Activity of neutral and alkaline ceramidases on fluorogenic N-acylated coumarin-containing aminodiols

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Abstract Ceramidases catalyze the cleavage of ceramides into sphingosine and fatty acids. Previously, we reported on the use of the RBM14 fluorogenic ceramide analogs to determine acidic ceramidase activity. In this work, we investigated the activity of other amidohydrolases on RBM14 compounds. Both bacterial and human purified neutral ceramidases (NCs), as well as ectopically expressed mouse neutral ceramidase hydrolyzed RBM14 with different selectivity, depending on the N-acyl chain length. On the other hand, microsomes from alkaline ceramidase (ACER3) knockdown cells were less competent at hydrolyzing RBM14C12, RBM12C14, and RBM14C16 than controls, while microsomes from ACER2 and ACER3 overexpressing cells showed no activity toward the RBM14 substrates. Conversely, N-acylthanolamine-hydrolyzing acid amidase (NAAA) overexpressing cells hydrolyzed RBM14C14 and RBM14C16 at acidic pH. Overall, NC, ACER3, and, to a lesser extent, NAAA hydrolyzed fluorogenic RBM14 compounds. Although the selectivity of the substrates toward ceramidases can be modulated by the length of the N-acyl chain, none of them was specific for a particular enzyme. Despite the lack of specificity, these substrates should prove useful in library screening programs aimed at identifying potent and selective inhibitors for NC and ACER3.—Casasampere, M., L. Camacho, F. Cingolani, J. Casas, M. Egido-Gabás, J. L. Abad, C. Bedía, R. Xu, K. Wang, D. Canals, Y. A. Hannun, C. Mao, and G. Fabrias. Activity of neutral and alkaline ceramidases on fluorogenic N-acylated coumarin-containing aminodiols. J. Lipid Res. 2015. 56: 2019–2028.

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Abbreviations: AC, acidic ceramidase; ACER, alkaline ceramidase; ACER1-TET-ON, HeLa T-REX cells stably transfected with ACER1; ACER2-TET-ON, HeLa T-REX cells stably transfected with alkaline ceramidase 2; ASAH1, acid ceramidase gene; ASAH2, neutral ceramidase gene; CerC12NBD, N-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-y1)amin0]dodecanoyl]-D-erythro-sphingosine; FD-pcDNA5/TO-ASAH2, Farber cells transfected with the pcDNA5/TO plasmid containing mouse ASAH2; FRET, fluorescence resonance energy transfer; hACER, human alkaline ceramidase; HCT116-shACER3, HCT116 cells transfected with shRNA against human alkaline ceramidase 3; HCT116-shsRNA, HCT116 cells transfected with nonspecific shRNA; hNC, human neutral ceramidase; HTS, high-throughput screening; mACER, mouse alkaline ceramidase; MEF, mouse embryonic fibroblast; mNC, mouse neutral ceramidase; NAAA, N-acylthanolamine-hydrolyzing acid amidase; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-y1; NC, neutral ceramidase; pCDase, Pseudomonas aeruginosa neutral ceramidase; shACER3, ACER3-specific shRNA; shCtrl, control shRNA; TBST, TBS-Tween.

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In this work, we show that RBM14 compounds are hydrolyzed by NC, ACER3, and NAAA, but not by ACER1 and ACER2. Each amidohydrolase exhibits different substrate selectivity depending on the nature of the N-acyl chain. The results presented underscore the usefulness of these compounds in massive screening programs aimed at identifying amidohydrolase inhibitors.

**EXPERIMENTAL PROCEDURES**

**Materials**

Dulbecco’s modified Eagle’s medium, FBS, penicillin/streptomycin solution, nonessential amino acids, tetracycline, and puromycin, were from Sigma. Zeocin was from Genaxxon Bioscience and blasticidin was from CalBiochem. Opti-MEM and lipofectamine 2000 were from Invitrogen. Antibodies were purchased from: anti-ACER2 (HPA014092) and anti-actin (A2228) (Sigma); anti-rabbit (NA934V) and anti-mouse (RPN4201V) secondary antibodies (GE Healthcare). Recombinant human NC (hNC) was obtained from R&D Systems. Bacterial CDase was obtained following the reported procedure (16). The latter protein was obtained at a 0.33 mg/ml final concentration and it was stored at −80°C. Once thawed, the enzyme solution was kept at 4°C until activity loss. pcDNA3.1(+) (empty vector) and pcDNA3.1(+) harboring the human NAAA gene were kindly provided by Professor Natsuo Ueda.

