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Molecular Regulation of MHC Class I Chain-Related Protein A Expression after HDAC-Inhibitor Treatment of Jurkat T Cells

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In this study, we characterize the molecular signal pathways that lead to MIC class I chain-related protein A (MICA) expression after histone deacetylase (HDAC)-inhibitor (HDAC-i) treatment of Jurkat T cells. Chelating calcium with BAPTA-AM or EGTA potently inhibited HDAC- and CMV-mediated MICA/B expression. It was further observed that endoplasmic reticulum calcium stores were depleted after HDAC treatment. NF-κB activity can be induced by HDAC treatment. However, nuclear translocation of NF-κB p65 was not observed after HDAC treatment of Jurkat T cells and even though we could effectively inhibit p65 expression by siRNA, it did not modify MICA/B expression. To identify important elements in MICA regulation, we made a promoter construct consisting of −3 kb of the proximal MICA promoter in front of GFP. Deletion analysis showed that a germininal center-box containing a putative Sp1 site from position −113 to −93 relative to the mRNA start site was important for HDAC and CMV-induced promoter activity. Sp1 was subsequently shown to be important, as targeted mutation of the Sp1 binding sequence or siRNA mediated down modulation of Sp1-inhibited MICA promoter activity and surface-expression. The Journal of Immunology, 2007, 179: 8235–8242.

The immune system is critically dependent on the ability to recognize or sense infected, stressed, and transformed cells. One of these sensing systems relies on NKG2D/NKG2D-ligand interaction, where NKG2D is an activating receptor constitutively expressed by several effector cells of the immune system and NKG2D-ligands are up-regulated on the surface of abnormal cells. This system is primarily regulated through increased expression of NKG2D-ligands, and it is thus of pivotal importance to understand the molecular signals that regulate NKG2D-ligand expression.

NKG2D is expressed by human NK cells and CD8 T lymphocytes and can be expressed by CD4 T lymphocytes after activation (1–3). Subsets of NK-T cells and γδ T cells can also express NKG2D (4).

Several diverse NKG2D-ligands exist primarily belonging to the MIC or RAET1 (ULBP) gene families. Healthy human cells express low levels of NKG2D-ligands. However, expression of NKG2D-ligands is up-regulated on many tumors and stressed or infected cells (1, 5). The up-regulation of NKG2D ligands on transformed or infected cells suggests that cells have sensing mechanisms that recognize changes associated with infection and transformation and activate pathways that up-regulate cell surface expression of NKG2D ligands.

We and others have previously shown that cancer cells can be stimulated to express the NKG2D-ligands MHC class I chain-related protein A (MICA)/B after exposure to histone deacetylase (HDAC)-inhibitors (HDAC-i) (6, 7), an occurrence that was not observed in healthy cells. Expression of functionally active MICA/B was not dependent on induction of cell death caused by HDAC-i treatment. MICA/B expression was however dependent on glycosyn proteinase kinase-3 activity, as down-regulation of glycosyn proteinase kinase-3 by different inhibitors or specific siRNA blocked HDAC-i-mediated MICA/B expression (6).

Various other forms of cell stress results in MICA/B up-regulation. It has thus previously been shown that heat shock, viral infection, inflammatory cytokines, retinoic acid, TLR signaling, and DNA damage response involving ataxia telangiectasia-mutated activation, can lead to increased MIC expression (1, 8–12).

Diefenbach et al. have previously shown that cancer cells engineered to over-express NKG2D-ligands can be targeted by an acquired T cell dependent immune attack; the memory response occurred even toward cancer cells not expressing NKG2D-ligands (13). These results imply the very interesting ability of NKG2D-ligand-expressing cells to induce lasting immunity, possibly in the context of suppressing tolerance toward the cancer.

Given the importance of NKG2D/NKG2D-ligand interaction, it is nearly inevitable that regulation of NKG2D-ligands will be targeted by some transformed cancer- or virus-infected cells to avoid immune recognition. It has thus been described that some cancers blunt the system by expressing large amounts of soluble NKG2D-ligand and that CMV hinders surface expression of MICB by affecting endoplasmic reticulum (ER)/Golgi transport (14–19).

