Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts

Short title for running head: rafts control ART2.2 enzyme activity

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**Abstract**

ADP-ribosyltransferases (ARTs) transfer ADP-ribose from NAD onto target proteins. T cells express ART2.2, a toxin-related, GPI-anchored ecto-enzyme. Following release of NAD from cells, ART2.2 ADP-ribosylates the P2X7 purinoceptor, lymphocyte function associated antigen (LFA-1), and other membrane proteins. Using lymphoma transfectants expressing either ART2.2 with its native GPI-anchor (ART2.2-GPI) or ART2.2 with a grafted transmembrane anchor (ART2.2-Tm) we demonstrate that ART2.2-GPI but not ART2.2-Tm associates with glycosphingolipid enriched microdomains (lipid rafts). At limiting substrate concentrations ART2.2-GPI exhibits a more than 10-fold higher activity than ART2.2-Tm. On intact cells ART2.2-GPI ADP-ribosylates a small number of distinct target proteins. Strikingly, disruption of lipid rafts by cyclodextrin or membrane solubilization by Triton X-100 increases the spectrum of modified target proteins. However, ART2.2 itself is a prominent target for ADP-ribosylation only when GPI-anchored. Furthermore, cholesterol depletion or detergent solubilization abolishes auto-ADP-ribosylation of ART2.2. These findings imply that ART2.2-GPI but not ART2.2-Tm molecules are closely associated on the plasma membrane and lend support to the hypothesis that lipid rafts exist on living cells as platforms to which certain proteins are admitted and others are excluded. Our results further suggest that raft association focuses ART2.2 on specific targets that constitutively or inducible associate with lipid rafts.
Introduction

ART2.2 is a GPI-anchored ecto-enzyme expressed on the surface of most terminally differentiated T cells.\(^1\) ART2.2 is related in structure and function to ADP-ribosylating bacterial toxins.\(^2-4\) Following the release of the ART substrate, NAD, from cells by lytic or nonlytic mechanisms, ART2.2 catalyzes the transfer of ADP-ribose from NAD onto arginine residues onto leukocyte function associated antigen LFA-1, the P2X7 purinoceptor and other cell surface proteins.\(^5,6\) Akin to protein phosphorylation, protein ADP-ribosylation usually profoundly affects the function of the modified target protein.\(^7,8\) ADP-ribosylation activates P2X7, triggering Calcium flux, flashing of phosphatidylserine, and formation of a nonselective pore in the cell membrane.\(^6\) ADP-ribosylation of LFA-1 and other cell surface proteins inhibits the binding of T cells to target cells and interferes with the clustering of the T cell receptor.\(^5,9\)

Lipid rafts, also known as GEMs (glycosphingolipid enriched membranes) or DIGs (Detergent-insoluble glycosphingolipid-enriched membranes) are plasma membrane microdomains enriched in gangliosides and cholesterol which form liquid ordered domains.\(^10-12\) Lipid rafts are postulated to segregate molecules in the plasma membrane and to regulate signaling through the spatial coordination of intermolecular interactions. Lipid rafts are characterized by insolubility in nonionic detergents and low buoyancy in sucrose density gradients. The posttranslational modification of proteins with saturated acyl groups can result in their localization within lipid rafts. Thus these microdomains are enriched in many signaling molecules such as the lck protein kinase, the adaptor protein LAT, heterotrimeric G proteins, and GPI-anchored proteins.\(^13\) In addition, the T cell receptor and other transmembrane receptors
including the IL2-receptor and LFA-1 are inducibly recruited or stabilized in lipid rafts. Activation of signaling molecules in lipid rafts is thought to facilitate signaling through the TCR and other immunoreceptors.

Some cell surface proteins lack a transmembrane-spanning domain and are anchored in the outer leaflet of the plasma membrane by a glycosylphosphatidylinositol (GPI) moiety. The physiological significance of the GPI anchor is not known. Despite the fact that GPI-anchored proteins are restricted to the outer leaflet of the membrane lipid bilayer and lack a cytoplasmic domain, many GPI-anchored proteins can mediate signaling events following the binding of specific antibodies. In case of Qa-2, CD55, and CD59 it has been shown that exchange of the GPI anchor by a transmembrane anchor abrogates antibody-induced signaling. The association of GPI-anchored proteins with Src family kinases in lipid rafts may explain the involvement of GPI-anchored proteins in signaling.

Considering that ART2.2 and other members of the ART-family carry a GPI-anchor, we hypothesized that the GPI-anchor mediates lateral segregation of ART2.2 in rafts and that this might be a mechanism to regulate the activity and specificity of ART2. In order to test these hypotheses, we have adopted an experimental approach that has been successfully used in previous studies addressing the lateral segregation of cell surface enzymes in membrane microdomains. By replacing the native transmembrane anchors of angiotensin converting enzyme (ACE) or β-secretase by a GPI-anchor Parkin et al. and Cordy et al. successfully sequestered these enzymes in lipid rafts. In this study, we employed the mirror image approach and have replaced the native GPI-anchor of ART2.2 with the transmembrane and cytosolic domains of CD8. Our results, indeed, indicate that sequestration within lipid rafts controls the activity and specificity of ART2.
Materials and Methods