**Cells**

Cells were cultured at 37°C/5% CO₂ in Dulbecco’s modified Eagle’s medium high glucose supplemented with 10% FBS and 1% penicillin/streptomycin solution. Nonessential amino acids (0.1 mM) were also added to HEK293T cells. Zeocin (25 μg/ml) and blasticidin (5 μg/ml) were also added to HeLa T-REX cells [HeLa T-REX-human ACER (hACER)2, HeLa T-REX-mouse ACER (mACER)1, and control vector HeLa T-REX pcDNA4], mACER1 and hACER2 gene expression were induced by adding 10 ng/ml tetracycline to the medium. Puromycin (2 μg/ml) was added to ACER3 knockdown HCT116 cells (HCT116-shACER3) and their controls (HCT116-shRNA). Antibiotics other than penicillin/streptomycin were removed during treatments.

**Overexpression of hACER2 and mACER1**

To overexpress ACER2, we used the stable cell line HeLa T-Rex/ACER2 that we constructed in our previous study (17). To overexpress ACER1, we applied the same expression system. The coding sequence of the mACER1 was cloned into the empty vector, pcDNA4, to construct the expression vector, pcDNA4-ACER1, which was used to generate the stable cell line, HeLa T-Rex/ACER1, as described in our previous study (17).

**Knockdown of ACER3 in HCT116 cells**

A lentiviral vector expressing a control shRNA (shCtrl) (CG-GCGAAACAGATGAAGGACCACTCGATGTCGTCGTGATCTTTGTTT) or ACER3-specific shRNA (shACER3) (CCCGTATACGCTGTTGTGCAATTTTGCTGGACAAATATGCAAGCTGTATATTTTTTG) from Sigma-Aldrich (St. Louis, MO) was packaged into lentiviral particles in HEK293T cells using a viral packaging kit (GeneCopeia, Rockville, MD) and lentiviruses expressing shCtrl or shACER3 were then transduced into HCT116 cells according to the manufacturer’s protocol. Forty-eight hours later, the transduced cells were replated and treated with 5 μg/ml puromycin (Sigma-Aldrich) for 2 weeks. Puromycin-resistant
clones were expanded before their ACER3 mRNA levels and ACER activity were determined, as described below.

**Overexpression of mouse NC.** Twenty-four hours before transfection, Farber cells were plated in 6-well plates (2.5 x 10^5 cells per well). Then cells were transected with 2.5 μg/well of pcDNAS1/TO (empty vector) or pcDNA5/TO harboring the mouse ASAH2 gene (pcDNA5/TO-ASAH2) using opti-MEM/lipofectamine, following the manufacturer’s instructions. Test compounds were added 24 h after transfection.

**Overexpression of NAAA.** Twenty-four hours before transfection, HEK295T cells were plated in 100 mm Petri dishes (3 x 10^5 cells per dish) and were transected with 8 μg/well of pcDNA3.1(+) (empty vector) or pcDNA3.1(+) harboring the human NAAA gene (pcDNA3.1-NAAA) using opti-MEM/lipofectamine following the manufacturer’s instructions. Test compounds were added 48 h after transfection.

**Cell lysates**

Cell pellets were resuspended in the appropriate volume of a 0.25 M saccharose solution. The suspension was submitted to three cycles of 5 s sonication (probe) at 10 watts/5 s resting on ice. The cell lysate was centrifuged at 600 g for 10 min. The supernatant was collected and protein concentration was determined as specified below.

**Microsomal preparations**

The above 600 g centrifugation supernatants were transferred to 1.5 ml ultracentrifuge tubes and were spun at 100,000 g for 45 min to 1 h at 4°C. The pellets were either stored at −80°C or protein concentration was measured, adjusted with a 0.25 M saccharose solution to the appropriate concentration, and used for activity determination or processed for quantitative (q)PCR or Western blot.

