Interaction of Two Classes of ADP-ribose Transfer Reactions in Immune Signaling*

CD38 is a bifunctional ectoenzyme predominantly expressed on hematopoietic cells where its expression correlates with differentiation and proliferation. The two enzyme activities displayed by CD38 are an ADP-ribosyl cyclase and a cyclic adenosine diphosphate ribose (cADPR) hydrolase that catalyzes the synthesis and hydrolysis of cADPR. T lymphocytes can be induced to express CD38 when activated with antibodies against specific antigen receptors. If the activated T cells are then exposed with NAD, cell death by apoptosis occurs. During the exposure of activated T cells to NAD, the CD38 is modified by ecto-mono-ADP-ribose transferases (ecto-mono-ADPRTs) specific for cysteine and arginine residues. Arginine-ADP-ribosylation results in inactivation of both cyclase and hydrolase activities of CD38, whereas cysteine-ADP-ribosylation results only in the inhibition of the hydrolase activity. The arginine-ADP-ribosylation causes a decrease in intracellular cADPR and a subsequent decrease in Ca\(^{2+}\) influx, resulting in apoptosis of the activated T cells. Our results suggest that the interaction of two classes of ecto-ADP-ribose transfer enzymes plays an important role in immune regulation by the selective induction of apoptosis in activated T cells and that cADPR mediated signaling is essential for the survival of activated T cells.

The type II transmembrane glycoprotein CD38 is the prototypic member of the class of adenosine diphosphate ribose (ADPR)\(^1\) transfer enzymes known as nicotinamide adenine dinucleotide (NAD) glycohydrolases (NADases) (1–3). CD38 is a bifunctional ectoenzyme that catalyzes the formation and hydrolysis of cyclic adenosine diphosphate ribose (cADPR) (4–6). Thus, this enzyme possesses both ADP-ribosyl cyclase and cADPR hydrolase, the net reaction of which is identical to that catalyzed by NADase.

cADPR is a potent Ca\(^{2+}\) mobilizing agent believed to be involved in Ca\(^{2+}\)-induced Ca\(^{2+}\) release in a variety of cells from plants to humans (7–9). cADPR has also been shown to augment the proliferative response of activated murine B lymphocytes (5), as well as to mediate the Ca\(^{2+}\) release associated with ATP-activated potassium currents in alveolar macrophages (10), suggesting that cADPR may function as a signaling second messenger in multiple hematopoietic cell types. Although the physiological ligand for CD38 has yet to be identified, CD38 was reported to undergo internalization through non-clathrin-coated endocytic vesicles upon incubating cells with thiol compounds or NAD (11). The mechanism by which endocytic vesicles can generate cADPR was suggested by the demonstration that the CD38-catalyzed conversion of NAD to cADPR can be achieved by the influx of cytosolic NAD into the endocytic vesicles and the cADPR into the cytosol for subsequent Ca\(^{2+}\) signaling (12).

Lymphocyte surface molecules are known to play an important regulatory role by modulation of their expression, binding activity, and/or signal transduction. The ecto-enzymes expressed on the lymphocyte surface also regulate the functions of lymphocytes. ADP-ribosylation of surface proteins on T cells by an ecto-mono-ADP-ribose transferase (ADPRT) results in the inhibition of cytotoxic T cell functions such as cell proliferation, cytotoxicity, and cytokine secretion (13), and the suppression of cytotoxic T cell function is closely correlated with the expression of the mono-ADPRT (14). Most ecto-NADases are known to be inhibited in the presence of their substrate, NAD (15, 16). Recently, we described the NAD-dependent inactivation of 65-kDa NADase from rabbit erythrocytes by auto-ADP-ribosylation (17). In the study described here, we have explored whether CD38 is also inactivated in the presence of NAD. Our results show that ADP-ribosylation of CD38 by mono-ADPRT in activated T cells results in apoptosis as well as CD38 inactivation, suggesting that ADP-ribosylation of CD38 plays an important role in immune regulation and that cADPR-mediated signaling is essential for the survival of activated T cells.

**EXPERIMENTAL PROCEDURES**

**T and B Cell Preparation**—A single cell suspension of splenocytes was prepared from normal BALB/c6–8-week-old mice as described previously (18). The T cells were purified by negative selection using goat anti-mouse IgG-coated Immulan beads (Biotecx Laboratories). The T cells were activated by incubation for 12 h with anti-CD3 antibody (1 μg/ml; Chemicon) and interleukin-2 (100 units/ml; Perkin-Elmer Cetus). B cells were prepared by eluting cells which were bound to goat anti-mouse IgG-coated Immulan beads.

