 ± 1.2 nmol NAD\(^+\) + NADH/mg protein in unwashed cells) may, however, require a much higher than 0.25 mM extracellular NAD\(^+\) concentration to create and maintain a gradient against NAD\(^+\) efflux. To circumvent these technical difficulties, the NAD\(^+\) analog 8-N\(_3\)-NAD\(^+\) was synthesized (28, 29). Addition of the NAD\(^+\) analog to the supernatant, at progressively increasing concentrations (the highest being 1.2 mM), markedly inhibited efflux of metabolically labeled [\(^{14}\)C] NAD\(^+\) from HeLa cells (Fig. 5). Upon removal of the analog, NAD\(^+\) efflux steadily resumed (Fig. 5). This reversible inhibition of NAD\(^+\) efflux by 8-N\(_3\)-NAD\(^+\), which was observed on 3T3 cells as well, rules out conclusively cell lysis as the source of extracellular NAD\(^+\) and indicates the existence of an inhibitable, bidirectional NAD\(^+\) transport in the membrane of these cells.

These results suggested an experimental setting to maximize NAD\(^+\) efflux from these cell lines by maintaining a near-zero extracellular [NAD\(^+\)]. Adherent HeLa cells were first washed to remove the intracellular exchangeable NAD\(^+\) and cells were subsequently cultured with or without medium changes for 7 h. As shown in Fig. 6, a much higher amount of NAD\(^+\) was released into the supernatant when the medium was changed every 2 h as compared to cell cultures where the medium was not replaced and the intracellular and extracellular NAD\(^+\) concentrations could equilibrate. Approximately 3% of the intracellular NAD\(^+\) was released.

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**Figure 4.** NAD\(^+\) efflux from wild-type HeLa cells. Adherent, exponentially growing HeLa cells (7 flasks; 10\(^6\) cells per 25 cm\(^2\) flask) were either extracted in alkaline buffer (see Materials and Methods) without washing (zero time) or subjected to sequential 5 min-washings at 37°C in DME. Each flask was washed for a different number of times, from 1 to 6, as shown on the abscissa. NAD\(^+\) + NADH content ([NAD\(^+\)+NADH])\(_i\) was estimated by the enzymatic cycling procedure (33) and the total amount of NAD\(^+\) + NADH released was calculated as the sum of the pyridine dinucleotide present in each individual medium change. The percentage of cell lysis in each washing was below 0.5% as determined by assay of G6PD and hexokinase activities in the corresponding supernatants. Results of a representative experiment are shown. Similar results were obtained with wild-type 3T3 cells.
into the medium per hour when the medium was changed. The \([\text{NAD}^+]_i\), at the beginning and end of the 7 h incubation period was not significantly different: thus, biosynthesis apparently prevented \(\text{NAD}^+\) depletion inside the cells. In fact, incubation of the cells under the same culture conditions (medium changes every 2 h) but without nicotinamide in the medium resulted in a 50% reduction of \(\text{NAD}^+\) release into the supernatant.

**Influx of pyridine dinucleotides into intact cells**

The results shown in Fig. 6 indicate that \(\text{NAD}^+\) transport depends on the concentration gradient across the plasmamembrane. To demonstrate unequivocally the bidirectional nature of dinucleotide transport and to investigate its kinetic properties, we analyzed the influx of pyridine dinucleotides into HeLa and 3T3 cells. A short incubation time was necessary to prevent intracellular conversions between the various dinucleotide pools. As shown in **Table 2**, incubation of the cells with each of the four pyridine dinucleotide species tested resulted in a two- to ninefold increase in intracellular concentration without any significant modifications of the levels of the other dinucleotides. Partial loss of the internalized nucleotides during washings was confirmed, for each incubation experiment, by HPLC analysis of the last washing, which indicated dinucleotide concentrations 10- to 20-fold higher than that present in the last washing of the control flask (see Materials and Methods). If this loss was taken into account and added to the intracellular amount of dinucleotides actually measured in cell extracts, the values of influx were closely comparable to those obtained with the silicon oil procedure. Similar results were also obtained with 3T3 cells. Finally, the kinetics of \(\text{NAD}^+\) influx were investigated both in CD38\(^-\) (HeLa and 3T3) cells and in CD38\(^+\) (Namalwa and Jurkat) cell lines. Influx of \(\text{NAD}^+\) into intact HeLa cells followed hyperbolic saturation kinetics (not shown). The apparent \(K_m\) for \(\text{NAD}^+\) transport was approximately 15 mM, similar for all cell types tested and close to values previously recorded on resealed human erythrocyte membranes (22). The initial rate of \(\text{NAD}^+\) influx was calculated to be approximately 10 nmol/(min \cdot mg cell protein) for HeLa and 3T3 cells. These values are the mean from results obtained with both experimental settings described under Materials and Methods. Influx of a number of \(\text{NAD}^+\) analogs was also investigated on both HeLa and 3T3 cells: while modifications in the adenine moiety of the molecule