**Ceramidase activities**

All in vitro assays were conducted in 96-well plates at a final volume of 100 μl/well. Reaction buffers were: Pseudomonas aeruginosa neutral ceramidase (pCPlase) [50 mM HEPES buffer, 1 mM CaCl_2 (pH 7.4) in the presence or absence of 0.3% (w/v) Triton X-100]; NC [50 mM HEPES, 150 mM NaCl, 1% sodium cholate (pH 7.4)]; ACER [50 mM HEPES, 1 mM CaCl_2 (pH 9.0)]. Final amounts of protein per well are indicated in the figure legends. For the determination of Kₜ and Vₘₐₓ (pCPlase and hNC), serial dilutions of substrates in the appropriate reaction buffer were made from 80 μM (pCPlase) or 200 μM (hNC) solutions prepared from a 4 mM stock solution in ethanol. Cell lysates: substrates were tested at 40 μM concentration by adding the appropriate volume of 0.1 mM solutions in the suitable buffer, prepared from 4 mM stock solutions in ethanol. The reaction mixtures contained: pCPlase (10 μl/well of protein, 40 μl/well of substrate at the required concentrations, and 50 μl/well of buffer); hNC (20 μl/well of protein, 40 μl/well of substrate at the required concentrations, and 40 μl/well of buffer); ASAH2(−/−) mouse embryonic fibroblasts (MEFs) and ACERS (25 μl/well of protein and 75 μl/well of 53 μM substrate). The reaction mixture was incubated at 37°C for 3 h, except for the determination of Kₜ and Vₘₐₓ (30 min). In all cases, reactions were stopped with 25 μl/well of methanol and then 100 μl/well of NaO₂ [2.5 mg/ml in 100 mM glycine-NaOH buffer (pH 10.6)] was added. After incubation at 37°C for 1 h in the dark, 100 μl/well of 100 mM glycine-NaOH buffer (pH 10.6) was added and fluorescence was measured spectrophotometrically at excitation and emission wavelengths of 355 and 460 nm, respectively. The same reaction mixtures without enzymes were used as blanks.

To determine ceramidase activity in intact cells, the day before the assay, 2 x 10⁴ cells per well were seeded in a 96-well plate. Cells were incubated for 24 h at 37°C and 5% CO₂. Medium was replaced by 100 μl of fresh medium to which the required volume of a 4 mM stock solution of the RBM14 substrate in ethanol had been added to obtain a 40 μM final concentration (1% final concentration of ethanol). The plate was incubated for 3 h at 37°C. 25 μl/well of methanol were added and the assay was continued as described above for cell lysates.

**NAAA activity**

The reaction mixtures contained 25 μl/well of protein (1 mg/ml, 25 μg/well), 40 μl/well of 0.1 mM substrate solution (40 μM final concentration) in reaction buffer [100 mM acetate/acetate buffer (pH 4.5)], and 35 μl/well of reaction buffer. The reaction mixture was incubated at 37°C for 3 h, stopped with 25 μl/well of methanol, and the assay was continued as described in the above section.

**Protein concentration determination**

Protein concentrations were determined with BSA as a standard using a BCA protein determination kit (Thermo Scientific) according to the manufacturer’s instructions.

**Western blot analysis**

Thirty micrograms of microsomal protein were combined with Laemmli sample buffer and heated at 55°C for 30 min. Samples were loaded onto a 12% polyacrylamide gel, separated by electrophoresis at 140 V/1 h, and transferred onto a polyvinylidene fluoride membrane (100 V/1 h). Unspecific binding sites were then blocked with 5% milk in TBS-Tween (TBST) (ACER2) or 3% BSA in TBST (actin). Anti-ACER2 antibody was diluted 1:1,000 in 5% milk in TBST. Anti-actin antibody was diluted 1:2,000 in 3% BSA in TBST. Membranes were incubated overnight at 4°C under gentle agitation. After washing with TBST, membranes were probed with the correspondent secondary antibody for 1 h at room temperature (ACER2, anti-rabbit diluted 1:1,000 in 3% BSA in TBST; actin, anti-mouse diluted 1:10,000 in 5% milk in TBST). Antibody excess was eliminated by washing with TBST and protein detection was carried out using ECL and membrane scanning with LI-COR Image Studio Lite software.

**qRT-PCR analysis**

Total RNA was isolated from cells using RNeasy® Mini kit from Qiagen protocol. RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-8000 spectrophotometer. RNA was treated with DNAse I to remove genomic DNA contamination. Quantities from 1 μg to 100 ng of DNAse I-treated RNA were retrotranscribed to cDNA using Superscript® II reverse transcriptase from Invitrogen and stored at −20°C. cDNA preparations were used to quantify specific transcripts in a LightCycler® 480 real-time PCR system, using SYBR® Green mix (Roche, Germany) and the following pairs of primers: mACER1 (forward, 5'-CAGGAGTACAGGAAGACCAGC-3'; reverse, 5'-CTCTTGCACACCCCGGATG-3'); hACER2 (forward, 5'-CTTCTGCCATGGACCGGGTA-3'; reverse, 5'-GCACTACACAGCCAGGTTAG-3'); hACER3 (forward, 5'-TGACCTGTAGGAATACATGG-3'; reverse, 5'-GGCCGATTCTGGAATACTG-3'); and GAPDH (forward, 5'-ACCATCTTCCAGGACGGAGA-3'; reverse, 5'-GATGGCATTGACTGTTGCA-3'). For all ACER, the initial PCR steps were: 10 min at 95°C, followed by 45 cycles of a 10 s melting at 95°C, and a 30 s annealing/extension at 60°C.
The final step was a 1 min incubation at 60°C. All reactions were performed in triplicate. Relative mRNA abundances of the different genes were calculated from the second derivative maximum of their respective amplification curves (Cp, calculated by duplicates). Cp values for target genes (Tg) were compared with the corresponding values for the GAPDH reference gene to obtain the \( \Delta C_{\text{P}} \) values (\( \Delta C_{\text{P}} = C_{\text{P,GAPDH}} - C_{\text{P,Tg}} \)).

