NICOTINIC ACID ADENINE DINUCLEOTIDE PHOSPHATE (NAADP) DEGRADATION BY ALKALINE PHOSPHATASE

Frederike Schmid¹, Ralf Fliegert¹, Tim Westphal¹, Andreas Bauche¹,² and Andreas H. Guse¹,²*

From ¹The Calcium Signalling Group, Department of Biochemistry and Signal Transduction, and ²Department of Biochemistry and Molecular Cell Biology, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany

Running head: NAADP degradation to NAAD

*Correspondence: Andreas H. Guse, The Calcium Signalling Group, Department of Biochemistry and Signal Transduction, University Medical Center Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany, Email guse@uke.de

Background: Biosynthesis and metabolism of nicotinic acid adenine dinucleotide phosphate (NAADP) are unclear.

Result: Alkaline phosphatase was identified and characterized as enzyme involved in NAADP degradation.

Conclusion: In cells not expressing CD38, alkaline phosphatase is a valid candidate enzyme for degradation of NAADP to nicotinic acid adenine dinucleotide.

Significance: First evidence for identity of the enzyme degrading NAADP in cells not expressing CD38.
from CD38 knock out mice, e.g. spleen and thymus, indicate a role for CD38 in NAADP degradation. Indeed, others and we have shown that metabolism of NAADP to 2’-phospho-adenosine diphosphoribose (2’-P-ADPR) takes place under physiological conditions in vitro (14,17).

In the present study we investigated the degradation of NAADP in HeLa cells. Expression of CD38 was not detectable in these cells. The degradation product was different from the product obtained in CD38 expressing cells, 2’-P-ADPR and was identified by HPLC as nicotinic acid adenine dinucleotide (NAAD). The NAADP phosphatase activity was characterized, identified and heterologous expression restored its activity.

EXPERIMENTAL PROCEDURES

Reagents. NAADP and 1,N6-etheno-nucleotides were obtained from Biolog (Bremen, Germany). Other compounds and chemicals were purchased from Sigma-Aldrich (Munich, Germany)

Cell Culture. Jurkat T lymphocytes (subclone JMP) were cultured in RPMI 1640 medium supplemented with Glutamax, HEPES (25 mM), 7.5% (v/v) newborn calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin. HeLa and HEK293 cells were cultured in DMEM medium supplemented with Glutamax, 10 % (v/v) fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

Analysis of endogenous NAADP. Endogenous NAADP was extracted and quantified by the enzymatic NAADP cycling assay (5). Briefly, 2×10⁸ HeLa cells were divided into identical twin samples and centrifuged (500xg, 5 min). One part was spiked with 15 pmol NAADP to be able to calculate recovery. Then, cells pellets were resuspended in 1 ml ice-cold trichloroacetic acid (20%, w/v) and frozen in liquid N₂. After two freeze-thaw cycles, samples were centrifuged (4400xg, 10 min, 4°C). The supernatant was neutralized by extraction with water-saturated diethyl ether. Samples were purified by anion exchange chromatography. NAADP was quantified by the enzymatic NAADP cycling assay (5). Increase in fluorescence was measured at λₖₐᵢᵣ₃ = 530 nm and λₑₓₑᵢ₃ = 590 nm on a fluorescence micro plate reader (Infinite M200 with Software i-control, Tecan, Grödig, Austria). Mean recovery of NAADP in control samples was 66%. Data were subjected to Grubbs outlier test (significance level < 0.01) and then tested using one-sample t-test vs the hypothetical value 0.

Analysis of mRNA. Total RNA was extracted from 1×10⁹ cells using the RNeasy Mini Kit with on-column DNase digest (Qiagen, Hilden, Germany). RT-PCR was performed with the Titan One Tube RT-PCR Kit (Roche, Mannheim, Germany) according to the manufacturer’s protocol. Primers for the amplification are listed in Tab. 1. Nested PCR was performed with diluted product of the RT-PCR, primers (Tab. 1) and Taq-DNA-Polymerase (Fermentas, St. Leon-Rot, Germany).

Preparation of subcellular fractions. Subcellular fractions were obtained by differential centrifugation as previously described (14,18). Briefly, cells were lysed with a Potter Elvehejm-homogenizer, cell debris and nuclei were discarded. Membrane protein fractions were obtained as pellet after 10,000xg centrifugation (P100 membranes) or after 100,000xg centrifugation (P100 membranes) and dissolved in buffer containing 140 mM NaCl, 20 mM HEPES and protease inhibitor complete EGTA free (Roche, Mannheim, Germany) at pH 7.4. Supernatant after 100,000xg centrifugation was termed S100.