**Enzyme Assay**—Activated T cells were harvested at the indicated times and washed with Hank’s balanced salt solution (HBSS) (2 mM CaCl\(_2\), 145 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 5 mM Na-glucose, 20 mM Hepes, pH 7.3). ADP-ribosyl cyclase activity was measured by fluorometric assay with nicotinamide guanine dinucleotide as a substrate (19). cADPR hydrolase was assayed as described previously (20).

**ADP-ribosylation**—Activated T cells were washed with HBSS and incubated with 10 μM [adenylate-\(^{32}\)P]NAD. The cells were lysed on ice in...
RESULTS AND DISCUSSION

ADP-ribosylation of CD38 Results In Inactivation of Its ADP-ribosyl Cyclase and cADPR Hydrolyase Activities—Most eucaryotic cell lines are known to be inactivated in the presence of NAD (15, 16). We tested whether T lymphocyte CD38 is also inactivated in the presence of NAD. We induced the expression of CD38 on T cells from Balb/c mice by activation with anti-CD3 antibodies, since CD38 on murine T cells prepared from splenocytes was not induced by other stimuli, such as concanavalin A, phytohemagglutinin, or phorbol 12-myristate 13-acetate and ionomycin (Fig. 1). When the activated T cells were incubated with various concentrations of NAD for 1 h, the ADP-ribosyl cyclase and cADPR hydrolyase activities of CD38 decreased in a dose-dependent manner with increasing concentrations of NAD (Fig. 2A). CD38 seemed to be the only enzyme responsible for the activities of both ADP-ribosyl cyclase and cADPR hydrolyase, since most of these enzyme activities in the activated T cells were recovered by immunoprecipitation with anti-CD38 antibodies and inactivated by the addition of NAD as in the intact T cells (Fig. 2A, inset).

This result indicates that both of the enzyme activities of CD38 are affected by the addition of NAD. To study the mechanisms responsible for the NAD-dependent inhibition of CD38 enzyme activities, we examined the transfer of ADPR from NAD to the CD38 molecule. We incubated the activated T cells with [adenylic-32P]NAD and immunoprecipitated with anti-CD38 antibodies. We found that the precipitate contained 32P-labeled protein, as shown in Fig. 2B, control lane. These results suggest that ADPR from NAD was transferred to CD38 and that the modification of CD38 is NAD-dependent.

Next, we tested whether CD38 was inactivated by mono-ADP-ribosylase by autoradiography. As it has been reported that mono-ADP-ribosylation is anchored on the cell membrane via a glycosylphosphatidylinositol-linkage (14, 28), we examined whether the treatment of T cells with PI-PLC would block the ADP-ribosylation of CD38. Pretreatment of T cells with phosphatidylinositol-specific phospho-
lipase C prevented NAD-dependent ADP-ribosylation of CD38 (Fig. 2B), but did not affect the amount of membrane-associated CD38 and the activities of CD38. Phosphatidylinositol-specific phospholipase C treatment might not damage the T cells because there was no significant ATP release from the cells and no changes of the cell viability. This result indicates that the ADP-ribosylation of CD38 was not due to automodification but was mediated by a glycosylphosphatidylinositol-anchored cell surface mono-ADPRPT.

Mammalian cells contain at least two types of mono-ADPRPTs that are either arginine- or cysteine-specific (29); thus, we determined the ADPR acceptor site(s) on CD38. We treated [32P]ADP-ribosylated CD38 with water (control), hydroxylamine (lane 1), or mercuric chloride (lane 4). The ratio of densities of each lane was measured with image analyzer as 1.0:0.47:0.53:0. The data are representatives of three experiments.