RT-PCR analysis

Total RNA isolation, reverse transcription, and cDNA PCR amplification were performed as indicated in the previous section. Primers were: mACER1, 5′-TTGGACGCTTCTGGCAGCGAGA-3′ (forward) and ACTTTGCATCCACCAGGGCCA-3′ (reverse); mACER2, 5′-GCTGTGATTGCAGGACCAAGCCTT-3′ (forward) and AGGCCGAAGACACACAGCCC-3′ (reverse); mACER3, 5′-ACTGAGAGCAGGATATTGCTG-3′ (forward) and 5′-GGGAGGGCATCCAACAGTCTGATT-3′ (reverse); and microglobulin, 5′-GCTATCAGAAAACCCCTCAAG-3′ (forward) and 5′-CATGTCTCGATCAGGATAGCCGT-3′ (reverse). Conditions for the three ACERs were: one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 57°C for 30 s, 2°C for 20 s, and 1 cycle of 72°C for 5 min. Conditions for β-2-microglobulin were: one cycle of 94°C for 2 min, 30 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 20 s, and 1 cycle of 72°C for 5 min. The PCR products were separated by 1% (w/v) agarose gel electrophoresis. The separated DNA fragments were visualized by ethidium bromide staining and photographed under UV light.

LC/MS

Lipid extracts and LC/MS analysis were carried out as previously described (18, 19).

Statistics

Comparison between two means has been carried out with the t-test and statistical differences are marked with asterisks. For comparison of more than two means, data have been analyzed by one-way ANOVA test followed by Bonferroni’s multiple comparison test. Statistical differences between means are pointed out with different letters atop each bar (same letter indicates no statistical difference).

The quality of the assays for NC and ACERs has been assessed by calculating the Z-factor, which is a valuable tool to evaluate the robustness and suitability of high-throughput screening (HTS) assays (20). This parameter is calculated using the equation: \( Z = 1 - \left[ \frac{3 \text{ SD of sample} + 3 \text{ SD of background}}{\text{mean of sample} - \text{mean of background}} \right] \). The SD values represent the standard deviation values (of sample and background). The Z-factors were calculated using 5 ng of protein (buffer in background) and 20 μM substrate (RBM14C16) for hNC and 25 μg of protein (buffer in background) and 20 μM substrate (RBM14C14) for ACER3. For both enzymes, data were obtained from 15 replicates for both sample and background.

RESULTS

Neutral ceramidases hydrolyze RBM14

Because different NCs have been reported, we tested and compared the activity of bacterial NC, mNC, and hNC on RBM14 compounds. The bacterial ceramidase from Pseudomonas aeruginosa was purified as previously reported (16). Prior to screening the enzyme activity on the fluorogenic substrates, the assay was optimized with this enzyme. Because an aldehyde is produced in the oxidation step of the development phase of the procedure (1), the presence of primary amines in the solution must be avoided. Therefore, Tris was substituted with HEPES, which was also compatible with the presence of Ca\(^{2+}\) in the solution, and so it could be used for ACER. Although Triton X-100 has been previously used in ceramidase activity measurements, we found that it decreased the fluorescence signal (Fig. 2A). Therefore, it was omitted in the assays. The higher hydrophilicity of the RBM14 compounds, as compared with other substrates used [i.e., 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labeled ceramides], allows avoiding the use of Triton X-100 in activity determinations. As concluded from the kinetic parameters, the best substrates for pCDase were RBM14C14 = RBM14C12 > RBM14C10 >> RBM14C8 (Fig. 2B; Table 1).

Determination of enzyme activity in vitro was also carried out with human recombinant enzyme (hNC). As shown in Fig. 2C and Table 1, as in the case of pCDase, the substrate affinity for hNC increased directly with increasing the Nacyl chain length, as concluded from the decrease of the \( K_m \) values. Kinetic parameters show that substrate preference is RBM14C16 = RBM14C14 >> RBM14C12 >> RBM14C10 > RBM14C8.