Western blot analysis of CD38. For determination of CD38 expression, membrane protein P10 (15 µg) was separated under non reducing conditions by SDS-PAGE using a 12.5% gel and transferred onto a PVDF membrane. One part of the membrane was incubated with anti-CD38 antibody ATM (1 µg/ml, 1 h; Santa Cruz Biotechnology, Heidelberg, Germany), the second part was incubated with anti- Hsc70 antibody (0.04 µg/ml, 1 h, Santa Cruz Biotechnology, Heidelberg, Germany) as loading control. Then the membranes were incubated with the secondary antibody (goat anti-mouse horseradish peroxidase conjugate, 0.2 µg/ml, 1 h, Dianova, Hamburg, Germany). Membranes were incubated for 1 min with ECL solution (Amersham Biosciences; Freiburg, Germany). Chemiluminescence was detected with Intelligent Dark Box, CAS 3000 (FujiFilm, Tokyo, Japan).

Western blot analysis of alkaline phosphatase. To analyze the expression of the different isozymes of alkaline phosphatase, 15 µg membrane protein (P10) were separated under reducing conditions by SDS-PAGE using a 10% gel and transferred onto a PVDF-membrane. One part of the membrane was incubated with antibody specific for each isozyme for 1 h (mouse anti hGCAP, 2.5µg/ml, AbdSerotec, Düsseldorf, Germany; rabbit anti human PLAP, 1:800, Abcam, Cambridge, UK; mouse anti human IAP, 0.6µg/ml, Abcam, Cambridge, UK; rabbit anti human TNAP, 0.6µg/ml, Abcam, Cambridge, UK), the other part was incubated with mouse anti-human
β-actin antibody (1:1200, Sigma-Aldrich, Darmstadt, Germany) as loading control. Then the membranes were incubated for 1h with the corresponding HRP-conjugated secondary antibody (goat anti-mouse, 1:2500 or goat anti-rabbit, 1: 5000; Dianova, Hamburg, Germany). After incubation with ECL solution (Amersham Biosciences; Freiburg, Germany) for 1 min, chemiluminescence was detected with Intelligent Dark Box, CAS 3000 (FujiFilm, Tokyo, Japan).

Analysis of NAD-glycohydrolase activity. NAD-glycohydrolase activity of CD38 was tested with a fluorescence assay. Membrane protein P10 (100 µg/ml) of Jurkat and HeLa cells was incubated with 100 µM 1,N6-etheno-NAD for 30 min. NAD-glycohydrolase converts the weakly fluorescent 1,N6-etheno-NAD into highly fluorescent 1,N6-etheno-ADPR. Increase in fluorescence was measured at $\lambda_{ex} = 300$ nm and $\lambda_{em} = 410$ nm on a fluorescence micro plate reader (Infinite M200 with Software i-control, Tecan, Grödig, Austria).

Determination of phosphate release with malachite green. P10 membrane protein (1 µg/ml) of HeLa cells were incubated with 10 µM NAADP in TEA buffer (50 mM triethanolamine, 1 mM MgCl$_2$, pH 8.0) for 30 min. Release of free phosphate was then detected by complexation with molybdate and malachite green (Malachite Green Phosphate Assay Kit, BioAssay Systems, Hayward, USA) according to manufacturer’s protocol. Absorption was measured at $\lambda = 600$ nm on a micro plate reader (Infinite M200 with Software i-control, Tecan, Grödig, Austria).

Determination of phosphatase activity with pNPP. P10 membrane protein (1 µg/ml) of HeLa cells were incubated with 5.5 mM para-nitrophenyl phosphate (pNPP) in DEA buffer (1 M diethanolamine, 1 mM MgCl$_2$, pH 9.8) for 20 min. Alkaline phosphatase dephosphorylates the colorless substrate pNPP to yellow para-nitrophenol. Absorption of the dephosphorylated product was measured at $\lambda = 405$ nm on a micro plate reader (Infinite M200 with Software i-control, Tecan, Grödig, Austria).

Determination of endogenous intracellular NAADP 2′-phosphatase activity in HeLa cells. HeLa cells were detached, washed twice with PBS, resuspended either in PBS (140 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1.5mM KH$_2$PO$_4$, pH 7.4) or PBS supplemented with 1 mg/ml bromelain (Sigma-Aldrich) and incubated for 30 min at 37°C. Subsequently cells from both conditions (± bromelain) were washed twice with assay buffer (140 mM NaCl, 5 mM KCl, 1 mM MgSO$_4$, 1 mM CaCl$_2$, 20 mM HEPES, 1 mM NaH$_2$PO$_4$, 5.5 mM glucose, pH 7.4), resuspended either in assay buffer or in assay buffer containing 60 µg/ml saponin and incubated for 5 min at 37°C. Afterwards cells were again washed twice and resuspended in assay buffer. Protein content was determined using BioRad protein assay. The different cell preparations were then incubated with 100 µM NAADP for 1, 3 and 10 min. Reaction was stopped by addition of ice-cold trichloroacetic acid (TCA) to a final concentration of 5% (v/v). TCA was removed by extraction with water-saturated diethyl ether. After neutralization of the samples by addition of phosphate buffer and removal of traces of residual TCA in a centrifugal evaporator, samples were analyzed for NAAD and NAADP content by HPLC.