Materials

AEBSF, etheno-NAD, G418, and cholera toxin B subunit (CT), were obtained from Sigma Chemical Cooperation (Deisenhofen, Germany). Monoclonal antibodies (mAbs) used in this study for immunofluorescence staining and/or immunoprecipitation include anti-FLAG (M2), anti-ART2.2 (AliA53), anti-LFA-1 (M17/4), and anti-etheno-adenosine (1G4). PE- and FITC-conjugated antibodies were purchased from Pharmingen/Becton-Dickinson, Heidelberg, Germany. Anti-ART2.2 AliA53 and P2X7-specific antiserum K1G were raised by gene-gun immunization as described previously. Antibodies were purified by affinity chromatography on protein G sepharose (Pharmacia) and conjugated to fluorochrome Alexa according to the manufacturer's instructions (Molecular Probes). Anti-ART2.2 specific mAbs 9-13 and 10-6 (rat IgG2a) were kindly provided by Dr. Lucienne Chatenoud, Paris. Anti-Gß polyclonal antibody (pAb) T20 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cells and generation of ART2.2 transfectants

The mouse lymphoma cell line DC27.10 was kindly provided by Dr. Bernhard Fleischer, Hamburg. Vectors for expressing mouse ART2.2 in mammalian cells were cloned as described previously, replacing sequences for the N-terminal leader and/or C-terminal GPI-signal sequence. Expression constructs were transfected into DC27.10 cells by electroporation (250 mV, 900 µF) using 10 µg DNA/10^7 cells in 700 µL RPMI and a Gene pulser (BioRad GmbH, München). Stable transfectants were obtained by selection in medium supplemented with G418 (1 mg/mL). Cells
were subcloned by limiting dilution and clones were analyzed for ART2.2 expression levels by FACS analyses.

**FACS-analyses and immunocytochemistry**

Cells (5 x 10^5/100 µL) were incubated with or without phosphatidylinositol-specific phospholipase C (PI-PLC, Molecular Probes, 0.01U/mL in PBS) for 1h at 37°C. For extraction of membrane proteins, cells were incubated in PBS containing 1% Triton-X-100 (TX-100) at 4°C for 10 min, diluted 10-fold in ice-cold PBS 2% PFA and fixed at 4°C for 30 min. Cells were stained with fluorochrome-conjugated antibodies and analyzed by flow cytometry on a FACS-Calibur (Becton Dickinson, Heidelberg, Germany) and the CellQuest software as described previously. Dead cells were excluded after staining with propidium iodide. For immunocytochemistry, stained and fixed cells were centrifuged onto microscope slides using a cytospin centrifuge and mounted in fluorescent mounting medium (DAKO, Hamburg). Cells were visualized using a Leica Confocal DM LSFA Microscope with a 40x objective lens and the Leica Confocal software.

**Incubation of cells with etheno-NAD and staining with 1G4**

Cells (1 x 10^6) were incubated with the indicated concentrations of etheno-NAD in 100 µL RPMI medium for the indicated times at 37°C. Cells were diluted 20-fold in medium and were washed twice. This was followed by incubation with 1G4-Alexa488 (1 µg/mL) or control antibodies for 30 min at 4°C. Cells were then analyzed by flow cytometry as described previously.27

**Isolation of lipid rafts by density gradient centrifugation**

Cells (2 x 10^7) were resuspended in 1 mL ice-cold lysis buffer (25 mM MES, 150 mM NaCl, 5 mM EDTA, 1 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF),
1% TX-100) at 4°C for 30 min. This was followed by six pulses (5 sec) of ultrasonication (Bender ultra, low power setting). An equal volume of an 80% sucrose solution (in lysis buffer) was mixed with the lysate, and a step gradient was formed by overlaying with 6 mL of 30% sucrose and 4 mL of 5% sucrose. Isopycnic equilibration was achieved by centrifugation at 200,000 g for 14 h in a SW40 rotor (Beckman) at 4°C. Twelve fractions (1 mL) were harvested from the top. Aliquots of each fraction (400 µL) or from pooled fractions were acetone precipitated and subjected to Western Blot analyses with the indicated antibodies. The pellet from the sucrose density gradients was dissolved directly in 2% SDS (100 µL) and a 5 µL aliquot was used for SDS-PAGE.

**Cell solubilization, immunoprecipitation, SDS-PAGE, and Western-Blot analyses**

Cells were incubated in RPMI with or without etheno-NAD (1-50 µM) or 1 µM 32P-NAD (5 µCi) for 10 min at 37°C. Cells were washed and resuspended in ice cold lysis buffer (1 x 10^7 cells/mL PBS containing 0.05% or 1% TX-100, 1 mM AEBSF). After solubilization for 5 min at 4°C, insoluble material was removed by centrifugation (5 min, 500g, 4°C). The supernatant was collected (4°C lysate). Pellets were resuspended in prewarmed (37°C) lysis buffer and incubated for 20 min at 37°C. Insoluble material was removed by centrifugation (10 min, 500g, room temperature). The supernatant was collected (37°C lysate). Pellets were resuspended in PBS, 2% SDS and solubilized on ice by two pulses (10 sec) of ultrasonication (Bender ultra, high power setting). For immunoprecipitation, anti-P2X7 antiserum K1G was conjugated to Aminolink matrix (Pierce) according to the manufacturer’s instructions. Anti-LFA-1 mAb M17/4 was immobilized on Protein G Sepharose (Amersham-Pharmacia, Braunschweig, Germany) (1 µg/10 µL beads). Lysates were cleared by
high speed centrifugation (15 min 15,000g) followed by incubation with Protein G Sepharose for 60 min at 4°C. Cleared lysates were incubated with immobilized antibodies (100 µL lysate/10 µL beads) for 60 min at 4°C. Immunoprecipitates were washed five times in lysis buffer. Bound material was eluted by heating in SDS-PAGE sample buffer for 10 min at 70°C. Proteins were size fractionated by electrophoresis on precast gels (Novex, Heidelberg, Germany) and blotted onto Nitrocellulose (Hybond-N, Amersham-Pharmacia) or PVDF (Immobilon, Applied Biosystems) membranes as described previously. Radioactivity incorporated into proteins was visualized by exposure of blots to Kodak X-Omat film for 24 h at –80°C.