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5 Abbreviations used in this paper: HDAC-i, histone deacetylase-inhibitors; EN, endoplasmic reticulum; WT, wild type; ChIP, chromatin immunoprecipitation; MICA, MHC class I chain-related protein A.
Calcium is a well-documented intracellular second-messenger; it is involved in a plethora of signals ranging from stimulation of proliferation to induction of cell death (20). The free cytoplasmic calcium concentration is low in resting cells (10^{-7} M), due to active transport out of the cells or into intracellular stores, primarily the ER (21). Increase in intracellular free calcium concentration can be initiated either by influx across the plasma membrane or by the emptying of the intracellular stores. The pathways are often connected and give rise to complex intracellular calcium waves (20, 21).

Calcium activates the serine/threonine phosphatase calcineurin that is involved in dephosphorylation/nuclear translocation of NFAT, which is critical for T cell activation (22). Calcineurin is effectively inhibited by the widely used immunosuppressant cyclosporine (23). Cyclosporine has been shown to inhibit MICA expression that is normally observed after prolonged CD3/CD28 stimulation of T lymphocytes (24). In contrast, cyclosporine treatment can also lead to increased MIC expression in human hepatocytes (25).

Several groups have found that the transcription factors Sp1 and Sp3 are activated after HDAC-i treatment (26–28). Both Sp1 and Sp3 can be acetylated and it is conceivable that acetylation can increase activity/DNA binding (29, 30). A recent study from Venkataraman et al. (31) has shown that heat shock activation of a MICA promoter construct is critically dependent upon a GC box motif that can interact with Sp1. The purpose of the current study is to delineate the signal pathway that is involved in MICA regulation after HDAC-i treatment of cancer cells.

**Materials and Methods**

**Cells and reagents**

Jurkat E6-1 T cells were from American Type Culture Collection; JTag-9 Jurkat T cells stably transfected with large T Ag from SV40 virus, was provided by C. Geisler (University of Copenhagen, Denmark). JTag-9 cells were primarily used for transient transfection studies. Cells were grown in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS, 2 mmol/L glutamine, 2 mmol/L penicillin, and streptomycin. BAPTA-AM and cyclosporine were from Sigma-Aldrich, FR901228 was provided by the National Cancer Institute (Bethesda, MD).

**Intracellular calcium measurement**

Changes in [Ca^{2+}]_{i} was measured on a Perkin-LS-50B fluorescence spectrophotometer after labeling 4 × 10^6 cells with 1 μM fura-2-AM essentially as described previously (32). Thapsigargin, 100 nM or ionomycin, 1 μM was added directly to the cuvette at the indicated time-points. Transient spikes seen directly after addition of the compounds are artifacts that sometimes occur due to opening and closing of the measurement chamber.

**Transient transfections**

JTag-9 Jurkat T cells were transiently transfected with the Nucleofector kit (Amamax) according to the manufacturer’s protocol. In brief, 1.5 × 10^6 cells were resuspended in 100 μl Cell Line Nucleofector Solution V, mixed with 2.4 μg plasmid or 800 pmol siRNA, and pulsed using the Nucleofector program G10. For siRNA transfections, the cells were retransfected with the same siRNA 24 h later.

**Flow cytometry**

For surface staining, cells were washed twice in cold PBS and stained with PE-coupled MICA/Ab Ab (558352; BD Biosciences) at a dilution of 1/100 for 30 min at 4°C, washed, resuspended and analyzed in PBS. For GFP expression, cells were washed, resuspended, and analyzed in PBS. Flow cytometry analysis and data acquisition were performed on a BD FACSCalibur using CellQuest software.