**HPLC analysis of nucleotides.** Reaction products were filtered through a 10 kDa cut-off filter (Microcon, Millipore, Billerica, MA, USA), diluted in elution buffer containing 20 mM KH$_2$PO$_4$ and 5 mM tetrabutylammonium dihydrogen phosphate at pH 6 and analyzed by ion pair RP-HPLC performed on a 250 x 4.6 mm C18-5 µm HYPURITY ADVANCE column (Thermo Fisher Scientific, Schwerte, Germany) or Multohyp BDS (Chromatographie Service, Langerwehe) equipped with a 10 x 4 mm guard cartridge filled with the same material (Thermo Fisher Scientific, Schwerte, Germany or Chromatographie Service, Langerwehe, Germany). Nucleotides were eluted with a nonlinear gradient with elution buffer and increasing amounts of methanol. Analysis of metabolism of 1,N6-etheno-NAAD was performed at 25°C at a flow rate of 1 ml/min with the following gradient (% methanol): 0 min (0), 3 min (0), 30 min (30), 32 min (30), 34 min (0) and 40 min (0). NAADP 2′-phosphatase assays were analyzed at 25°C at a flow rate of 0.8 ml/ml with the following gradient (% methanol): 0 min (15), 3,5 min (15), 17 min (40), 27 min (45), 31 min (50), 33 min (50), 35 min (15), 40 min (15). Nucleotides were detected by UV absorption at $\lambda = 260$ nm with a diode array detector (1200 series, Agilent Technologies, Böblingen, Germany) while fluorescent nucleotides were detected at $\lambda_{ex} = 300$ nm and $\lambda_{em} = 410$ nm with a fluorescence detector (1200 series, Agilent Technologies, Böblingen, Germany). Product peaks were analyzed with ChemStation Software (Agilent technologies, Böblingen, Germany). The different peaks were detected with ChemStation Software (Agilent technologies, Böblingen, Germany). Quantification was performed with external standards.

**Recombinant expression of alkaline phosphatase in HEK293 cells.** cDNA of placental alkaline phosphatase (PLAP) was obtained from imaGenes (Berlin, Germany) and cloned into the vector...
RESULTS

Analysis of endogenous NAADP levels in wt HeLa cells using an enzymatic cycling assay revealed basal NAADP concentration of 9.9 ± 2.2 nM (mean ± SEM, n=13, p=0.0007 vs hypothetical value 0; see also Suppl. Fig. 1). Recently, CD38 has been implicated in both NAADP formation (17,19) and degradation (14,17). However, expression of CD38, as analyzed at the mRNA and protein level (Fig. 1A,B), was not detectable in HeLa cells. In comparison to Jurkat T cells that do express CD38 (Fig. 1A,B), in protein extracts of HeLa cells only minor degradation of 1,N⁶-etheno-NAAD was observed (Fig. 1C). HPLC analysis of the reaction products revealed negligible pyrophosphatase activity and lack of detectable NAD glycohydrolase activity in Hela cells.

When NAADP was incubated with either membranes or cytosolic protein extracts from HeLa cells, the major reaction product was identified as NAAD indicating a phosphatase activity degrading NAADP (Fig. 2A). This enzyme activity was prominent in P10 membrane fraction and weakly also in P100 membranes and the cytosolic fraction S100 (Fig. 2). The NAADP 2’-phosphatase activity revealed a pH optimum between 8 and 9 (Fig. 3A). Different buffers used at the same pH showed only minor effect on NAAD formation (Suppl. Fig. 2). Michaelis-Menten kinetics resulted in $K_m = 37 \mu M$. The specific activity in P10 membranes was 0.8 µmol/mg/min. Since endogenous NAADP levels are in the range of approximately 10 to 100 nM (5,20), a $K_m$ of 37 µM appeared somewhat large and raised the question whether this enzymatic activity might be of physiological relevance. Thus, in the next set of experiments, physiological concentrations of NAADP were used as substrate. In fact, incubation of NAADP concentrations as low as 50 nM with P10 membranes from HeLa cells resulted in NAAD formation (Fig. 4A,B). Furthermore, even at pH 7.2 the 2’-phosphatase reaction was observed; here the fluorescent 1,N⁶-etheno-NAADP was used as substrate and metabolism to 1,N⁶-etheno-NAAD was monitored by HPLC and fluorescence detection (Fig. 5). The second metabolic product observed at pH 9 only, 1,N⁶-etheno-ADPR also was formed in the absence of protein, obviously due to non-enzymatic degradation of 1,N⁶-etheno-NAADP via 1,N⁶-etheno-2’-phospho-ADPR, visible as shoulder of the 1,N⁶-etheno-NAADP peak.

Since degradation of NAADP to NAAD preferentially occurred at alkaline pH, alkaline phosphatase (AP) appeared a suitable candidate for the NAADP 2’-phosphatase. Thus, expression of isoforms of AP was analyzed in HeLa cells. On both mRNA and protein level the AP isoforms PLAP, intestinal alkaline phosphatase (IAP) and tissue-nonspecific alkaline phosphatase (TNAP) were detected (Fig. 6A,B).