For immunoblot-analyses, PVDF membranes were blocked with 5% dry milk (Nestle) and proteins on the blots were detected by specific antibodies and the the enhanced chemiluminescence system (Amersham, Braunschweig, Germany). Blots were first probed with mAb 1G4 (0.5 µg/mL in TBS, 1% dry milk, 0.05% Tween-20) and peroxidase-conjugated goat anti-mouse IgG. Subsequently blots were stripped with Re-Blot Plus stripping solution (Chemicon, Temecula, CA), and probed with peroxidase-conjugated mAb M2 and peroxidase-conjugated CT. Blots were stripped again and probed further with pAb T20 and peroxidase-conjugated anti-rabbit IgG. Quantification of labeled bands was performed by densitometric scanning analyses using the AIDA 2.11 software (Raytest, Straubenhardt, Germany).
Results

Expression of ART2.2-GPI and ART2.2-Tm in DC27.10

DC27.10 mouse lymphoma cells, which do not express detectable levels of ART2, were stably transfected with cDNA encoding either GPI-anchored ART2.2 or ART2.2-Tm in which the GPI-signal sequence was replaced with the distal juxtamembrane stalk, the transmembrane domain, and cytoplasmic tail of CD8 (Fig. 1A). Both constructs were epitope tagged at the N-Terminus with a FLAG-tag. FACS analyses with ART2.2-specific mAb AliA53 and FLAG-tag specific mAb M2 confirmed cell surface expression of both constructs. Stable transfectants were selected and subcloned by limiting dilution. Subclones were tested for level of cell surface ART2.2 expression and clones with intermediate but similar expression levels were selected for further analyses (Fig. 1B). Lysates from untransfected and ART2.2-transfected cells were subjected to immunoblot analyses with ART2.2-specific mAbs 9-13/10-6 or with anti-FLAG mAb M2. ART2.2-Tm migrated with a molecular weight of 45 kd, whereas ART2.2-GPI migrated with an apparent molecular weight of 29 kd (see Fig. 3 below).

ART2.2-GPI but not ART2.2-Tm is released from cells by PI-PLC while ART2.2-Tm but not ART2.2-GPI is released from cells by treatment with TX-100 at 4°C

GPI-anchored proteins can be released from the cell surface by treatment with bacterial PI-PLC, while transmembrane proteins are not affected by PI-PLC treatment. Conversely, most transmembrane proteins can be released from the cell surface by treatment with the nonionic detergent Triton X-100 at 4°C, while GPI-
anchored proteins are resistant to this treatment.\textsuperscript{30} To confirm the nature of the membrane anchors of the ART2.2 constructs, ART2.2-transfected cells were treated with PI-PLC or TX-100, and then subjected to FACS analyses with ART2.2-specific mAb AliA53. For control, cells were also stained for cell surface expression of the type 1 transmembrane protein LFA-1 and for the raft marker GM1 (Fig. 2A). Treatment of cells with PI-PLC resulted in an 84\% reduction of the cell surface staining level for ART2.2-GPI (Fig. 2A). In contrast, staining levels for ART2.2-Tm, LFA1 and GM1 were not affected by PI-PLC. Immunoblot analyses of cell supernatants from PI-PLC treated cells confirmed that most of ART2.2-GPI but no detectable ART2.2-Tm was released into the cell supernatant by treatment of cells with PI-PLC (Fig. 3B lanes 4 and 5).

Conversely, treatment of cells with TX-100 at 4°C led to a dramatic (97-98\%) reduction in cell surface staining of ART2.2-Tm and LFA1 (Fig. 2B). Similar results were obtained when cells were subjected to immunocytochemistry (Fig. 2C). When the cells were incubated with 1\% TX-100 at 4°C prior to fixation, the level of fluorescence in ART2.2-Tm expressing cells was dramatically reduced (Fig. 2C, panel 8) consistent with this construct residing in detergent-soluble regions of the plasma membrane. In contrast, ART2.2-GPI and GM1 retained a punctuate staining pattern after pretreatment of cells with TX-100 (Fig. 2C, panels 5 and 6), consistent with the localization of ART2.2-GPI in detergent-resistant rafts.

**Differential segregation of ART2.2-Tm and ART2.2-GPI following a two step lysis protocol in Triton X-100**

Although resistant to TX-100 solubilization at 4°C, GPI-anchored proteins reportedly can be solubilized by TX-100 at 37°C.\textsuperscript{31,32} In order to test the solubility of ART2.2-
GPI and ART2.2-Tm by TX-100, we subjected cells to a two step lysis protocol, in which cells were first lysed in TX-100 for 10 minutes at 4°C (Fig. 3). Insoluble material was pelleted by centrifugation and the supernatant (4°C lysate) was harvested for analysis. Pellets were resuspended in prewarmed TX-100 and incubated for 20 min at 37°C. Insoluble material was again pelleted by centrifugation and the supernatant (37°C lysate) was harvested for analysis. Proteins in TX-100 resistant pellets were solubilized by ultrasonication in SDS. Size fractionation of proteins by SDS-PAGE and staining for total protein (Fig. 3A) revealed that the 4°C lysate contains most (> 80%) of the cellular protein (lane 1), whereas the 37°C lysate contains < 5% of total cell protein (lane 2). Histones appear as prominent bands only in the TX-100 resistant pellet fraction (lane 3). Immunoblot analyses (Fig. 3B) reveal that ART2.2-Tm is almost completely solubilized by TX-100 at 4°C, whereas little if any ART2.2-GPI is contained in the 4°C lysate (lane 1). ART2.2-GPI, however, is readily solubilized at 37°C (lane 2). The traditional raft marker GM1 behaves like ART2.2-GPI: little if any GM1 is solubilized by TX-100 at 4°C while a prominent GM1 band is obtained in the 37°C lysate. The heterotrimeric G-protein β subunit segregates partially into the 4°C and 37°C lysates. Upon treatment of cells with PI-PLC, most of ART2.2-GPI is released into the cell supernatant (lane 4), while ART2.2-Tm, GM1, and Gß are resistant to PI-PLC (lane 5).