**Plasmids, generation of promoter constructs, and mutagenesis**

The plasmids pEQ336 (encoding CMV IE promoter, but without any IE coding sequences) and pEQ266 (encoding CMV IE2) were provided by A. Geballe (Fred Hutchinson Cancer Research Center, Seattle, WA) (33). To generate the 3.2-kb wild-type (WT)-GFP plasmid we first produced a 3207 bp PCR product using PfuUltra II Fusion HS DNA polymerase (Stratagene), human genomic DNA as template and the primers: MICA_-3168_Fwd 5’-AGCTCAGACAACTGCAACTGATG-3’; MICA_+39_Rev 5’-GGCCCCGCGACGTCGCCACCCTCAG-3’. The PCR product was then cloned into the GFP reporter vector pGlow-TOPO (Invitrogen Life Technologies) by using TOPO technology. The 3.2-kb-GC-mut-GFP plasmid was made by using the QuickChange II site directed mutagenesis kit (Stratagene) with the primers MICA_-107-107sR: 5’-CAA GGTGCGCGCGAAATCTCCGCGAGG GGGCCCGGGCGAGGGACGCGG-3’ and MICA_-107sR: 5’-CAATCCAGGAGGAGATTTCGGGGCCACGCTTGAG-3’. The PCR product was then cloned into pGlow-TOPO. For the generation of promoter constructs, we first produced a 3207 bp fragment of the 5′ untranslated region of a 3.2-kb-GC-mut-GFP plasmid that we made from a 3.2-kb-GC-mut-GFP plasmid using the PfuUltra II Fusion HS kit. The 3.2-kb-WT-GFP as template, PfuUltra II Fusion HS as the polymerase and the reverse primer MICA_+39_Rev in combination with one of the following forward primers: MICA_-270_Fwd: 5’-CGCGGCGCTGATGTTGGA-3’; MICA_-230_Fwd: 5’-AAAGGCTTTGCTTGCCTTGAGG-3’; MICA_-173_Fwd: 5’-GGGCCGCCGACGCTCTCAG-3’; MICA_-144_Fwd: 5’-CAGCCACCAT CGGCGCGGAGCTCTC-3’; MICA_-115_Fwd: 5’-TGGCCCGCGCCCTCTCGGC TC-3’; MICA_-97_Fwd: 5’-TGTGATTTGCGACTTAACTTT-3’; MICA_-84_Fwd: 5’-TAAGTCCGCGCCGACGTCGCC-3’; MICA_-63_Fwd: 5’-ATT GATGGACGCGGCGGGC-3’; MICA_-41_Fwd: 5’-CGGCCAGCGGTAGC TAATGG-3’; MICA_-25_Fwd: 5’-GTTCTCCGCGCCGACGTC-3’; MICA_-2_Fwd: 5’-GACTCTTGACGGCGTCGAG-3’. Each PCR product was then TOPO cloned into pGlow-TOPO. All constructs were sequenced (MWG-Biotech) to verify correct orientation and sequence of the insert. Promoter-activity was calculated by multiplying percentage of GFP expressing cells with the mean fluorescence of these cells.

**siRNA sequences**

The following siRNAs were purchased at MWG-Biotech: si-sr: 5’-UAG UGUAUUUGGAAUCAUA-3’; si-Sp1: 5’-GAUCAUCUACUGUAUAAA-3’; si-Sp3: 5’-GCUGCAGUGGGGAUCCUACU-3’. The si-p65 was purchased at Qiagen (Hs_RELA (1) validated siRNA).

**Immunoblotting**

Cells were solubilized in lysis buffer (1% NP40, 20 mmol/L Tris-HCl (pH 8.0), 140 mmol/L NaCl, 10% glycerol, 1 mmol/L PMSF, 1 mmol/L Na3VO4, 10 mmol/L NaF, 1 mmol/L IAA, 5 mmol/L EDTA, and 7.5 Ag/ml aprotinin) for 30 min at 4°C and cell lysates were clarified by centrifugation at 13,000 rpm for 10 min. Nuclear and cytoplasmic proteins were separated by Nuclear Extraction Kit (Active Motif) according to the protocol of the manufacturer. Mixtures were tested with NuPage sample buffer (Invitrogen Life Technologies) and incubated at 70°C for 10 min. Proteins were resolved on NuPage gels (Invitrogen Life Technologies) and transferred to nitrocellulose. Proteins were visualized by the indicated primary Ab and HRP-conjugated secondary Ab followed by ECL detection. Primary Abs used were: rabbit anti-p65 (SA-171) and rabbit anti-p50 (SA-170) were from BIOMOL. Rabbit anti-Topoisomerase I (ab3825) was from Abcam. Rabbit anti-Erk2 (K-23; sc-153), rabbit anti-Spi (PEP2; sc-59), and rabbit anti-Sp3 (D-20; sc-644) were all from Santa Cruz Biotechnologies.