The hydrolysis of RBM14 compounds by NC was also determined in intact cells. Farber cells, which do not hydrolyze RBM14, were transfected with mNC (Farber cells transfected with the pcDNA5/TO plasmid containing mouse ASAH2 (FD-pcDNA5/TO-ASAH2)). In these experiments, in which all substrates were tested simultaneously at the same concentration (40 μM), NC showed a slight preference for the N-C10 analog (Fig. 2D). This was also the case in cell lysates (Fig. 2E). Interestingly, the N-C8 analog was also hydrolyzed by mNC, in contrast to AC, which does not accept this compound as substrate (11), and the hNC and bacterial NC, for which the C8 and the C10 derivatives are poor substrates.

To confirm the robustness of the assay, we calculated the Z-factor, a statistical parameter used to evaluate the suitability of a HTS assay based on the range of the signal and the internal variation of the measurements. A score of 1 > \( Z \geq 0.5 \) indicates a good assay, and a score of \( Z = 1 \) indicates a perfect assay. Our calculated Z factor for the NC assay using the pure human protein was 0.69 ± 0.17 using the best substrate, RBM14C16. This result demonstrated that the assay is suitable for HTS applications.

Hydrolysis of RBM14 by ACERs

As mentioned in the introduction, three different ACERs have been reported, all of them exhibiting an optimum activity at pH around 9.0. Because NC exhibits residual ceramidase activity at basic pH, the effectiveness of ACERs to hydrolyze RBMC14 was first explored using MEFs deficient in the ASAH2 gene (ASAH2\(^{-/-}\) MEFs). As expected, lysates from these cells were significantly less competent at hydrolyzing RBM14C12 than wild types at neutral pH values (Fig. 3A). As shown in Fig. 3B, all the analogs except for RBM14C8 were hydrolyzed in intact cells, with...
We then determined the levels of mRNA for the different ACERs in ASAH2 (H11002/H11002) MEFs. As shown in Fig. 3D, these cells have high levels of ACER3 transcript. In contrast, levels of ACER2 mRNA are very low and ACER1 mRNA is undetectable. These results are in agreement with reported data (http://biogps.org) and suggest that ACER3 is the ACER involved in the hydrolysis of RBM14 at basic pH in ASAH2 (H11002/H11002) MEFs.

RBM14C14 producing the highest fluorescence values. Hydrolysis of RBM14 was also analyzed in ASAH2 (−/−) MEF lysates at different pH. As shown in Fig. 3C, the highest activity occurred at basic pH, at which RBM14C14 gave the highest hydrolysis rates. The activity observed at neutral pH is likely to correspond to residual activity of ACERs. These results suggested that RBM14s, mainly the C16 analog, were hydrolyzed by at least one of the three ACERS.

We then determined the levels of mRNA for the different ACERS in ASAH2 (−/−) MEFs. As shown in Fig. 3D, these cells have high levels of ACER3 transcript. In contrast, levels of ACER2 mRNA are very low and ACER1 mRNA is undetectable. These results are in agreement with reported data (http://biogps.org) and suggest that ACER3 is the ACER involved in the hydrolysis of RBM14 at basic pH in ASAH2 (−/−) MEFs.
detected in ACER3 knockdown cells than in mock cells, these increases were low (Fig. 4C). However, amounts of 16:0, 24:0, and 24:1 ceramide monohexosides and lactosylceramides were significantly increased in ACER3 knockdown over controls, suggesting metabolization of the augmented ceramide via glycosylation. No difference was observed for sphingomyelins between the two cell lines (data not shown).

To evaluate whether ACER2 hydrolyzes the fluorogenic substrates, we used a stable HeLa-based cell line expressing ACER2 in HeLa T-REx cells under the control of a tetracycline-inducible promoter system (3). This inducible expression system allows the expression of ACER2 to be turned on/off by adding/removing tetracycline to the medium. As expected, addition of tetracycline to the culture medium provoked a dramatic increase in the levels of ACER2 mRNA in HeLa T-REX cells stably transfected with ACER2 (Fig. 3A). Moreover, amounts of 16:0, 24:1, and 24:2 ceramides were significantly lower in ACER2 knockdown cells than in mock cells, these increases were low (Fig. 4C). However, amounts of 16:0, 24:0, and 24:1 ceramide monohexosides and lactosylceramides were significantly increased in ACER3 knockdown over controls, suggesting metabolization of the augmented ceramide via glycosylation. No difference was observed for sphingomyelins between the two cell lines (data not shown).