GPI-anchored but not transmembrane-anchored ART2.2 associates with glycosphingolipid-enriched microdomains (lipid rafts)

In order to determine whether ART2.2-GPI segregates with lipid rafts, cells were solubilized by ultrasonication in TX-100 at 4°C and lipid rafts were isolated by sucrose gradient centrifugation. The position of lipid rafts was evident by an opaque
band at the 5%/30% sucrose interface and was confirmed by immunoblotting for
GM1 (Fig. 4A, B). Only a small portion of the solubilized proteins was present in this
fraction (Fig. 4C). Most cellular proteins were located in the high-density fraction 12
and pellet (P) of the sucrose gradient (Fig. 4C). Similar to GM1, ART2.2-GPI was
strongly enriched in fraction 4, whereas only a small portion of ART2.2-Tm was
found in this fraction (Fig. 4A, B). These results further support the notion that most
of ART2.2-GPI is raft associated whereas little if any ART2.2-Tm is raft associated.

GPI-anchored ART2.2 mediates faster and more efficient etheno-ADP-
ribosylation of cell surface proteins than ART2.2-Tm

Using etheno-NAD as substrate and mAb 1G4 specific for etheno-adenosine,
(etheno)-ADP-ribosylated cell surface proteins can be detected by flow cytometry.27
Following a 10 minute incubation of cells with 50µM etheno-NAD, ART2.2-GPI and
ART2.2-Tm transfected cells showed similar labeling with 1G4, indicating that both
forms of ART2.2 are enzymatically active (Fig. 5A). Untransfected DC27.10 cells
showed only background staining with 1G4, confirming that ART2.2 is required for
ADP-ribosylation of cell surface proteins. Dose response analyses revealed a much
stronger incorporation of etheno-NAD by ART2.2-GPI transfected cells than by
ART2.2-Tm transfected cells at low substrate concentrations (Fig. 5B). Half maximal
staining was obtained with 1µM etheno NAD in case of ART2.2-GPI transfected cells
vs. 20µM etheno-NAD in case of ART2.2-Tm transfectants. Similarly, kinetic
analyses (Fig. 5C) revealed much faster etheno-ADP-ribosylation of cell surface
proteins by ART2.2-GPI transfected cells than by ART2.2-Tm transfectants (half
maximal staining < 1 min vs. > 20 min).
Disruption of lipid rafts by MCD inhibits cell surface protein ADP-ribosylation catalyzed by ART2.2-GPI

To further investigate the influence of raft association on ART2.2-activity, we used an established protocol for disrupting lipid rafts on living cells, MCD-mediated extraction of cholesterol. Pretreatment of cells with MCD, indeed, strongly inhibited the incorporation of etheno-ADP-ribose by ART2.2-GPI transfected cells, but had no detectable effect on etheno-ADP-ribosylation catalyzed by ART2.2-Tm (Fig. 6A). To determine whether MCD treatment affected the solubility of proteins in TX-100, cells treated with MCD were subjected to sequential lysis in TX-100 and immunoblot analysis. As shown in Figure 6B, pretreatment of cells with MCD, indeed, markedly enhanced the solubility of ART2.2-GPI, GM1, and Gß in TX-100 at 4°C in a dose dependent manner, consistent with the disruption of rafts by MCD treatment. Densitometric analyses showed that only 14% of ART2.2-GPI was solubilized by TX-100 at 4°C in untreated cells, whereas 45% of ART2.2-GPI was solubilized in MCD treated cells (Fig. 6B lanes 1 and 7).

Remarkably, MCD treatment also strongly affected the pattern of target proteins etheno-ADP-ribosylated by ART2.2-GPI (Fig. 6C). Immunoblot analyses with mAb 1G4 reveals several prominent bands in case of untreated ART2-GPI transfectants following incubation with 1µM etheno-NAD (lanes 1 and 2). 1G4 staining intensity of these bands is markedly reduced after MCD treatment and additional etheno-ADP-ribosylated protein bands become visible (lanes 4 and 5). Overall, the pattern of etheno-ADP-ribosylated bands is diffuse and lighter in MCD-treated than in untreated cells.

Note that the size of the prominent 29kd target protein band in the 37°C lysate of untreated cells corresponds to that of ART2.2-GPI (Fig. 6C, lane 2).
Immunoprecipitation confirmed that this band represents etheno-ADP-ribosylated ART2.2-GPI (not shown). Note that ADP-ribosylated ART2.2-GPI is no longer detectable in the 37°C TX-100 lysates of MCD treated cells (Fig. 6C, lane 5). Evidently, MCD treatment of cells results in weaker labeling and increased solubility of ART2.2-GPI. This is consistent with trans-ADP-ribosylation of ART2.2-GPI occurring in intact lipid rafts, but not after disruption of rafts with MCD. A similar phenomenon is observed for other detergent-resistant, etheno-ADP-ribosylated bands in untreated vs. MCD treated cells (Fig. 6C, lane 2 vs. lane 5). Note also that in case of ART2.2-Tm transfectants incubated with 1µM etheno-NAD, 1G4 immunoblot analyses do not reveal any detectable bands (Fig. 6C, lanes 7-9); consistent with the notion that the label incorporated by ART2.2-Tm transfectants is distributed over many weakly labeled proteins rather than a few prominent targets.