**RT-PCR analysis**

RNA was isolated using a RNeasy mini kit (Qiagen) or TRizol (Invitrogen Life Technologies) and reverse-transcribed using SuperScript III reverse transcriptase enzyme (Invitrogen Life Technologies). PCR was performed using standard conditions. MICA primer sequences were: MICA_523F: 5’-GCGTCGAGGAGGACCGAT-3’; MICA_107sR: 5’-CCGCTCAGTGTGATA-3’; MICA_-25_Fwd: 5’-GGTCCCGCGCCGACGTCGCC-3’; MICA_-2_Fwd: 5’-GACTCTTGACGGCGTCGAG-3’. The PCR product was then cloned into pGlow-TOPO. All constructs were sequenced (MWG-Biotech) to verify correct orientation and sequence of the insert. Promoter-activity was calculated by multiplying percentage of GFP expressing cells with the mean fluorescence of these cells.

**Chromatin immunoprecipitation (ChIP)**

For ChIP studies we used a protocol provided by T. H. Leung and D. Baltimore (34). In brief, we used 1 × 10^6 Jurkat E6-1 T cells pr. Ab. Cells were fixed in 1% formaldehyde for 10 min at room temperature and fixation was stopped by adding gylene to a final concentration of 125 mM. Nuclei was isolated and resuspended in 150 μL lysis buffer. After complete lysis, we added 250 μL dilution buffer and sonicated samples four times with 30 s pulses on a Sonicor 600 Ultrasonic Cell Disruptor (VirTis). Samples were then diluted to a final volume of 1.5 mL and immunopre- cipitated at 4°C overnight with specific Ab and protein A agarose. The recovered complexes were transferred to fresh tubes and crosslinking re- versed at 65°C overnight. The next day samples were treated with proteinase K for 1 h and the DNA was extracted using Qiaquick PCR purification
FIGURE 1. HDAC-i-mediated expression of MICA/B is dependent on intracellular calcium. A. Intracellular calcium measurement: Jurkat E6-1 cells were treated for 4 h with vehicle or 20 ng/ml FR901228, cells were subsequently labeled with fura-2-AM and stimulated with 100 nM thapsigargin or 1 μM ionomycin at the indicated time-points. Note that the peaks seen directly after addition of thapsigargin or ionomycin in the right panel are technical artifacts. B. Jurkat E6-1 cells were loaded with different concentrations of BAPTA-AM as indicated for 30 min before treatment with 0 (Control) or 20 ng/ml FR901228 for 18 h. Then cells were stained with anti-MICA/B Ab and analyzed by flow cytometry. The bar graph shows mean ± SD from two independent experiments. C. Jurkat E6-1 cells were loaded with different concentrations of BAPTA-AM as indicated for 30 min before treatment with 0 (Control) or 20 ng/ml FR901228 for 4 h. Then total RNA was extracted and used for quantitative RT-PCR. MICA mRNA expression was normalized to the expression of a housekeeping gene (RPLP0) and displayed as fold expression relative to control. The bar graph shows mean ± SD from two independent experiments. D. JTag-9 Jurkat T cells were transfected with p3.2k-WT-GFP and allowed to rest for 24 h. Then the cells were loaded with BAPTA-AM as indicated for 30 min and subsequently treated with 0 (Control) or 20 ng/ml FR901228. The next day, cells were analyzed for GFP fluorescence by flow cytometry. Data shown is representative of two experiments. E. JTag-9 Jurkat T cells were transfected with pEIQ336 (Control) or pEIQ326 (IE2) and allowed to rest for 6 h. Then the cells were incubated with EGTA as indicated. The next day, cells were analyzed for MICA/B expression by flow cytometry. Data shown is representative of two experiments. F. JTag-9 Jurkat T cells were incubated with cyclosporine (CsA) as indicated 30 min before addition of 20 ng/ml FR901228. Eighteen hours later, the cells were stained with anti-MICA/B Ab and analyzed by flow cytometry. The graph displays mean ± SD from two independent experiments.