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Table 1. $K_{m}$ and $V_{max}$ of RBM14 hydrolysis by NC.

| Substrate | $K_{m}$ (µM) | $V_{max}$ (nM/min/mg) | $K_{m}/V_{max}$ | $K_{m}$ (µM) | $V_{max}$ (nM/min/mg) | $K_{m}/V_{max}$ |
|-----------|--------------|-----------------------|----------------|--------------|-----------------------|----------------|
| RBM14C8   | 46 ± 18.1    | 101 ± 20.4             | 0.45           | 262 ± 32.1   | 2,201 ± 321          | 0.119          |
| RBM14C10  | 33 ± 4.2     | 408 ± 22.6             | 0.080          | 181 ± 21.2   | 807 ± 82             | 0.225          |
| RBM14C12  | 15 ± 3.0     | 470 ± 35.9             | 0.032          | 55 ± 4.8     | 1,186 ± 122          | 0.047          |
| RBM14C14  | 22 ± 2.9     | 469 ± 24.5             | 0.048          | 39 ± 4.9     | 4,716 ± 453          | 0.0083         |
| RBM14C16  | n.d.         | n.d.                  | —              | 16 ± 3.3     | 2,572 ± 298          | 0.0069         |
| C16Cer    | 139 (23)     | 5300 (23)              | 0.026          | 33 (29)      | 826 (29)             | 0.040          |
| CerC12NBD | —            | —                     | —              | —            | —                     | —              |

$K_{m}$ is given in micromoles and $V_{max}$ as nanomoles per minute per milligram protein. Data are mean ± SD of three to five independent experiments with triplicates. Protein amounts were: pCDase, 50 ng; hNC, 5 ng. Experiments were carried out as detailed in the Experimental Procedures. In the case of pCDase, the assays were performed in the absence of Triton X-100. C16Cer, N-palmitoylsphingosine. n.d., not determined.

Fig. 3. Hydrolysis of RBM14 compounds by intact (B) and lysed (A, C) ASAH2−/− MEFs. In (A), experiments were performed at pH 7.4 and data correspond to the mean ± SD of two experiments with triplicates using 17 and 12 µg of protein. The asterisk denotes statistical significance at $P < 0.00005$ (unpaired two-tailed t-test, n = 6). In (B), data (mean ± SD) were obtained from three different experiments with triplicates (3.7, 5.1, and 6.2 × 10^4 cells/well); In (C), incubations were carried out with 25 µg of protein at acid, neutral, or alkaline pH. Data correspond to the mean ± SD of three to five experiments with triplicates. Data were analyzed by one-way ANOVA test [$P < 0.0001$ (B); $P < 0.0001$ (C, pH 7.4); $P < 0.0001$ (C, pH 9.0)] followed by Bonferroni’s multiple comparison test. Different letters denote a statistically significant difference between groups ($P < 0.05$). D: Agarose gel electrophoresis ACER analysis of the RT-PCR products obtained from mRNA isolated from ASAH2−/− MEFs. Primers and PCR conditions are detailed in the Experimental Procedures. A representative image is shown.
ACER2 (ACER2-TET-ON cells) over those of cells cultured in tetracycline-free medium (Fig. 5A). In agreement, Western blot analysis with antibodies against ACER2 showed that expression of ACER2 was induced with tetracycline (10 ng/ml, 24 h) in ACER2-TET-ON cells (Fig. 5B). In contrast, microsomes of ACER2-TET-ON cells cultured in the presence of tetracycline had no activity on the fluorogenic substrates (Fig. 5C). Conversely, tetracycline provoked a low, but significant, decrease in fluorescence from all substrates tested as compared with tetracycline-free control cells. That the expressed enzyme was active was proven by LC/MS analysis. Thus, microsomes from ACER2-TET-ON cells treated with tetracycline contained significantly lower levels of 16:0, 18:0, 22:1, 24:0, 24:1, and 24:2 ceramides than microsomes from cells cultured in the absence of tetracycline (controls) (Fig. 5D). The higher difference was observed for the 24:1 and 24:2 species. In agreement with the activity of the overexpressed ACER2,
microsomes from tetracycline-treated ACER2-TET-ON cells had significantly higher levels of free bases than controls (Fig. 5D).

The same tetracycline-inducible enzyme expression system was used to evaluate the ability of ACER1 to hydrolyze the fluorogenic substrates. Addition of tetracycline to the culture medium provoked an increase in the levels of ACER1 mRNA in HeLa T-REx cells stably transfected with ACER1 (ACER1-TET-ON cells) over controls (cells incubated in tetracycline-free medium) (Fig. 6A). However, microsomes of ACER1-TET-ON cells cultured in the presence of tetracycline had no activity on the fluorogenic substrates (Fig. 6B). Contrarily, tetracycline provoked a low, but significant, decrease in fluorescence from RBM14C14 and RBM14C16, as compared with tetracycline-free controls. Evidence that the overexpressed enzyme was active was obtained by LC/MS analysis of microsomes isolated from both cells treated with or without tetracycline. Thus, tetracycline induced a significant decrease in the levels of 22:0, 22:1, 24:0, 24:1, and 24:2 ceramides and an increase in the amounts of free bases, mainly sphingosine (Fig. 6C).