To assess which isofom is responsible for the degradation of NAADP, isofom-specific inhibitors were tested. L-Homo-arginine has been reported to mainly inhibit TNAP and has very little effect on the other isoforms (21). In contrast, L-phenylalanine especially inhibits the tissue specific isoforms and shows little impact on TNAP (22). The third inhibitor, L-leucine, does not affect TNAP activity, but strongly reduces activity of PLAP and GCAP 21,23. Using the non-specific AP substrate paranitrophenyl phosphate (pNPP), degradation was mainly sensitive to L-homo-arginine and L-leucine confirming expression of TNAP and PLAP in HeLa cells (Fig. 6C). Interestingly, L-homo-arginine did not inhibit degradation of NAADP by AP suggesting that TNAP is not involved in this process (Fig. 6D). However, since L-leucine was the best inhibitor of NAADP degradation to NAAD (Fig. 6D) and since GCAP was not expressed in HeLa cells (Fig. 6B), PLAP appeared the best candidate for the enzymatic activity observed.

HeLa cells are known for their high expression of PLAP (22,24,25). Furthermore, the enzymatic characteristics observed in HeLa cells in our study indicate that PLAP is the most prominent isoform in these cells. Phosphodiesterase activity has been reported for TNAP, but not for PLAP (26,27). Importantly, in the present study phosphodiesterase activity using 3’,5’-cyclic AMP as substrate was not detected in HeLa cells (data not shown). In addition, IAP and TNAP show lower specific activity in the presence of highly phosphorylated substrates (28,29).

On the contrary, no correlation between the degree of phosphorylation and phosphatase activity was seen with PLAP (30). In the present study the degree of substrate phosphorylation had no influence on the phosphatase activity (data not shown) indicating that the observed NAADP 2’-phosphatase activity is mainly due to the expression of PLAP.

In Jurkat T cells NAADP was metabolized to 2’-P-ADPR (14) whereas degradation of NAADP to NAAD was never observed in those cells (Fig. 7).
C.D). Consistently, expression of the AP isozymes was not detectable in Jurkat T cells (Fig. 7 A,B). Taken together our data indicate specific degradation of physiological concentrations of NAADP to NAAD by AP, likely by the isozyme PLAP. To confirm these results we aimed at recombinant expression of AP in a cell in which expression of endogenous AP is not detectable. Thus, HEK293 cells were characterized towards expression and enzymatic activity of AP isozymes (Fig. 8). No endogenous expression of any of the AP isozymes (Fig. 8 A,B) and no AP activity was detected (Fig. 8 C,D). Thus, HEK293 cells appeared a suitable cell system to express AP and to analyze protein extracts for recombinant AP activity towards the substrate NAADP. Indeed, expression of PLAP in HEK293 cells resulted in dephosphorylation of NAADP to NAAD while no such enzymatic activity was observed in EGFP-transfected HEK293 cells (Fig. 9).

AP is localized on the cell surface as a GPI-anchored ectoenzyme (31), but expression in intracellular membranes, e.g. ER, Golgi, lysosomes and mitochondria (32-36) has been described. Interestingly, cytosolic AP enzyme activity was detected in saponin-permeabilized HeLa cells (35) indicating correct localization for potential involvement in cytosolic signal transduction. To test for intracellular NAADP 2’-phosphatase activity in HeLa cells, bromelain was used to proteolytically cleave surface protein. NAADP 2’-phosphatase activity decreased under such conditions (Fig. 10). Subsequent permeabilization by saponin in the absence of bromelain significantly increased NAADP 2’-phosphatase activity (Fig. 10) confirming intracellular localization of PLAP, as described for AP 31 years ago (35). Similar results were obtained in HEK293 cells overexpressing PLAP; however, PLAP was removed from the cell surface using phosphoinositide-specific phospholipase C instead of bromelain (data not shown).

DISCUSSION

In the current study we demonstrate (i) the presence of endogenous NAADP in the HeLa cell line, a cell type in which expression of CD38 is not detectable, (ii) degradation of NAADP to NAAD in HeLa cell extracts, (iii) identification of alkaline phosphatase (AP) as NAADP degrading enzyme, and (iv) intracellular localization of the NAADP degrading enzyme.

Although NAADP appears as a very important and probably ubiquitous second messenger, several aspects of its metabolism are unknown. Though the base-exchange reaction, catalyzed mainly, if not exclusively, by CD38 is the only reaction shown in vitro to produce NAADP (19), a growing number of reports suggests that endogenous NAADP is detected in cells or tissues even when the gene for CD38 is silenced, e.g. using an shRNA approach, or in CD38 knock-out mice (14,15). The current study adds another example, the human cell line HeLa. Though expression of CD38 is not detectable in HeLa cells, endogenous NAADP in a similar concentration range as compared to other cell types (5, 20) was clearly detected. To better understand synthesis of NAADP in HeLa cells, a potential phosphorylation of NAADP was tested in our lab. Attempts to generate NAADP by incubation of NAAD and ATP with NAD kinase failed. Furthermore, no NAAD phosphorylating activity could be detected using protein extracts from HeLa cells (F. Schmid and A.H. Guse, unpublished results). Another possibility, deamidation of NAADP, was also assayed. Various experiments with protein fractions from HeLa cells did not reveal an NAADP deamidase activity (F. Schmid and A.H. Guse, unpublished results). However, the presence of endogenous NAADP suggests that there must be an enzymatic activity for formation of NAADP other than CD38. The fact that neither kinase nor deamidase activity has been detectable so far indicates a more complex situation than hitherto acknowledged. It might well be that coenzyme(s) or co-substrate(s) necessary for these reactions have not been added in the right way in the in vitro assays carried out so far.