**FIG. 2.** Inactivation of CD38 activities by ADPRPT-mediated ADP-ribosylation. A, effect of NAD on enzyme activities of CD38. Activated T cells (1 × 10⁶ cells/ml) were incubated at 37 °C for 1 h in the presence of various concentrations of NAD and the enzyme activities were measured. Inset, activated T cells (1 × 10⁶ cells/ml) were incubated at 37 °C for 1 h in the presence of various concentrations of NAD, CD38 was immunoprecipitated and the enzyme activities were measured. The data shown are the mean ± S.D. of three independent experiments. B, effect of PI-PLC on the ADP-ribosylation of CD38. Cells that had been incubated for 1 h with 1 μg/ml B. cereus phosphatidylinositol-specific phospholipase C (PIPLC) or vehicle (control) in the presence of 2 mg/ml bovine serum albumin were further incubated with 10 μM [32P]NAD for 1 h. CD38 was immunoprecipitated, separated on a SDS-polyacrylamide gel, and subjected to autoradiography (top), or the intact cells were biotinylated, immunoprecipitated with anti-CD38 mAb, and blotted with avidin-alkaline phosphatase (bottom). C, specificity of the ADPR-CD38 bond. The [32P]ADP-ribosylated CD38 was treated with water (lane 1), hydroxylamine (lane 2), or mercuric chloride (lane 3), or hydroxylamine plus mercuric chloride (lane 4). The ratio of densities of each lane was measured with image analyzer as 1.0:0.47:0.53:0. The data are representatives of three experiments.
Fig. 3. Apoptosis of activated T cells and CD38-transfected cell lines by ADP-ribosylation of CD38. A. effect of NAD on T cell viability. The number of viable cells was determined by trypan blue exclusion after T cells were cultured for the indicated times following their incubation for 1 h in the presence (•) or absence (○) of 100 μM NAD. The data shown are the mean ± S.D. of three independent experiments. B. Flow cytometric analysis of apoptosis in activated T cells. For activation of T cells, T cells were prepared from mouse splenocytes as described under “Experimental Procedures” and incubated for 12 h in the medium supplemented with anti-CD3 antibody (1 μg/ml). Cells were analyzed with flow cytometry after the addition of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide 12 h after incubation for 1 h in the presence or absence of 100 μM NAD. The percentage of apoptotic cells is indicated in the upper right of each panel. C, NAD-dependent apoptosis of CD38-transfected cells. Wild-type (Mock) and CD38-transfected Jurkat or HeLa cells were incubated with 100 μM NAD in RPMI medium containing 10% fetal bovine serum for 16 h, following serum deprivation for 24 h. In the case of HeLa cells, 60 units/ml arginine-ADPRT purified from splenocytes were supplemented for ADP-ribosylation. The cells were centrifuged onto slides, fixed with 1% paraformaldehyde, and stained with 8 μg/ml Hoechst 33342 to identify cells that lost nuclear structure.

significantly decreased as compared with control T cells that were incubated under the same conditions except that NAD was omitted (Fig. 3A). To determine whether the reduction of viable T cells was a result of apoptosis, we examined annexin V binding, a method for the early detection of apoptosis (30), in activated T cells incubated in the presence or absence of NAD. The percentage of apoptotic cells was significantly increased in the activated T cells by the treatment with NAD (Fig. 3B).

To determine if ADP-ribosylation of CD38 plays an important role in inducing apoptosis in T cells, we examined whether ADP-ribosylation of CD38 could induce apoptosis in two cell lines. First CD38-transfected Jurkat cells, a cloned human T cell leukemia cell line that does not normally express CD38 but has both arginine- and cysteine-specific mono-ADPRTs, was examined. When the CD38-transfected and non-transfected Jurkat cells were exposed to NAD, we found that only the CD38-transfected Jurkat cells underwent apoptosis (Fig. 3C). Next, HeLa, a human epithelial cell line that does not normally express CD38 or mono-ADPRT activity, we tested the effects of the metabolites such as adenosine, AMP, ADP, ADP-ribose, and cADPR on the apoptosis of activated T cells, but no significant changes were found in these cells (data not shown).

Arginine-specific ADPRT Is Responsible for Apoptosis of Activated T Cells—Since CD38 is expressed on both T and B lymphocytes, we determined whether B cells also undergo apoptosis through ADP-ribosylation of CD38. We incubated B cells with NAD and apoptosis was tested by annexin V binding. We found that B cells showed no detectable apoptosis (Fig. 4A), although CD38 was ADP-ribosylated. To determine whether there are differences between the ADP-ribosylation of CD38 in T cells and B cells, we examined the ADP-ribosylation sites on the CD38 of B cells and found that the ADP on CD38 in B cells occurs only on the cysteine residue. We further examined the expression of arginine-specific mono-ADPRT activity in B cells and T cells by RT-PCR and Southern blot. We found that B cells did not express arginine-specific mono-ADPRT, whereas T cells did express this enzyme (Fig. 4B).

We then asked whether there are differences between arginine- and cysteine-ADP-ribosylation on the enzyme activity of CD38. We immunoprecipitated CD38 from B cells with anti-CD38 antibodies, incubated the immune complex with 100 μM
NAD and either arginine- or cysteine-specific mono-ADPRTs purified from T cells, and examined the ADP-ribosyl cyclase and cADPR hydrolase activities. We found that arginine-ADP-ribosylation of CD38 resulted in the inactivation of both the cyclase and hydrolase activities of CD38, while cysteine-ADP-ribosylation resulted in the inactivation of hydrolase activity but did not affect the cyclase activity (Fig. 4C). Therefore, we asked whether arginine-specific ADPRT plays an essential role in the induction of apoptosis. When B cells were incubated with NAD and arginine-specific mono-ADPRT purified from splenocytes, the cells underwent apoptosis similar to T cells after incubation with NAD (Fig. 4A and 3B). This result suggests that the ADP-ribosylation of an arginine residue(s) on CD38 plays a critical role in the induction of apoptosis, and that this may be due to reduced cyclase activity, which presumably results in a reduced intracellular cADPR concentration ([cADPR]). Thus, we examined the effect of CD38 ADP-ribosylation on [cADPR]. As predicted, prior incubation of T cells with NAD significantly reduced [cADPR], compared with cells that were not treated with NAD (Fig. 4D). Taken together, these results suggest that the inhibition of the cyclase activity of CD38 through ADP-ribosylation by arginine mono-ADPRT blocks intracellular cADPR production, which subsequently results in decreases in intracellular free Ca$^{2+}$ in activated T cells and culminating in apoptosis.