Because RBM14C14 is the best substrate for ACER3, the Z factor was calculated using cell lysates from ASAH2−/− MEFs. A Z factor of 0.74 ± 0.05 was found, demonstrating that the assay is suitable for HTS applications.

Hydrolysis of RBM14 by NAAA

Because NAAA has been shown to have a low, but significant, ceramide hydrolyzing activity over N-lauroyl-sphingosine at pH 4.5 (15), we tested to determine whether NAAA was able to hydrolyze the RBM14 compounds. To this end, HEK293T cells were transiently transfected with an expression vector harboring the human NAAA gene, and the activity over RBM14 was determined in cell lysates at acidic pH. As shown in Fig. 7, lysates from NAAA-transfected cells produced significantly more fluorescence from RBM14C14 and RBM14C16 than lysates from cells transfected with the empty vector. These increases were 1.6 and 2.7 for RBM14C14 and RBM14C16, respectively.

DISCUSSION

Ceramidases are amidohydrolases that catalyze the cleavage of ceramides and dihydroceramides to release fatty acids and sphingosine and sphinganine, respectively. Fluorescence spectroscopy-based methods to determine ceramidase activity have gained attention due to their high sensitivities and signal-to-noise ratios. In this context, different fluorophores, such as NBD (21–23), BODIPY, (24), lissamine-rhodamine (24), and Nile Red (25) have been incorporated into either the fatty acyl or the sphingoid base moiety to produce fluorescent (dihydro)ceramides as ceramidase substrates. Despite the advantage of fluorescent over radioactive methods, the former are not amenable for high-throughput formats. In contrast, fluorogenic substrates are susceptible for high-throughput configurations. Reported ceramidase fluorogenic substrates include FRET-based dually labeled ceramides (12, 26) in which donor and
acceptor FRET pairs are located as part of the acyl chain and/or the sphingoid base of the substrate, and fluorescence is displayed by the ceramidase hydrolysis-promoted fluorescence quencher release. On the other hand, a series of fluorogenic coumarin-containing ceramidase substrates has also been described (10, 11). These compounds (RBM14 compounds, Fig. 1) were first reported as substrates of the AC (10, 11), but the studies described herein show that they are also hydrolyzed by other amidohydrolases such as NC, ACER3, and, to a lesser extent, NAAA.

In the first case, NC includes bacterial, human, and mouse enzymes. Comparison of the $K_m/V_{max}$ ratios indicate that the bacterial enzyme exhibits a preference for RBM14C12 and RBM14C14 ($K_m/V_{max} = 0.032$ and $0.048$, respectively), while the human enzyme hydrolyzes RBM14C14 and RBM14C16 preferentially ($K_m/V_{max} = 0.0083$ and $0.0069$, respectively). In both cases, the substrate affinity increases directly with increasing the N-acyl chain length, as concluded from the decrease of the $K_m$ values. This correlation is particularly evident with the human enzyme, which has, in general, less affinity for the coumarinic substrates than the bacterial enzyme. In contrast, the latter hydrolyzes RBM14 with lower reaction rates than the human enzyme. As compared with reported substrates, hydrolysis of $N$-hexadecanoylsphingosine by pure pCDase occurs with a $K_m$ of 139 µM and a $V_{max}$ of 5,300 nmol/min/mg of protein ($K_m/V_{max} = 0.026$) (23) (Table 1). Thus, the enzyme exhibits a lower affinity for $N$-hexadecanoylsphingosine than for the best fluorogenic substrate, RBM14C12, but the reaction rate is ten times faster with the former than with the latter. On the other hand, pure hNC hydrolyzes $N$-[12-[(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl]-D-erythro-sphingosine (CerC12NBD) with a $K_m$ of 33.41 µM and a $V_{max}$ of 826 nmol/min/mg of protein ($K_m/V_{max} = 0.040$) (29) (Table 1). Therefore, RBM14C16 is a better substrate of hNC than CerC12NBD, as its $K_m$ value is one-half lower (16 µM) and its $V_{max}$ is almost three times higher, which affords a $K_m/V_{max}$ ratio 5.8 times lower (0.0069) (Table 1).