![Figure 5](image-url)  **Inhibition of \(\text{NAD}^+\) efflux from HeLa cells by 8-N3-\(\text{NAD}^+\)** Intracellular \(\text{NAD}^+\) was metabolically labeled by culturing HeLa cells for 5 days at 37°C in DME, 10% FCS containing \([^{14}\text{C}]\)-nicotinamide (5 \(\mu\)Ci per sample). At the time points indicated the supernatants (0.4 ml) were replaced with the media listed below and the amount of radioactive \(\text{NAD}^+\) released was determined by (■) HPLC. Control (■), DME containing 8-N3-\(\text{NAD}^+\) at a final concentration of 0.4, 0.8, and 1.2 mM in the first three medium changes, respectively. The arrow indicates removal of the inhibitor. The total amount of \([^{14}\text{C}]\)-\(\text{NAD}^+\) released was calculated as the sum of the radioactive dinucleotide present in each individual supernatant. Results are expressed as percentage of the total \([^{14}\text{C}]\)-\(\text{NAD}^+\) (intracellular + extracellular) released into the medium. Results of a representative experiment, out of 4 different ones, are shown.

![Figure 6](image-url)  **NAD\(^+\) efflux from HeLa cells cultured with or without medium changes.** Adherent, exponentially growing HeLa cells (10\(^6\) per sample) were washed in DME and incubated at 37°C in the same medium with (■) or without (■) medium changes (indicated by the arrows). The \(\text{NAD}^+\) concentration in the medium was determined by the cycling assay procedure (33) and the total amount of \(\text{NAD}^+\) released (mean ± SD of 4 experiments) was calculated as the sum of the \(\text{NAD}^+\) found in each supernatant. Similar results were obtained with 3T3 cells. The percentage of cell lysis in each supernatant was below 0.3%, as determined by assay of G6PD and hexokinase activities.
Influx of NAD\(^+\), NADH, NADP\(^+\), and NADPH into HeLa cells

<table>
<thead>
<tr>
<th>Extracellular dinucleotide</th>
<th>Intracellular pyridine dinucleotide (^*)</th>
<th>(% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAD(^+)</td>
<td>220 ± 20</td>
<td>109 ± 10</td>
</tr>
<tr>
<td>NADH</td>
<td>113 ± 10</td>
<td>300 ± 30</td>
</tr>
<tr>
<td>NADP(^+)</td>
<td>121 ± 11</td>
<td>101 ± 10</td>
</tr>
<tr>
<td>NADPH</td>
<td>112 ± 10</td>
<td>91 ± 8</td>
</tr>
</tbody>
</table>

\(\*\) Wild-type HeLa cells (10\(^4\) per sample) were washed once in DME and incubated for 5 min at 37°C in DME containing the indicated pyridine dinucleotide at 10 mM final concentration. Each incubation was performed in duplicate on adherent and on suspended cells. At the end of the incubation, adherent cells were washed with DME at 0°C; cells in suspension were briefly centrifuged through three volumes of silicon oil. The intracellular pyridine dinucleotide concentrations were determined by cycling assay (33) on alkaline cell extracts; the amount of dinucleotide lost in the last washing was determined by HPLC (34) and added to the intracellular dinucleotide concentration measured in adherent cells. Results shown are the mean ±S.D. of the values obtained from both experimental settings in three independent experiments, and are expressed as percentage of control values obtained from cells incubated without any addition (NAD\(^+\), 7.04 ±0.7 nmol/mg; NADH, 0.9 ±0.07 nmol/mg; NADP\(^+\), 0.16 ±0.01 nmol/mg; NADPH, 1.4 ±0.01 nmol/mg). Similar results were obtained with 3T3 cells.