Membrane compartmentalization restricts the specificity of target protein ADP-ribosylation by ART2

To further extend the above findings we performed comparative 1G4 immunoblot analyses of untransfected, ART2.2-GPI transfected and ART2.2-Tm transfected DC27.10 cells following incubation in the presence of low (1 µM) (Fig. 7A) and very high substrate concentrations (50 µM etheno-NAD) (Fig. 7B). In order to monitor the TX-100 solubility of ART2.2 and its target proteins, cells were again lysed by the two-step solubilization procedure. Control protein staining (not shown) and immunoblot analyses with CT and M2 (Fig. 7. top panels) confirmed the segregation of type 1 membrane proteins in the 4°C lysates, GM1 and GPI-anchored proteins in the 37°C lysates, and nuclear proteins in the TX-100 resistant pellet (P). In accord with the results of the FACS analyses (Fig. 5), labeling of target proteins was much more
efficient in ART2.2-GPI transfectants than in ART2.2-Tm transfectants. Note that in striking contrast to ART2.2-GPI, little if any ART2.2-Tm is etheno-ADP-ribosylated (lane 5 vs. lane 7 in Fig. 7A and 7B). Even at 50µM etheno-NAD, no labeling was observed in untransfected cells (Fig. 7B, lanes 1-3), confirming that target protein etheno-ADP-ribosylation requires ART2.

Comparing the patterns of bands obtained with 1µM vs. 50µM etheno-NAD, ART2.2-GPI evidently labels a much broader spectrum of bands at higher NAD concentrations (e.g. compare Fig. 7B vs. 7A, lanes 4-6). Note that at high etheno-NAD concentrations ART2.2-GPI and ART2.2-Tm apparently also label bands that are resistant to TX-100 solubilization even at 37°C (Fig. 7B, lanes 6 and 9).

We further compared the extent of ADP-ribosylation of LFA-1 and P2X7, two known target proteins of ART2.2, following incubation of ART2.2-GPI and ART2.2-Tm transfectants with radiolabeled NAD. The results of immunoprecipitation analyses show that both proteins are modified much more efficiently by ART2.2-GPI than by ART2.2-Tm (not shown). FACS analyses (not shown) revealed that P2X7 expression on DC27.10 cells is much lower than on primary T cells, consistent with the relatively weak radiolabeling of P2X7 in Fig. 7D compared to that by primary lymphocytes. Note that both target proteins, were efficiently solubilized at 4°C by 1% TX-100 (Fig. 7D, lanes 3). Takei and coworkers reported that a substantial fraction of LFA-1 associated with lipid rafts on EL4 and T28 mouse lymphoma cells and further that the association of LFA-1 with lipid rafts was disrupted when cells were lysed with 1% TX-100 but not - or only partially - when using 0.05% TX-100 which is thought to better preserve the integrity of lipid rafts. Similarly, when DC27.10 cells were lysed with 0.05% TX-100, LFA-1, P2X7 and most other ADP-ribosylated proteins were found largely in the 37°C fraction (Fig. 7D, lanes 2). In contrast, most of ART2.2-
Tm (not shown) and most of the total protein were found in the 4° fraction (Fig. 7D) under these conditions.

**Disruption of membrane integrity results in a dramatic increase in the extent of ART2.2-catalyzed protein ADP-ribosylation**

In order to test whether association with the membrane restricts the access of ART2.2 to certain targets, we compared the ADP-ribosylation of target proteins on intact cells vs. cell lysates (Fig. 7C). Indeed, when 1 µM etheno-NAD was added to cell lysates rather than to intact cells, a dramatic (> 50 fold) increase in the extent of protein etheno-ADP-ribosylation was observed (compare Fig. 7C vs. 7A). Promiscuous etheno-ADP-ribosylation was now observed for both ART2.2-GPI and ART2.2-Tm even at this low etheno-NAD concentration. This implies that membrane association focuses ART2.2 onto specific targets.
Discussion

Lipid rafts are thought to play an important role in mediating signal transduction through the cell membrane. Many lymphocyte ecto-enzymes, including alkaline phosphatase, 5' nucleotidase and ADP-ribosyltransferases are GPI-anchored. Since the GPI anchor is thought to mediate raft association it is conceivable that the sequestration within lipid rafts controls the activity of GPI-anchored ectoenzymes. Here we have addressed this hypothesis for T cell ecto-ADP-ribosyltransferase ART2.2 by exchanging its native GPI-anchor with the transmembrane anchor of CD8, a classic type I membrane protein. In contrast to native ART2.2-GPI, ART2.2-Tm was insensitive to cleavage by PI-PLC (Fig 2) and showed little if any association with the lipid raft fraction upon sucrose density gradient centrifugation (Fig. 4). Moreover, in contrast to native ART2.2-GPI, ART2.2-Tm was readily solubilized by TX-100 at 4°C (Figs. 2 & 3). These results strongly suggest that ART2.2-GPI and ART2.2-Tm are associated with distinct membrane (micro) domains.

The results of our solubilization assays are in accord with those of previous studies on the temperature-dependent solubilization of GPI-anchored associated proteins by TX-100. Filatov et al. showed that resistance of raft-associated proteins to solubilization by TX-100 at low temperature could be assessed by FACS analyses. Our results confirm this observation for ART2.2-GPI and extend it also to the classic raft marker asialo GM1 (Fig. 2). Similarly, resistance of ART2.2-GPI and asialo GM1 to low temperature detergent solubilization can be monitored by immunofluorescence microscopy (Fig. 3B). Moreover, we show that despite its resistance to solubilization by TX-100 at 4°C, ART2.2-GPI is readily solubilized at 37°C (Figs. 2 & 3). Indeed, by treating cells sequentially with TX-100 first at 4°C and then at 37°C, GPI-
anchored proteins and asialo GM1 could be separated from transmembrane-anchored proteins (Fig. 3A). Therefore, differential solubilization of cells by TX-100 at 4°C vs. 37°C may provide a convenient and rapid evaluation of the possible association of membrane proteins and glycolipids with lipid rafts.