Results

FR901228 treatment leads to depletion of ER sequestered calcium

We have made a genechip analysis of Jurkat T cells treated with or without the HDAC-i, FR901228 and found evidence for ER stress in the form of calcium depletion. Several genes associated with ER calcium depletion or elevated intracellular calcium concentration were induced after 4 h of FR901228 exposure, including GADD45β, Hsp70, and GADD153; notably, GADD153 is regarded as a relative specific determinant of ER stress.

To assess if HDAC-i modulated the intracellular free calcium concentration ([Ca2+]), Jurkat T cells were labeled with fura-2 and treated with FR901228. Somewhat to our surprise we could not detect a rise in [Ca2+]i within 30 min of FR901228 exposure (data not shown). However, a calcium rise can be difficult to measure if it is sustained and short, intervals relatively small; this has particularly been observed with other stress pathways dependent on intracellular calcium release (36). In this regard, it is more sensitive to detect sustained calcium release originating from ER indirectly using Thapsigargin that specifically release calcium from ER. As expected, Thapsigargin induced a typical rapid and transient [Ca2+]i rise in control treated Jurkat T cells. However pretreatment for 4 h with

Kit (Qiagen). DNA was eluted in 50 μl Elution Buffer (10 mM Tris-HCL (pH 8.5)). Recovered DNA concentration was measured using Quant-iT PicoGreen dsDNA Reagent (Invitrogen Life Technologies). We used the Mx3000P qPCR system (Stratagene) to analyze specific binding. Two nanograms of DNA (from IP sample or input sample) was used per qPCR reaction. MICA mRNA expression was normalized to the expression of a housekeeping gene (RPLP0) and displayed as fold expression relative to control. The bar graph shows mean ± SD from two independent experiments.
FR901228 nearly completely inhibited the \([\text{Ca}^{2+}]\) rise, showing that FR901228 treatment causes a sustained depletion of calcium from the ER (Fig. 1A).

These results indicate that FR901228 treatment of Jurkat T cells induces a rise in intracellular calcium through emptying of the ER calcium stores. The rise in intracellular calcium can however not be measured directly, probably due to the slow sustained nature that cannot be effectively detected with the method used.

**HDAC-i-induced MICA/B expression is dependent on intracellular calcium**

To further assess the role of calcium in HDAC-i-induced MICA expression we loaded Jurkat T cells with the calcium chelator BAPTA-AM. After treatment with FR901228 the cells were stained with MICA/B Ab and analyzed by flow cytometry. As shown in Fig. 1B depletion of intracellular calcium completely abrogated MICA/B expression in a dose dependent manner. To reveal whether this finding was due to an inhibition at the transcriptional level we measured MICA mRNA production by qPCR on BAPTA-AM-loaded Jurkat T cells 4 h after FR901228 treatment. As shown in Fig. 1C BAPTA-AM reduced HDAC-i-induced MICA mRNA level 5-fold in the same dose-dependent manner as observed with the surface expression. This reduction could be due to either reduced mRNA production caused by lower promoter activity or reduced mRNA stability. Hence, to measure MICA promoter activity we constructed a MICA promoter reporter consisting of 3.2k bp of the MICA 5’-untranslated region inserted upstream of the GFP gene (see Materials and Methods for further details). Jurkat T cells were then transfected with p3.2k-WT-GFP reporter and 24 h later loaded with BAPTA-AM and subsequently treated with FR901228. The next day the cells were analyzed for GFP expression by flow cytometry. As shown in Fig. 1D, removal of intracellular calcium with BAPTA-AM markedly reduced MICA promoter activity. BAPTA-AM (10 \(\mu\text{M}\)) also inhibited the smallest active –115-MICA-GFP reporter (described later in Fig. 3A) activation after FR901228 treatment (data not shown), suggesting that calcium affects transcription downstream from bp –115 relative to the MICA transcription start site. Identical experiments with the extracellular calcium chelator EGTA lead to similar inhibition profiles (data not shown).