Regarding degradation of NAADP our enzymatic assays combined with HPLC analysis of reaction products revealed 2 main reactions: (i) release of nicotinic acid to produce 2’-P-ADPR by CD38, or (ii) release of inorganic phosphate to generate NAAD. The latter reaction was catalyzed by the placental alkaline phosphatase isozyme (PLAP) in cell extracts from HeLa cells. An involvement of AP enzyme activity has not yet been implicated in NAADP metabolism, but a NAADP phosphatase activity from brain membranes was described (37). This NAADP 2’-phosphatase was characterized by a $K_m = 7 \mu M$, a specific activity of approx. 160 pmol/min/mg protein, and a dependency on free $[Ca^{2+}]$. Free $[Ca^{2+}] \geq 0.1 \mu M$ increased enzymatic activity substantially (37). Interestingly, Berridge et al. (37) have compared their NAADP 2’-phosphatase activity to commercially available alkaline phosphatase activity, but instead of analyzing any pH-dependency rather focused on the $Ca^{2+}$ dependency of their enzymatic activity (37). In our present study, cells were homogenized in the presence of 1 mM $[Ca^{2+}]$. However, though membranes obtained after 2 centrifugation steps were dissolved in a nominally $Ca^{2+}$ free buffer (containing...
110 mM KCl, 20 mM HEPES, pH 7.2, protease inhibitors complete EGTA free [Roche, Mannheim, Germany]), it is very likely that the free $[Ca^{2+}] \geq 0.1$ µM in our experiments. Reports from the literature indicate a Ca$^{2+}$-dependency of AP. Firstly, AP prepared from femur of chicken was stimulated by calmodulin and 10 µM Ca$^{2+}$ (30). Secondly, AP from basolateral membranes of rat mucosal cells was dependent on Ca$^{2+}$ with optimum enzyme activity at 40 µM [Ca$^{2+}$] (38). However, whether the enzyme initially characterized by Bertrige et al. (37) is an AP, remains to be investigated. The present study aimed far beyond characterization of the enzyme activity. In fact, we used the characterization, e.g. pH optimum between 8 and 9 and specific inhibition by L-leucine, to narrow down a suitable candidate enzyme. Most importantly, though the $K_M$ for NAADP was in the micromolar range, we demonstrated sufficient 2'-phosphatase activity also for NAADP concentrations at 50 and 100 nM, even at pH 7.2. Finally, expression analysis of AP isozymes in HeLa, Jurkat, and HEK293 cells allowed heterologous expression of the AP isozyme PLAP in HEK cells resulting in NAADP degradation to NAAD in cell extracts. Is there anything known about an involvement of PLAP in (NAADP) signaling? In vitro, PLAP is a promiscuous enzyme that degrades different adenine and uridine nucleotides, glucose 6-phosphate, β-glycerophosphate, and others (39). Phosphatidates with long fatty acid chains have been described as substrates, too (40). However, the physiological substrate of AP isozymes has remained unclear. In the present study, we present evidence that endogenous, very small NAADP concentrations are degraded by AP and that AP might be involved in this process under physiological conditions. However, PLAP is expressed on the cell surface as a GPI-anchored enzyme (31). Thus, in this sense PLAP resembles the ectoenzyme CD38 that can be detected on the surface of many cell types (reviewed in 41). This “topological paradox” has been solved for CD38 and its ADP-ribosyl cyclase enzymatic activity since De Flora and colleagues demonstrated transport pathways for both the substrate NAD via connexin 43 and also for the product cADPR via nucleoside transporters, respectively (42-44). For AP, expression in the cytosol and in intracellular membranes was described in earlier publications (32-36). Here, we successfully reproduced an increase of AP activity upon permeabilization of HeLa cells using NAADP as substrate confirming correct localization for potential involvement in cytosolic signal transduction, as reported earlier (35). Taken together, we present the AP isozyme PLAP as a candidate enzyme for degradation of NAADP to NAAD. In cells that lack CD38, like the HeLa cell line, this degradation pathway may be important to terminate NAADP responses.