Interestingly, our results indicate that the lateral distribution of ART2.2 within the cell membrane dramatically affects its capacity to ADP-ribosylate other cell surface proteins. Using a recently established immunoassay for monitoring ART-activity on living cells,27 we found that both ART2.2-GPI and ART2.2-Tm could catalyze ADP-ribosylation of cell surface proteins (Fig. 5A). However, ADP-ribosylation catalyzed by ART2.2-GPI was much faster than that by ART2.2-Tm (Fig. 5C). Moreover, ADP-ribosylation by ART2.2-GPI was much more effective at lower - and presumably more physiological - NAD concentrations than that by ART2.2-Tm (Fig. 5B). Only at very high NAD concentrations and long incubation times, did ADP-ribosylation reach similar levels of saturation for ART2.2-GPI and ART2.2-Tm transfected cells (Fig. 5).

Steady state levels of extracellular NAD in biological fluids, e.g. in serum, are in the submicromolar range, whereas intracellular NAD-concentrations reach 200-500µM.35 Following the lytic disruption of the plasma membrane, e.g. during tissue damage, hypoxia, or inflammation, local extracellular NAD concentrations can rise dramatically. Moreover, NAD reportedly can also be released nonlytically from some cells in a regulated manner through Connexin-43 hemichannels.36 Extracellular NAD, however, is rapidly metabolized by ecto-NADases (such as CD38) and ecto-phosphodiesterases (such as PC-1).34 Most likely, then, the relevant in vivo NAD concentrations and NAD exposure times are in a range (< 10 µM, < 20 minutes) in
which ART2.2-GPI shows more than tenfold higher activity than ART2.2-Tm in lymphoma cells (Fig. 5).

It has been proposed that GPI-anchorage may influence the lateral mobility within the cell membrane. Our findings that ART2.2-GPI mediates cell surface ADP-ribosylation with much faster kinetics than ART2.2-Tm, but that both forms reach similar saturation levels at long incubation times, in principle, is consistent with a faster lateral mobility of ART2.2-GPI vs. ART2.2-Tm. Furthermore, it is conceivable that the length and flexibility of the juxtamembrane stalk affects access of ART2.2 to arginine residues in potential target proteins. In ART2.2-Tm, there are 20 amino acids between the hydrophobic transmembrane segment and the fourth conserved cysteine residue (which is engaged in disulfide bond to the first cysteine in the 3D structure of rat ART2). This corresponds to the distance from the cell membrane in ART2.2-GPI, encompassing the hydrophilic sugar residues of the GPI-anchor and 16 amino acids between the site of the GPI-anchor attachment on the first Ser residue in the Ser-Gly-Ser motif (ω, ω + 1, ω + 2) and the fourth conserved cysteine (Fig. 1). The finding that both forms of ART2.2 can ADP-ribosylate known target proteins (LFA-1 and P2X7) (Fig. 7) and the finding that both forms of ART2.2 reach similar saturation levels of cell surface protein ADP-ribosylation at high NAD concentrations and long incubation times indicate that the observed striking differences in activity of ART2.2-GPI vs. ART2.2-Tm at low substrate concentrations and short incubation times (Fig. 5) are not due to differences in the lengths of the juxtamembrane stalk.

Strikingly, when relieved from the restraint of the cell membrane, both ART2.2-GPI and ART2.2-Tm show dramatically enhanced enzyme activities (Fig. 6C), implying that membrane association restricts the accessibility of ART2.2 to certain targets. When in solution, both ART2.2-GPI and ART2.2-Tm evidently are extremely
promiscuous and ADP-ribosylate many different proteins. This is in accord with previous reports showing that soluble recombinant ART2.2 can ADP-ribosylate many nonphysiologic targets including histones, antibodies and even free arginine. Moreover, the lateral distribution within the cell membrane - orchestrated by its membrane anchor - appears to further restrict the access of ART2.2 toward its targets. This interpretation is supported by the finding that disruption of lipid rafts by MCD-treatment alters the pattern of proteins ADP-ribosylated by ART2.2-GPI (Fig. 6). In untreated cells ART2.2-GPI modifies a small set of distinct target proteins. MCD-mediated depletion of cholesterol leads to a much broader and more diffuse pattern of ADP-ribosylated target proteins (Fig. 6C). Interestingly, two prominent targets of ART2-catalyzed ADP-ribosylation, LFA-1 and P2X7, at least partially localize to the raft fraction (Fig. 7D), suggesting that raft association facilitates ADP-ribosylation of these target proteins. However, raft association of a particular protein alone is not sufficient for it to be targeted by ART2 (e.g. compare total vs. radiolabeled protein bands in Fig. 7D). There must be other unknown factors besides membrane composition which influence the ability of ART2 to target particular proteins. For example, accessibility of arginine residues on a protein could determine whether it can be ADP-ribosylated or not. Moreover, surface loops adjacent to the NAD binding site of ART2 could confer target specificity, as was shown recently for exoenzyme S from *P. aeruginosa*, the closest ART-homologue from the bacterial world, which - like ART2 - can promiscuously ADP-ribosylate many different targets. Since much of the evidence for the existence and function of rafts has relied on biochemical analyses following detergent extraction rather than on studies with living cells, there has been considerable recent debate concerning the presence and size of rafts in living cells. Our results bear some relevance to this debate, since they
show that the activity and specificity of a cell surface enzyme on living cells depends
on the nature of its membrane anchor (Fig. 5) and the cholesterol content of the cell
membrane (Fig. 6). These results support the notion that membrane proteins, indeed,
are sequestered by lipids in different (micro)-domains on the cell surface of living
cells. In this context it is important to note that ART2.2 itself is a prominent target for
(trans) ADP-ribosylation only when it is GPI-anchored on the cell surface of living
cells but not when it is anchored by a transmembrane domain (Figs. 6 and 7).
Furthermore, ADP-ribosylation of ART2.2 is markedly reduced after cholesterol
depletion (Fig. 6C) or detergent extraction (Fig. 7). These findings imply that at least
two or more ART2.2-GPI molecules are in close vicinity on the intact cell surface and
lend support to the hypothesis that lipid rafts exist as platforms, to which certain
proteins are admitted and others are excluded.