Role of cADPR in the increased [Ca\(^{2+}\)]\(_i\) induced by CD38 internalization

8-N\(_3\)-NAD\(^+\) is a substrate of both the enzymatic and of the cyclic nucleotide-transporting activities of CD38 (L. Franco, unpublished data), but 8-N\(_3\)-cADPR is a potent antagonist of cADPR-induced calcium release (28, 29). We took advantage of the fact that 8-N\(_3\)-NAD\(^+\) is also internalized by the pyridine dinucleotide-transporting system (see above) to devise an experiment aimed to causally correlate endocytosis of CD38, intracellular production of cADPR and [Ca\(^{2+}\)]\(_i\) increase. Adherent CD38\(^+\), HeLa cells were first depleted of their cytosolic free NAD\(^+\) by continuous perfusion with DME in the recording chamber described in Materials and Methods. Cells were incubated with either 10 \(\mu\)M NAD\(^+\) or 20 \(\mu\)M 8-N\(_3\)-NAD\(^+\) for 30 min under continuous flow, and the [Ca\(^{2+}\)]\(_i\) and the surface cyclase activity on NGD\(^+\) were determined on the same sample. The decrease of surface cyclase activity was closely comparable in NAD\(^+\)- and 8-N\(_3\)-NAD\(^+\)-incubated cells, down to 48% and 50% of starting values, respectively. In addition, confocal fluorescence microscopy confirmed a comparable extent of CD38 internalization with both dinucleotides (not shown). The [Ca\(^{2+}\)]\(_i\), however, increased only in the cells incubated with NAD\(^+\) while keeping fairly stable in the cells exposed to the NAD\(^+\) analog (Fig. 7). These results demonstrate a causal correlation between the intracellular cADPR production by internalized CD38 and the [Ca\(^{2+}\)]\(_i\) increase. Moreover, the experimental setting of the continuous flow enabled us to demonstrate that micromolar concentrations of NAD\(^+\) are sufficient to elicit both internalization of CD38 and enhanced [Ca\(^{2+}\)]\(_i\), provided fresh NAD\(^+\) is continuously supplied to the CD38\(^+\) cells.

DISCUSSION

The original aim of this study was to investigate the effect of ligand (NAD\(^+\) or GSH) -induced internalization of CD38 on [Ca\(^{2+}\)]\(_i\). A link between the two processes was suggested, but not yet demonstrated, by our earlier finding that the NAD\(^+\)-or GSH-induced specific internalization of CD38 through non clathrin-coated endocytotic vesicles in Namalwa B-lymphocytes results in increased [cADPR]\(_i\) (21).

The preliminary demonstration (Fig. 1 and Table 1) that CD38 internalization occurs in different cell types (Namalwa, CD38\(^+\), HeLa and 3T3 cells, and PBL), with a similar mechanism (NAD\(^+\)- or GSH-induced, vesicle-mediated endocytosis) and with comparable kinetics (approximately 50% being internalized within 1 h incubation), supports the conclusion that internalization is an intrinsic property of
CD38 itself and does not depend on cell type or membrane protein environment.

After CD38 internalization, the \([\text{Ca}^{2+}]_i\) increased to maximal values (i.e., approximately twice the initial concentration) within 45–60 min incubation in all cell types tested and with either internalizing agent used—NAD\(^+\), GSH, or NAC (Table 1 and Fig. 2). These kinetics of calcium increase closely parallel the process of CD38 internalization and are different from the transient (seconds) and more limited (around 50%) increase of cytosolic calcium that immediately follows exposure of CD38\(^+\)-transfected HeLa cells to NAD\(^+\) (22). The rapid elevation of \([\text{Ca}^{2+}]_i\) triggered in the latter case is due to the recently demonstrated property of CD38 to behave as an active channel for generation and selective influx of cADPR across membranes (22). On the contrary, the sustained increase of \([\text{Ca}^{2+}]_i\) after exposure of cells to NAD\(^+\), GSH, or NAC is mechanistically related to 1) specific CD38 internalization, as demonstrated by blockade of both processes by prior cross-linking of membrane proteins with glutaraldehyde (Fig. 3), and 2) the consequently enhanced formation of intracellular cADPR, as shown by the experiments with 8-N\(_3\)-NAD\(^+\) (Fig. 7).