REFERENCES


**FOOTNOTES**

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Abbreviations used: AP, alkaline phosphatase; GCAP, germ cell alkaline phosphatase; IAP, intestinal alkaline phosphatase; NAAD(P) nicotinic acid adenine dinucleotide (phosphate), 2’-P-ADPR, 2’-phospho-adenosine diphosphoribose; PLAP, placental alkaline phosphatase; pNPP, para-nitrophenyl phosphate; RyR, ryanodine receptor(s); TCA, trichloroacetic acid; TNAP, tissue-nonspecific alkaline phosphatase; TPC, two-pore channel; TRP, transient receptor potential; wt, wild type
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<td>CD38</td>
<td>5’ GGC TCT CTA GGA GAG CCC AAC 3’</td>
<td>5’ CAC ACT CCC AAA AGT GCT GTT T 3’</td>
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<tr>
<td>CD38 (nested)</td>
<td>5’ CAC CAA GCG CTT TCC CCG 3’</td>
<td>5’ GAA TAC TGA AAC AGG GTT G 3’</td>
</tr>
<tr>
<td>GCAP</td>
<td>5’ AGC TCA TAC TCC ATA CCT G 3’</td>
<td>5’ CAC CCC CAT CCC GTC A 3’</td>
</tr>
<tr>
<td>PLAP</td>
<td>5’ CTC ATA CTC CAT GCC CA 3’</td>
<td>5’ CAC CCC CAT CCC ATC G 3’</td>
</tr>
<tr>
<td>IAP</td>
<td>5’ CTG CAG CCG GTT CCT GG 3’</td>
<td>5’ GCA CCC CCA ACC CAT CG 3’</td>
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<tr>
<td>TNAP</td>
<td>5’ ACA TCT GAC CAC TGC CA 3’</td>
<td>5’ GAG ACA CCC ATC CCA TC 3’</td>
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Primers used for RT-PCR and nested PCR. Primers specific for alkaline phosphatase isozymes were adapted from Schär et al. (45).
FIGURE LEGENDS

Fig. 1: Expression of CD38 is not detectable in HeLa cells.
(A) Total RNA was extracted from Jurkat and HeLa cells. RT-PCR was performed with CD38 specific intron spanning primers followed by nested PCR. PCR product was expected to be 400 bp in size; marker: gene ladder 100 bp. (B) P10 membrane protein of Jurkat or HeLa cells (15 µg) was separated by SDS-PAGE under non-reducing conditions and transferred onto a PVDF membrane. One part of the membrane was incubated with an antibody detecting the heat shock protein Hsc70 as loading control. CD38 was detected using the anti-CD38 antibody AT1 on the second part of the membrane. A representative experiment out of 3 is shown. (C) P10 membrane protein of Jurkat and HeLa cells (100 µg/ml) was incubated with 100 µM etheno-NAD for 30 min. Increase in fluorescence was measured at λ_ex = 300 nm and λ_em = 410 nm. The background fluorescent signal obtained with HeLa cells is due to pyrophosphatase producing etheno-AMP and nicotinamide ribose phosphate (19). Data shown are mean values ± SD (n = 7-14), ** p < 0.01 student’s t-test. (D) Shown are representative chromatograms obtained with HeLa or Jurkat membrane incubated with 100 µM etheno-NAD for 30 min. Fluorescence signal was measured at λ_ex = 300 nm and λ_em = 410 nm.

Fig. 2: NAADP is degraded to NAAD in HeLa cells.
Cytosolic protein (S100), light membrane fraction (P100) and heavy membrane fraction (P10; each at 1 mg/ml) were incubated with 20 µM NAADP at 37°C for 15 min. Reaction products were analyzed by HPLC. (A) Shown are representative chromatograms before and 15 min after incubation with substrate. Incubation of NAADP with membrane fractions of HeLa cells resulted in dephosphorylation of NAADP and formation of NAAD. (B) The reaction product NAAD was quantified after 15 min of incubation. NAAD production by P10 membrane fractions was much higher than by P100 membrane fractions. Incubation of NAADP with cytosolic fractions (S100) resulted in relatively little NAAD formation. Data shown are mean values ± SD (n = 7-14), ** p < 0.01, *** p < 0.001 ANOVA.

Fig. 3: Characterization of the NAADP degrading activity in HeLa cells.
(A) pH dependency of NAADP degrading activity: membrane protein of HeLa cells (1 mg/ml) was incubated with 20 µM NAADP at 37°C for 5 min at different pH. Reaction products were analyzed by HPLC. Buffers used: pH = 5 - 6: MES; pH = 7 - 9: TEA; pH = 10 – 11: DEA. Data shown are mean values ± SD (n = 2 - 4), p < 0.001. (B) Kinetic characterization of NAADP degrading activity: membrane protein of HeLa cells (0.5 mg/ml) was incubated with increasing concentrations of NAADP in TEA at pH = 9. Reaction products were analyzed by HPLC. Data shown are mean values ± SD (n = 1 - 6).

Fig. 4: Physiological concentrations of NAADP are metabolized by NAADP degrading activity.
Physiological concentrations of NAADP in HeLa cells are 10 nM to 100 nM. Membrane protein of HeLa cells (0.5 mg/ml) was incubated with nanomolar concentrations of NAADP at 37°C and pH = 9. Samples were freeze-dried and reconstituted in water. NAAD formation was analyzed by HPLC. (A) Shown are representative chromatograms at 0 min, 5 min and 15 min after incubation with 50 nM NAADP. (B) Formation of NAAD was quantified after incubation with varying concentrations of NAADP.