On the basis of these observations we propose that raft association provides a
topological isolation for native GPI-anchored ART2.2 and focuses this enzyme onto
specific-targets, e.g. resident raft proteins or proteins that become transiently
associated with rafts. A large body of evidence indicates that rafts play an important
role during T cell activation, where rafts are thought to establish a signal transduction
platform within the 'immunological synapse' between T cell and antigen presenting
cell. It is tempting to speculate that ART2.2 responds to NAD released from the
antigen presenting cell and/or from lysed cells in an inflammatory environment and
regulates the function and geography of raft associated proteins via ADP-ribosylation.
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**Figure legends**

Fig. 1. Schematic diagram of wild-type ART2.2-GPI and ART2.2-Tm (A) and stable expression of ART2.2-GPI and ART2.2-Tm by DC27.10 lymphoma cells (B)

(A) The domain structures of GPI-anchored ART2.2 (ART-GPI) and transmembrane-anchored ART2.2 (ART2.2-Tm) are shown. The N-terminal signal peptide of ART2.2 was replaced by the signal peptide of CD8 (SP) followed by a FLAG-tag. The wild-type GPI-signal sequence of ART2.2-GPI is underlined and the predicted GPI-anchor attachment site is indicated (*). In the ART2.2-Tm construct, the C-terminal GPI anchor signal sequence was replaced by the 21 residue transmembrane domain of CD8 (underlined) and 5 flanking extracellular and 17 cytosolic amino acid residues.

(B) DC27.10 lymphoma cells were stably transfected with either ART2.2-GPI or ART2.2-Tm. Stable transfectants were selected by growth in the presence of G418 and subcloned by limiting dilution. Levels of ART2.2 expression on the cell surface of parental untransfected lymphoma cells and of ART2.2-transfectants were determined by FACS-analysis after staining with Alexa488-conjugated ART2.2-specific mAb AliA53 (solid lines). Dashed lines show cells treated with Alexa488-conjugated isotype control mAb. Numbers indicate mean fluorescence intensity of ART2.2 staining.

Fig. 2. ART2.2-GPI but not ART2.2-Tm is released from cells by exogenous PI-PLC treatment (A) whereas ART2.2-Tm but not ART2.2-GPI is released from cells by treatment with TX-100 at 4°C (B, C)

(A) DC27.10 cells stably transfected with ART2.2-GPI or ART2.2-Tm were incubated for 60 min at 37°C in the absence (solid lines) or presence (dashed lines) of
PI-PLC. Cells were then washed and stained for cell surface expression of GM1, ART2.2 or LFA-1 before FACS analysis. Number indicates the percent reduction of the mean fluorescence intensity of PI-PLC treated vs. untreated ART2.2-GPI transfectants. (B) DC27.10 cells stably transfected with ART2.2-GPI or ART2.2-Tm were stained for cell surface expression of GM1, ART2.2 or LFA1. Cells were then washed and incubated for 10 min at 4°C with or without 1% TX-100 before fixation and FACS analysis. Numbers indicate percent reduction of mean fluorescence intensity of TX-100 treated vs. untreated cells. (C) DC27.10 cells stably transfected with ART2.2-GPI or ART2.2-Tm were stained for cell surface expression of GM1 and ART2. Cells were then washed and incubated for 10 min at 4°C with (+) or without (-) 1% TX-100 before fixation and confocal microscopic analysis. Results are representative of 3 independent experiments.

**Fig. 3. Solubilization of ART2.2-GPI but not ART2.2-Tm by TX-100 is temperature sensitive.**

DC27.10 cells stably transfected with ART2.2-GPI or ART2.2-Tm were pooled (1 x 10⁶ of each cell type) and lysed in 1% TX-100 for 10 min at 4°C (lane 1). Insoluble material was pelleted by centrifugation and solubilized again in 1% TX-100 for 20 min at 37°C (lane 2). Insoluble material was pelleted by centrifugation. Pellets were solubilized by ultrasonication in 2% SDS (lane 3). A separate aliquot of pooled ART2.2-GPI and ART2.2-Tm transfected DC27.10 cells (1 x 10⁶ of each) were incubated for 60 min with PI-PLC. PI-PLC treated cells were pelleted by centrifugation and supernatant collected (lane 4). The cell pellet was solubilized directly by ultrasonication in SDS (lane 5). Proteins were size fractionated by SDS-PAGE and stained with Coomassie brilliant blue (A). The histone bands in lane 3 are indicated (hi). An identical gel was subjected to immunoblot analyses (B). Immunodetection was performed sequentially by incubation of the blot with PO-conjugated cholera toxin (GM1), PO-conjugated anti-FLAG mAb M2 (ART2), rabbit
antiserum T20 followed by PO-conjugated anti rabbit IgG (Gβ). Results are representative of 3 independent experiments.
Fig. 4. ART2.2-GPI segregates with the lipid raft fraction in sucrose gradients
DC27.10 cells stably transfected with ART2.2-GPI (A) or ART2.2-Tm (B) were
solubilized by ultrasonication in 1% Tx-100 at 4°C. Lysates were subjected to sucrose
gradient centrifugation. Isopycnic equilibrium was achieved by centrifugation at
200,000g for 14h. Twelve fractions were harvested from the top of gradient. Fractions
1-3, 6-7, and 8-9 were pooled. Fraction 4 contains the visible opaque band at the
5%/30% sucrose interface. The pellet at the bottom of the tube was resuspended by
ultrasonication in 2%SDS. Proteins in individual or pooled fractions were precipitated
and were subjected to SDS-PAGE-immunoblot analyses using anti-ART2.2 mAbs 9-
13/10-6, anti-Gß pAb T20 and CT. Total protein in each fraction was visualized by
silver staining (C). Results are representative of 3 independent experiments.