A significant outcome of the present investigation was the demonstration of a hitherto unrecognized transport system for NAD\(^+\) in cell membranes and of its role in the regulation of cytosolic calcium related to the ligand-induced CD38 internalization. Evidence for this transport emerged from the attempt to explain a topological inconsistency of the CD38/cADPR system during either internalization (this study) or export (23) of membrane-bound CD38. In fact, due to the reverse polarity of asymmetrically oriented CD38 molecules in the plasmamembrane, both endocytotic and exocytotic membrane vesicles display the carboxyl-terminal catalytic region of CD38 in the intravesicular space, therefore not directly accessible to its cytosolic substrate NAD\(^+\). Accordingly, for the endocytotic or exocytotic vesicles to behave as cADPR-dependent calcium-releasing systems in cells, as actually observed in different experimental conditions (21,23), two requirements should be met: 1) availability of NAD\(^+\) to the apparently sealed active site of CD38, and 2) release of catalytically generated cADPR from both types of vesicles into the cytosol, allowing the cyclic nucleotide to reach its receptor-operated calcium stores (24–27). As mentioned, membrane-embedded CD38 was shown to meet the latter requirement since it proved to be a catalytically active transporter of cADPR, but not of NAD\(^+\), across membranes (22), by virtue of its oligomeric (dimeric and tetrameric), channel-generating structure (22, 35). The need to identify an NAD\(^+\)-translocating system prompted us to investigate NAD\(^+\) fluxes across the plasmamembrane, since the endocytotic vesicles derive from the plasmamembrane itself. These studies were carried out on intact cells, mostly using the wild-type human HeLa and murine 3T3 cells, which do not exhibit any NAD\(^+\)-cleaving enzyme activity at their outer surface. Therefore, both influx and efflux of NAD\(^+\) from these cells could be investigated without any interference by NAD\(^+\) degradation.

The present findings, besides conclusively demonstrating that cell membranes are permeable to NAD\(^+\), also identify some properties of this novel transport system. This proves to be a passive transport, because the direction of pyridine dinucleotide flux depends on the concentration gradient only and no apparent energy source is required. The transport system is characterized by low dinucleotide specificity and follows hyperbolic saturation kinetics. No significant effect of temperature was observed in influx and efflux experiments (not shown), this suggesting that dinucleotide translocation occurs across a channel rather than involving a transporter oscillating between different conformations (36).

The demonstration of NAD\(^+\) fluxes through the plasmamembrane of intact mammalian cells is, to our knowledge, without precedent, although slow NAD\(^+\) leakage across the mitochondrial membranes has been described both in plant (37) and mammalian cells (38). We found that approximately 25% of intracellular NAD\(^+\) can be lost from HeLa or 3T3 cells upon repeated washings: this ‘exchangeable’ NAD\(^+\) (over 90% oxidized) is probably the cytosolic, free (i.e., non enzyme-bound) dinucleotide (Fig. 4). Efflux of NAD\(^+\) can be reversibly inhibited by the NAD\(^+\) analog 8-N\(_3\)-NAD\(^+\) (Fig. 5), but attempts to irreversibly inhibit NAD\(^+\) efflux from HeLa cells by photolysis were unsuccessful. However, photoaffinity labeling does not necessarily yield stoichiometric labeling: values between 0.01 and 1.0 mol to mol are usually reported (39).