Fig. 5: 1,N^6-etheno-NAADP is metabolized by NAADP degrading activity at neutral pH.
Membrane protein of HeLa cells (0.05 µg/ml) was incubated with 100 nM of 1,N^6-etheno-NAADP (e-NAADP) at 37°C and pH = 7.2 and 9. Samples were deproteinized using 10 kD cut-off filters (Vivaspin). Product formation was analyzed by HPLC. Shown are representative chromatograms from samples incubated with 100 nM 1,N^6-etheno-NAADP for 0, 5, 15, 40 and 60 min. Peak area for 1,N^6-etheno-2-phospho-ADPR (e-ADPRP) and 1,N^6-etheno-ADPR (e-ADPR) are considerably larger due to the higher fluorescence intensity upon cleavage of the intramolecular quencher, the nicotinic acid moiety.

Fig. 6: Expression and isoform-specific inhibition of AP isozymes in HeLa cells.
Expression analysis: (A) Expression analysis of alkaline phosphatase at mRNA level. Total RNA was extracted from HeLa cells and subjected to reverse transcriptase PCR using primers specific for the different isoenzymes. Size of PCR products was analyzed by gel electrophoresis. Sequence of PCR products was verified by TA cloning and sequencing (data not shown). mRNA coding for PLAP (259 bp), IAP (250 bp) and TNAP (281 bp) but not GCAP (251 bp) was detected. Marker: pUC (MspI). (B) Expression analysis of alkaline phosphatase at protein level. 10 µg membrane protein of HeLa cells were separated by SDS-PAGE and transferred onto a PVDF
membrane. One part of the membrane was incubated with an antibody detecting β-actin as loading control. Alkaline phosphatase was detected with antibodies specific for the different isoenzymes. Alkaline phosphatase is highly glycosylated and therefore the apparent molecular weight is larger than the predicted molecular weight (57 kDa). **Isoform-specific inhibition:** (C) Membrane protein of HeLa cells (1 µg/ml) was preincubated with 10 mM inhibitor for 10 min on ice. Then 5.5 mM para-nitrophenyl phosphate (pNPP) were added and incubated for 20 min. Absorption of the dephosphorylated reaction product was measured at λ = 405 nm. (D) Membrane protein of HeLa cells (1 µg/ml) was preincubated with 10 mM inhibitor for 10 min on ice. Then, 10 µM NAADP were added and incubated for 30 min. Release of phosphate was then assessed with the malachite green assay. Data shown are mean values ± SD (n = 4 - 8); *** p < 0.001 ANOVA.

**Fig. 7: In Jurkat cells expression or activity of alkaline phosphatase was not detectable.**  
(A) Expression analysis of alkaline phosphatase at mRNA level. Total RNA was extracted from HeLa and Jurkat cells subjected to reverse transcriptase PCR using primers specific for the different isoenzymes. (B) Expression analysis of alkaline phosphatase at protein level. Membrane protein of HeLa or Jurkat cells (10 µg) was separated by SDS-PAGE and transferred onto a PVDF membrane. One part of the membrane was incubated with an antibody detecting β-actin as loading control. Alkaline phosphatase was detected with antibodies specific for the different isozymes. (C) Membrane protein from HeLa or Jurkat cells (5 µg/ml) was incubated with 5.5 mM para-nitrophenyl phosphate (pNPP). Absorption of the dephosphorylated reaction product was measured at λ = 405 nm. No alkaline phosphatase activity could be detected in Jurkat cells. (D) Membrane protein of HeLa or Jurkat cells (5 µg/ml) was incubated with 40 µM NAADP. Release of phosphate was then assessed with the malachite green assay. Data shown are mean values ± SD (n = 4 - 8); n.s. not significant, *** p < 0.001 ANOVA.

**Fig. 8: In HEK293 cells expression or activity of alkaline phosphatase was not detectable.**  
(A) Expression analysis of alkaline phosphatase at mRNA level. Total RNA was extracted from HeLa and HEK293 cells subjected to reverse transcriptase PCR using primers specific for the different isoenzymes. Size of PCR products was analyzed by gel electrophoresis. (B) Expression analysis of alkaline phosphatase at protein level. 10 µg membrane protein of HeLa and HEK293 cells were separated by SDS-PAGE and transferred onto a PVDF membrane. One part of the membrane was incubated with an antibody detecting β-actin as loading control. Alkaline phosphatase was detected with antibodies specific for the different isozymes. (C) Membrane protein of HeLa or HEK293 cells (5 µg/ml) was incubated with 5.5 mM para-nitrophenyl phosphate (pNPP). Absorption of the dephosphorylated reaction product was measured at λ = 405 nm. (D) Membrane protein of HeLa or HEK293 cells (5 µg/ml) was incubated with 40 µM NAADP. Release of phosphate was then assessed with the malachite green assay. Data shown are mean values ± SD (n = 4 - 8); n.s. not significant, *** p < 0.001 ANOVA.