Regarding the mNC, studies were carried out using acid ceramidase-deficient Farber cells transiently transfected with the mouse ASAH2 gene. In this case, a preferential trend for the short acyl chain substrates was observed both in intact and lysed cells. The similarity between both sets of results excludes the possibility that the substrate preference observed in intact cells is due to putative transacyla-
tion of the administered substrate. Intriguingly, the mNC also hydrolyzes RBM14C8, which is not accepted as a substrate by any other ceramidase so far studied. The different substrate preferences between bacterial NC, hNC, and mNC are likely due to differences in their amino acid sequences that may impart different 3D structures to the substrate binding pockets.

Among the three ACERs, only ACER3 has been shown to hydrolyze RBM14, with a preferential activity over the C14 derivative. This conclusion was drawn from results obtained in $ASAHA2^{-/-}$ MEFs, which exhibited an expected lower capacity to hydrolyze RBM14C12 than wild-type MEFs at neutral pH. These cells, containing ACER3 as the almost exclusive ACER, were able to hydrolyze the fluorogenic substrates. As expected, lipid analysis of microsomes from ACER3 knockdown HCT116 cells contained lower amounts of free sphingoid bases than control cells. However, although they also had a higher content of C16:0, C24:1, and C24:2 ceramides, they were not as high (less than 30%) as we expected based on the decrease in ACER3 mRNA levels. Because accumulation of ceramide has been reported to induce cell death, further metabolism of ceramide to more complex lipids could explain the low levels found in these cells. The fact that ACER3 knockdown HCT116 cells contained significantly higher levels of ceramide monohexosides and lactosylceramide supports this possibility.

As mentioned in the Results section, microsomes from ACER1 and ACER2 overexpressing cells produced less fluorescence from the substrates than control microsomes, suggesting a lower capacity of the former to hydrolyze RBM14 compounds. These intriguing results suggest that overexpression of ACER1 and ACER2 provokes a downregulation of other ceramidases. Because the assay was conducted in microsomes at basic pH, ACER3, which hydrolyzes RBM14, is the most plausible candidate. Furthermore, ACER3 is highly expressed in HeLa T-Rex cells (3). Experimental evidence has been reported that ACER3 knockdown upregulates the expression of ACER2 (4). However, no data have been reported that overexpression of one ACER affects the expression of other ceramidases. Nevertheless, transcript analysis showed that tetracycline did not affect ACER3 mRNA levels in either ACER1-TET-ON or ACER2-TET-ON cells (data not shown). Whether the enzyme activity is reduced by ACER1 and ACER2 overexpression is not known.

In this regard, it is possible that activity is decreased by product (sphingoid bases) inhibition, because the total sphingosine/sphinganine levels in cells grown in the presence of tetracycline increased 2- and 3-fold over controls for ACER1 and ACER2 overexpressing cells, respectively. In any case, the overall results shown indicate that neither ACER1 nor ACER2 hydrolyze RBM14.

The lack of hydrolysis of RBM14 compounds by ACER1 and ACER2 could be explained by their reported preference for very long chain ceramides (1, 2, 17, 27), while ACER3 hydrolyzes long chain, but not very long chain, unsaturated ceramides (4). Considering that RBM14 bears a modification in the sphingoid base moiety, another plausible explanation is that ACER3 tolerates this modification, while ACER1 and ACER2 do not. In this regard, ACER3 can hydrolyze ceramides, dihydroceramides, and phytoceramides, while ACER1 and ACER2 are more restrictive toward the ceramide sphingoid base portion (28).

Bioactive N-acylethanolamines, including the endocannabinoid anandamide and the anti-inflammatory and neuroprotective N-palmitoylethanolamine, are hydrolyzed to fatty acids and ethanolamine by fatty acid amide hydrolase and NAAA. The latter, discovered by Ueda and collaborators (14), was shown to work only at acidic pH. NAAA has no homology to fatty acid amide hydrolase, but shares homology with AC and was reported to hydrolyze N-lauroyl-sphingosine at acidic pH (15). In agreement with the AC activity of NAAA, in this article, we show that NAAA hydrolizes...
RBM1C414 and RBM1C416, although both appear to be poor substrates.

In summary, besides AC, NC, ACER3, and NAAA also hydrolyze fluorogenic RBM14 compounds. Although the selectivity of the substrates toward ceramidases can be modulated by the length of the Nacyl chain, none of them is specific for a particular enzyme, except for RBM1C48, which seems to be accepted only by the ectopically expressed mNC. Despite the lack of specificity, the excellent Z factors obtained for the assays with both NC and ACER3 warrant the suitability of these substrates in high-throughput library screening programs aimed at identifying potent and selective inhibitors of NC and ACER3, which are currently unknown.

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