Influx of all pyridine dinucleotides (NAD\(^+\), NADH, NADP\(^+\), and NADPH) occurs across the plasmamembrane of intact HeLa and 3T3 cells: although some degree of intracellular reequilibration between the various oxidized and reduced dinucleotide pools takes place at longer incubation times (not shown), selective increase of the intracellular concentration of each extracellularly added nucleotide was obtained by reducing the incubation time to 5 min (Table 2). NAD\(^+\) influx into both CD38\(^-\) HeLa and 3T3 cells and CD38\(^+\) Namalwa and Jurkat cells displays hyperbolic saturation kinetics: over the short incubation time sufficient for NAD\(^+\) influx (below 2 min), a very limited NAD\(^+\) degradation occurred with CD38\(^+\) cells. Incubation of these cells with concentrations of nicotinamide and ADPR as low as those produced ectocellularly from NAD\(^+\) during the incubation time failed to determine any
increase of the [NAD+]i. This control confirms the existence of NAD+ transport also in the plasma membrane of CD38+ lymphocytic cell lines.

It is significant that micromolar NAD+ concentrations were effective in inducing both CD38 internalization and calcium increase (Figs. 1, 7). Although reported extracellular NAD+ concentrations are approximately 10 nM in rat cerebellar interstitial fluid (6) and 50–60 nM in human blood serum (T. F. Walseth, personal communication), these values represent average levels of the dinucleotide in the extracellular compartment: micromolar NAD+ concentrations in the extracellular fluids may be reached in specific conditions (e.g., apoptosis in lymphnodes; see ref 40) and in selected districts, also in view of the demonstrated ability of fibroblast and epithelial cell lines to steadily release NAD+ (Fig. 6). It is likely that also micromolar concentrations of GSH or reducing agents can induce internalization of CD38 in vivo; a relevant observation is that oral administration of therapeutic doses of N-acetylcysteine for 3 days resulted in a 30% decrease of ectocellular ADP-ribosyl cyclase activity in PBL (L. Guida, E. Zocchi, and A. De Flora, unpublished data).

Extracellular GSH proved not to affect the efflux of [14C]-NAD+ from metabolically labeled HeLa cells (not shown). Accordingly, the presence of high GSH concentrations in the endocytotic vesicles generated by GSH-induced internalization does not restrict the influx of cytosolic NAD+ into these vesicles. The high intracellular concentrations of NAD+ in the cell lines investigated (HeLa and 3T3) and the high rate of NAD+-transporting activity are consonant with a remarkable efficiency of cytosolic NAD+ influx into the vesicles.

The identification of a still unrecognized dinucleotide transport system, responsible for bidirectional NAD+ flux across membranes, and the recently established nature of CD38 as a selective and unidirectional cADPR transporter (22) allow us to rationalize the complex interplay of intracellular trafficking of NAD+ and of cADPR as well. Both transport systems seem to operate sequentially inside cells so as to bypass apparent processes of compartmentalization and to produce functionally significant, cytosolic cADPR concentrations during endocytosis or exocytosis, effective on intracellular calcium levels. As previously postulated (22), this subcellular type of structural and functional organization centered on opposite polarity of NAD+ and cADPR transports may provide the dual advantage of preventing unrestricted NAD+ consumption, while representing a flexible means for finely tuning the NAD+-dependent regulation of [Ca2+]i. Unequivocal confirmation of the regulatory role of NAD+ on [Ca2+]i should come from elucidation of the patterns of NAD+-induced, cADPR-related [Ca2+]i increases in specific cell types (e.g., oscillatory vs. sustained) and from identification of selected molecular targets of these calcium movements, e.g., expression of specific genes (41–43). Dissection between distinctive functional consequences of oscillatory and sustained increases of [Ca2+]i, as possibly elicited by different schedules of exposure and by different concentrations of internalizing compounds, will be important in this respect, since CD38 per se is suitable to elicit both types of effects as a steady cADPR channel (22) and as a catalytic component of slowly cADPR-generating vesicles, respectively.

The recognized efflux of NAD+ from the plasma membrane of intact cells and the properties of transmembrane CD38 suggest that the CD38-related regulation of [Ca2+]i is not restricted to a single CD38+ cell. Rather, it may occur through transfer of NAD+ from any cell type to CD38+ cells, according to paracrine mechanisms whereby NAD+ could act as a hormone, involved in cell-to-cell interactions, and cADPR as its intracellular second messenger. These results, besides accounting for a hitherto unknown cross-talk of signal metabolites between different cell compartments or between different cells, also identify potential targets for the development of new drug molecules affecting intracellular calcium levels in various diseases characterized by imbalanced calcium homeostasis.

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