**Fig. 9: Recombinant expression of alkaline phosphatase in HEK293 cells results in NAADP degradation.**  
HEK293 cells were transiently transfected with pIRES2-EGFP-PLAP and control plasmid (pIRES2-EGFP). 24 h after transfection cells were incubated with 100 µM NAADP and formation of NAAD was analyzed by HPLC. (A) Shown are representative chromatograms before and 2 h after incubation with substrate. Transient expression of PLAP in HEK293 cells led to dephosphorylation of NAADP and formation of NAAD. (B) Formation of NAAD was quantified after 2 h. Data shown are mean values ± SD (n = 5).

**Fig. 10: Intracellular NAADP degrading activity in wildtype HeLa cells.**  
(A) HeLa cells were incubated for 30 min with the protease bromelain (1 mg/ml) to release AP from the cell surface. Then, the cells were washed to remove both released AP and bromelain. Afterwards the cells were permeabilized using saponin. Protein content of cells was determined and portions equivalent to 0.2 to 1.0 µg total protein were incubated with 100 µM NAADP. (B) NAAD content was analyzed by HPLC after 1, 3 and 10 min to determine NAADP 2’-phosphatase activity. Data shown are mean values ± SD (n = 4), * p < 0.05 (Student’s t test).
Figure 2

A

NAAD

NAADP

Abs. $\lambda = 260$ nm (mV)

0 min

15 min

ADPR

P10

P100

S100

B

NAAD (nmol)

0.0

0.1

0.2

P10

P100

S100

NAADP $\rightarrow{\text{ NAAD + P}_i}$
Figure 3

A

B

pH

% max. activity

0% 25% 50% 75% 100% 125%

0 5 6 7 8 9 10 11

NAAD (nmol)

NAAD (nmol/min)

[NAADP] (µM)

0 0.0 0.02 0.04 0.06

0 50 100 150 200
Figure 4

A

B

NAAD

Abs. $\lambda = 260$ nm (mV)

0 min
5 min
15 min

NAAD (pmol)

100 nM NAADP
50 nM NAADP
20 nM NAADP
Figure 5

The figure shows chromatograms for pH 7.2 and pH 9.0 conditions. The peaks labeled e-NAAD, e-ADPR, and e-NAADP are observed at different time points (0 min, 5 min, 15 min, 40 min, and 60 min) for each pH condition. The chromatograms illustrate the changes in peak intensity over time, indicating the metabolic activity of the samples under the respective pH conditions.
Figure 6

A

B

C

D

phosphatase activity
substrate pNPP

NAADP degrading activity
substrate NAADP
Figure 7

A

B

C

D

phosphatase activity
substrate pNPP

OD \( \lambda = 405 \, \text{nm} \)

phosphatase degrading activity
substrate NAADP

HeLa Jurkat buffer

0.00 0.25 0.50 0.75 1.00

*** n.s.

0 10 20 30 40

*** n.s.

100 kDa 75 kDa 50 kDa 37 kDa

phosphatase

\( [\text{Pi}] \) (\( \mu \text{M} \))

H J H J H J H J

\( \beta \)-actin

HeLa Jurkat buffer

HeLa Jurkat buffer

Downloaded from http://www.jbc.org/ by guest on November 18, 2017
Figure 8

A

[Image of gel electrophoresis showing bands at 500 bp and 300 bp for HEK and HeLa samples labeled M, GCAP, PLAP, IAP, TNAP.]

C

Phosphatase activity substrate pNPP

- OD$_\lambda$ = 405 nm
- *** n.s.

- HeLa
- HEK
- buffer

B

[Image of Western blot showing bands for GCAP, PLAP, IAP, TNAP at 100 kDa, 75 kDa, 50 kDa, and 37 kDa with arrows labeled phosphatase and β-actin for HEK and HeLa samples.]
Figure 9

A

![Graph A](image1)

B

![Graph B](image2)

Abs. λ = 260nm (mV)

NAADP

NAAD

PLAP

EGFP

t (min)

0 5 10 15 20

0 2 4 6

PLAP EGFP

NAAD (pmol/min)
Figure 10

A) Specific activity (nmol/min/mg)

<table>
<thead>
<tr>
<th>Bromelain</th>
<th>-</th>
<th>-</th>
<th>+</th>
<th>+</th>
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<tbody>
<tr>
<td>Saponin</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>wash</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

B) Graph of NAADP turnover (%)

- untreated
- w Bromelain
- w Bromelain + Saponin

Graph shows the effect of Bromelain and Saponin on NAADP turnover over time (t minutes).
Nicotinic acid adenine dinucleotide phosphate (NAADP) degradation by alkaline phosphatase
Frederike Schmid, Ralf Fliegert, Tim Westphal, Andreas Bauche and Andreas H. Guse

J. Biol. Chem. published online July 31, 2012

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