It has previously been shown that CMV infection also leads to MICA/B expression (5). We obtained plasmids encoding the critical regulatory gene IE2 and a control plasmid containing IE2 promoter/enhancer, but no coding region (33). Transfection of these plasmids into Jurkat T cells showed that IE2, but not the control, induced MICA/B expression 24 h after transfection, although not to the level observed with HDAC-inhibitors (data not shown). As shown in Fig. 1E, chelating of calcium with EGTA effectively inhibited MICA/B expression induced by IE2 transfection. These results imply that calcium could be a more general regulator of MICA/B expression.

To identify the calcium-dependent molecular signaling pathway leading to MICA/B expression, we used cyclosporine, an inhibitor of the calcium/calcineurin/NFAT pathway. Although cyclosporine was functionally active in Jurkat T cells, as it could potently inhibit CD3 Ab mediated CD154 promoter activity (data not shown and Ref. 32), cyclosporine was not able to inhibit FR901228-induced MICA/B expression in Jurkat T cells (Fig. 1F), suggesting that an unknown calcium-dependent pathway is regulating HDAC-i-induced MICA expression.

**HDAC-i-induced MICA/B expression is not dependent on NF-κB**

Previous studies have shown that HDAC-i treatment of some cell lines induce expression of the NF-κB target gene IL-8 (37). How-
ever, semiquantitative RT-PCR did not reveal any increase in IL-8 mRNA level after FR901228 treatment of Jurkat T cells (Fig. 2A). Furthermore, pretreatment of Jurkat T cells with the IκB inhibitor sulfasalazine, which has been found to inhibit MICA expression in activated T cells (24), was only able to inhibit FR901228 induced MICA/B expression in doses that seriously affected cell viability (data not shown). A key event in NF-κB activation is the translo-
cation of the activated p65/p50 heterodimer from the cytoplasm to the nucleus. Western blotting of cytoplasmic and nuclear fractions of Jurkat T cells treated with FR901228 did not, however, reveal any changes in subcellular localization nor overall levels of p65 or p50 (Fig. 2B).

Finally, siRNA-mediated down-regulation of p65 had no effect on HDAC-i-induced MICA/B expression (Fig. 2C), although siRNA treatment clearly inhibited p65 protein levels (Fig. 2D). For these studies, we conclude that NF-κB p65 activity is not essential for HDAC-i-induced MICA/B expression.

Identification of a regulatory element within the MICA promoter

To identify important HDAC-i regulatory elements within the MICA 5′ untranslated region, we made several 5′ deletion constructs of the 3.2k-WT-GFP reporter. Jurkat T cells were then transiently transfected with these constructs and treated with 20 ng/ml FR901228 the following day. The cells were analyzed for GFP fluorescence after 18 h. The result presented in Fig. 3A revealed a HDAC-i sensitive region spanning from −115 to −84 relative from the transcription start site. Computer analysis of this region with PROMO, an internet based program for identification of putative transcription factor binding sites (38, 39), revealed a GC-box that is a prominent target for Sp1-family binding at position −113 to −93 (Fig. 3B).

To further reveal the functional importance of the GC-box element, we generated a substitution mutant of the 3.2k-WT-GFP reporter and compared the HDAC-i-inducible activity of the mutant reporter with the WT. As shown in Fig. 3C, mutation of the GC-box decreased FR901228-inducible promoter activity by 85% when compared with WT activity. A similar analysis, using the CMV IE2 construct that lead to MICA/B expression (data not shown), showed that IE2-induced promoter activity was inhibited by ~50% by mutation of the GC-box (Fig. 3C).