We further determined which arginine residue of CD38 is ADP-ribosylated to inactivate the cyclase activity. We converted residues Arg-66 to Gly-66 (R66G), Arg-78 to Gly-78 (R78G), Arg-269 to Gly-269 (R269G), and Arg-280 to Gly-280 (R280G) by site-specific mutagenesis and transfected HeLa cells with the mutated cDNAs (R66G, R78G, R269G, or R280G). The transfected cells were incubated with NAD and arginine mono-ADPRT and the ADP-ribosylation of CD38 was examined. We found that Arg-269 was the only acceptor of ADPRT (Fig. 5A).

We then asked whether R269G mutant-transfected cells are resistant to NAD-induced apoptosis because this CD38 mutant would not be ADP-ribosylated upon addition of NAD. Indeed, R269G mutant-transfected HeLa cells did not undergo apoptosis by NAD treatment. With regard to Arg mutant that retains WT levels of ADP-ribosyl cyclase but cannot serve as a substrate for the Arg-ADPRT, we generated a R269K mutant and its transfectant cell line. These cells had 70% ADP-ribosyl cyclase activity of the control WT, and the cells' CD38 could not serve as a substrate for the Arg-ADPRT, therefore these cells did not undergo any apoptosis by NAD treatment (data not shown). This result also indicates that ADP-ribosylation of CD38 is an important signal that can lead to apoptosis.

The ligation of CD38 by agonistic CD38 antibodies has earlier been shown to elicit cellular responses, including elevation in cytoplasmic Ca$^{2+}$ due to Ca$^{2+}$ influx (1, 31). Also, Guse et al. (32) recently demonstrated sustained increases in [cADPR] and Ca$^{2+}$ entry by the stimulation of TCR-CD3 complex in T lymphocytes. The mechanism by which cADPR regulated Ca$^{2+}$ entry was suggested to be similar to that of inositol 1,4,5-trisphosphate in the capacitative Ca$^{2+}$ model, because cADPR mediated depletion of the Ca$^{2+}$ pool by releasing Ca$^{2+}$ from its target storage (32). Therefore, in order to examine the effect of ADP-ribosylation on CD38 ligation-induced signaling in control WT- and R269K mutant-transfected cells, we measured the [cADPR], and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]), following CD38 ligation in cells pretreated with or without NAD. A prior incubation of WT-transfected HeLa cells with NAD completely abolished CD38 ligation-induced increase of cADPR production (Fig. 5B) and [Ca$^{2+}$] (Fig. 5C), whereas cells without NAD treatment had increased [cADPR], and [Ca$^{2+}$]. The
The first requirement for this pathway is likely CD38 expression (possessing cyclase activity) and the second requirement is ADP-ribosylation of CD38 (inactivation of cyclase). The present studies using mutant enzymes can be extrapolated to activated T cells by assuming that CD38 expression (cyclase activity) can accelerate cell cycling necessary for proliferation (12). In activated T cells, CD38 may be essential for the proliferation via Ca$^{2+}$ influx by the action of cADPR which is generated by the ADP-ribosyl cyclase activity of CD38. However, ADP-ribosylation of CD38 results not only in cessation of cell proliferation but also in apoptosis of the cells, which the mechanism by which apoptosis is induced by ADP-ribosylation of CD38 may be related to the cell cycle, because actively proliferating cells are sensitive to the apoptotic insults such as storage of Ca$^{2+}$ supply.

For our ex vivo observations to have direct physiologic relevance, NAD, an intracellular metabolite, should be available extracellularly to support the ADP-ribosylation of CD38. Extracellular NAD concentrations have been reported to be submicromolar in the plasma of mammals including mice (33), whereas micromolar NAD concentrations are likely to be needed for effective ADP-ribosylation of CD38 in the extracellular compartment (Fig. 2A). However, a recent finding by Zocchi et al. (12) that NAD is steadily released from fibroblast and epithelial cells supports the physiologic relevance of our observations as they suggest that micromolar NAD concentrations, especially in the vicinity of the outer surface, can be reached. Furthermore, conditions, such as massive cell lysis during inflammatory immune reactions may also lead to an increase in the concentration of NAD in the plasma, consequently enhancing the ADP-ribosylation of CD38.

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