Fig. 5. Dose response and kinetics of etheno-ADP-ribosylation of cell surface
proteins by ART2.2-GPI and ART2.2-Tm transfected cells
(A) Untransfected, ART2.2-GPI transfected, or ART2.2-Tm transfected DC27.10
cells were incubated for 10 minutes in the absence (dashed lines) or presence (solid
lines) of 50µM etheno-NAD. Cells were then washed, stained with Alexa488-
conjugated mAb 1G4, and subjected to FACS analysis. Numbers indicate mean
fluorescence intensity of cells incubated with etheno NAD. (B) ART2.2-GPI
transfected (black diamonds), or ART2.2-Tm transfected (open squares) DC27.10
cells were incubated for 3 minutes in the presence of the indicated concentrations of
etheno-NAD. Cells were then subjected to FACS analysis as in A. (C) ART2.2-GPI
transfected (black diamonds), or ART2.2-Tm transfected (open squares) DC27.10
cells were incubated for the indicated times in the presence of 1 µM etheno-NAD.
Cells were then subjected to FACS analysis as in A. Results are representative of 3
independent experiments.

Fig. 6. Disruption of lipid rafts by ßMCD inhibits the activity of ART2.2-GPI
(A) DC27.10 cells stably transfected with ART2.GPI (GPI) or ART2.2-Tm (Tm)
were incubated for 60 min in the absence or presence of 10 mM MCD. Cells were
washed and incubated further in the presence of 1µM etheno-NAD for the indicated times. Cell surface protein ADP-ribosylation was detected by FACS-analysis with Alexa488-1G4 as in Fig. 5. (B) Equal aliquots of ART2.2-GPI and ART2.2-TM cells were mixed and incubated for 60 min in the absence or presence of 5-10 mM MCD. Cells were then lysed in two steps in 1% TX-100 and proteins in cleared lysates were analyzed by immunoblot analysis as in Fig. 3. (C) Cells were incubated for 60 min in the absence or presence of 10mM MCD. Cells were then washed and incubated further for 10 min in the presence of 1µM etheno-NAD. Immunodetection was performed by incubation of blots with mouse anti etheno-adenosine mAb 1G4 followed by PO-conjugated anti-mouse IgG (1G4). Bound antibody was detected with the enhanced chemiluminescence system (ECL).

Fig. 7. Membrane compartmentalization restricts the specificity of target protein (etheno)-ADP-ribosylation by ART2.2

(A, B) Untransfected (Untr), ART2.2-GPI-transfected (GPI), and ART2.2-Tm-transfected (Tm) DC27.10 cells were incubated for 10 min in the presence of 1µM (A) or 50 µM etheno-NAD (B). Cells were washed and then lysed in two steps in 1% TX-100 for 10 min at 4°C and then for 20 min at 37°C as in Fig. 3. TX-100 resistant pellets (P) were solubilized by sonication in 2% SDS. Proteins in cell lysates were acetone precipitated and subjected to size fractionation by SDS-PAGE followed by Western-blotting onto PVDF membranes. Immunodetection was performed as in Fig. 6. (C) Cells were first lysed by TX-100 and SDS as above. Then, 1µM etheno-NAD was added to each lysate and lysates were incubated for 10 min at 37°C. Reactions were stopped by addition of acetone and proteins were analyzed by immunoblotting as in (A). (D) ART2.2-GPI-transfected DC27.10 cells were incubated for 10 min in the presence of 1µM 32P-NAD. Cells were washed and lysed in two steps in either 0.05% TX-100 (lanes 1 and 2) or 1% TX-100 (lanes 3 and 4) for 10 min at 4°C and then in 1% TX-100 for 20 min at 37°C. Lysates were subjected to immunoprecipitation with immobilized anti-LFA1 or anti-P2X7. Total protein in cell
lysates was visualized by Coomassie staining and ART2.2 was visualized by immunoblotting with PO-conjugated anti-FLAG mAb M2 (left panels). Radioactivity incorporated into proteins was determined by autoradiography; exposure times were 6 h for LFA-1, 6 h for total proteins, and 36 h for P2X7 (right panels).

References


Figures

Fig. 1.
Fig. 2.
Fig. 3.
Fig. 4.

A
Fraction: 1-3 4 5 6-7 8-9 10 11 12 P
GM1
GPI
Gβ

B
Fraction: 1-3 4 5 6-7 8-9 10 11 12 P
GM1
Tm
Gβ

C
Fraction: 1-3 4 5 6-7 8-9 10 11 12 P
hi
Fig. 5.

A Untransfected ART2-GPI ART2-Tm
log MFI rel. cell num 11 1322 1381
eNAD eNAD 1 10 100 1000

B etheno NAD µMART2-GPI ART2-Tm
log MFI 0 10 20 30 40 50 60

C

0 5 10 15 20 25 30
Fig. 6.
Fig. 7.
Activity and specificity of toxin-related mouse T cell ecto-ADP-ribosyltransferase ART2.2 depends on its association with lipid rafts

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