Targeted down-regulation of Sp1 decreased HDAC-i-induced MICA/B expression

Because a GC-box is a known target for members of the Sp1-family, we performed siRNA-mediated targeted down-regulation of Sp1 and Sp3. As shown in Fig. 4B, both siRNAs were able to down-regulate their respective targets. However, as illustrated in Fig. 4A, flow cytometric analysis of MICA/B expression from siRNA-transfected cells showed that Sp1 siRNA treatment could decrease HDAC-i-induced MICA/B expression from 76% positive cells to 49% positive cells whereas only a small inhibition of MICA/B expression was observed on Sp3 siRNA-treated cells. The siRNA treatment did not affect expression of MHC class I molecules (data not shown).

Fig. 4C shows FR901228-induced MICA promoter activity after cotransfection with siRNA against Sp1 or Sp3. In agreement with the above results, Sp1 down-regulation inhibited promoter activity, whereas siRNA against Sp3 had little effect. These data strongly suggest that Sp1 affects MICA promoter activity.

To see whether Sp1/3 directly binds to the MICA promoter region, we made a ChIP on Jurkat T cells treated for 2 h with or without FR901228. Our ChIP analysis showed a low basal level of Sp1 and Sp3 binding to MICA promoter with a minor increase after FR901228 treatment (Fig. 4D). In contrast, acetylation of histone H4 was markedly increased after FR901228 treatment in concordance with activation of the MICA gene.

In summary, we conclude that Sp1 is a transcriptional inducer in HDAC-i-induced MICA/B expression.

Discussion

It is important to delineate the molecular signal pathways that regulate MICA/B expression, especially given the highly regulated nature of expression that is furthermore readily targeted by infection or transformation.

Our data suggest that an increase in intracellular calcium concentration is important for MICA/B expression after treatment with the HDAC-i FR901228. Jurkat T cells treated 4 h with FR901228 had limited amounts of calcium sequestered in the ER compartment, suggesting that calcium was mobilized. We were not able to directly observe an increase in intracellular calcium after FR901228 treatment, most likely due to technical limitation of the measurement. These results indicate that FR901228 treatment induce a slow but sustained rise in intracellular calcium concentration.

HDAC-i-induced MICA/B promoter-activity, mRNA, and surface expression was strongly inhibited by chelating either the intracellular or extracellular calcium with BAPTA-AM or EGTA respectively. The fact that EGTA (which does not cross the plasma membrane) potently inhibits MICA/B suggests that an effective calcium increment is dependent on extracellular calcium influx.

MICA/B expression induced by Trichostatin A, a structurally different HDAC-i, was also inhibited by BAPTA-AM and EDTA (data not shown). This suggests that the calcium involvement in MICA/B expression is linked to HDAC-i and not to a secondary effect of FR901228 treatment. This correlates with previous studies showing that MICA/B was induced by different HDAC-i, e.g., Valproic acid, FR901228, Trichostatin A, suberoylanilide hydroxamic acid, and PXD-101 (6, 7). Chelating calcium also blocked MICA/B expression after transfection with the MICA/B-inducing CMV promoter IE2, suggesting that calcium involvement in MICA/B expression is not confined to HDAC-i treatment.

We found that cyclosporine did not inhibit HDAC-i-induced MICA/B expression on Jurkat T cells, suggesting that the calcium activated Ser/Thr phosphatase calcineurin is not involved in MICA/B expression. Molinero et al. (24) have reported that MICA expression on CD3/CD28 activated T lymphocytes can be inhibited by cyclosporine. It is indeed possible that different signal pathways regulate MICA expression, however in the study by Molinero et al., cyclosporine was added together with the CD3/CD28 stimulation and MICA expression was assayed 3 days later. We believe that this setup will inhibit nearly every molecule induced after T lymphocyte activation, due to IL-2 and IL-2R inhibition and subsequent lack of further activation/differentiation.

We are currently trying to elucidate how calcium is linked down-stream to MICA/B expression. Our genechip analysis strongly points to induction of ER stress, however preliminary experiments using siRNA suggest that GADD153 is not involved in HDAC-i-mediated MICA/B expression. Likewise the putative ER stress inhibitor, salubrinal, that inhibits elf2α dephosphorylation (40), is without effect (data not shown).

Previous studies have shown that the HDAC-i’s MS-275 and SAHA can induce hyperacetylation and nuclear translocation of the NF-κB subunit p65 in leukemia cells (41, 42). In contrast, trichostatin A has been shown to inhibit cyclin D1 expression through ablation of p65 dimer interaction with the cyclin D1 promoter (43). It was thus of interest to study the involvement of NF-κB activity in HDAC-i-mediated MICA/B expression. We did not detect a nuclear translocation of p65 after treatment of Jurkat T cells with FR901228. In line with this, FR901228 did not activate the NF-κB responsive gene IL-8, which was activated by the
NF-κB activator PMA. These results imply that p65 is not effectively activated in Jurkat T cells treated with FR901228. Because we do observe a robust induction of MICA/B promoter-activity, mRNA expression, and surface expression, these results suggest that p65 activity is not crucially important for HDAC-i-induced MICA/B expression. To support these data, we down-modulated p65 expression by specific siRNA and despite an effective inhibition of p65 expression, this did not affect the induced MICA/B expression. We conclude that NF-κB p65 activity is not essential for HDAC-i-mediated MICA/B expression in Jurkat T cells. It has been shown that NF-κB activity is important for MICA expression of activated peripheral blood T lymphocytes (44) and our data does not argue against the ability of NF-κB activity to further regulate MICA expression.

The MICA promoter analysis revealed a strong regulatory region spanning from −115 to −84 (Fig. 3, A and B) relative to mRNA start site. Within this region we identified a GC-rich region: a GC-box, which is a known target of members of the Sp1/Krüppel-like factor family (45). Mutation of the GC-box motif inhibited HDAC-i-induced MICA promoter activity by −85%. This mutation also inhibited CMV IE2 induced MICA promoter activity, however this inhibition was not as effective (−50%). Interestingly, Venkataraman et al. (31) have recently shown that this GC-box-motif is an important transcriptional regulator in heat-shock induced MICA/B expression. In contrast, Venkataraman et al. did not find that CMV induced MICA promoter activity was affected by mutation of the GC-box. There are several differences in the experimental setup that can account for the differences: 1) different length of the cloned MICA promoter construct: Venkataraman et al. −400 bp, ours −3200 bp; 2) the mutation in the GC-box motif is not similar: Venkataraman et al. CCCC -> AGTT, ours CCCC -> AAAA; 3) differences in the cells and transfection/infection: Venkataraman et al. used human fibroblasts and CMV infection; we used Jurkat T cells transfected with CMV IE2 gene. Different promoter-reporter gene: Venkataraman et al. luciferase, ours cumulated-GFP. Any way the weaker inhibition of the GC-box mutated reporter we observed with IE2 compared with HDAC-i clearly suggests that other factors are important during CMV induced MICA promoter activation. In this regard, it is worth to note that several proteins can bind to the GC-box possibly causing redundancy. An explanation for the observed differences could be that full infection with CMV induces activation of Sp1 redundant factor(s) that does not bind to the mutated sequence, factor(s) that is then not induced by IE2 alone. There may also be negative regulatory factors bound to the upstream promoter which affect promoter-activity if the GC-box is not fully functional.

The GC-box motif can interact with members of the Sp1/Krüppel-like factor family of transcription factors and especially Sp1 and Sp3 have been implicated in HDAC-i-induced signal transduction (46). Sp1 and Sp3 have been shown to compete for the same DNA binding sites (47). siRNA down-modulation showed that Sp1 is involved in HDAC-i mediated MICA/B expression, whereas Sp3 down-modulation only had weak effect. We observed a weak binding of Sp1/3 to the MICA promoter by ChIP analysis, which was not substantially altered by gene-activation. In this regard it is notable that Sp1/3 activity can be regulated by other means than increased binding for example by acetylation (JBC 278:35775, 2003; MCB 26:1770, 2006).

Even though it is impossible directly to compare data from the siRNA experiments with the GC-box mutated promoter construct, it is notable that the mutated promoter construct inhibits MICA activity more effectively than the siRNA targeting, implying that other factors may be involved in MICA regulation through binding to the GC-box motif.

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References


