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Regulation of SIRT 1 mediated NAD dependent deacetylation: A novel role for the multifunctional enzyme CD38

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Abstract

The SIRT 1 enzyme is a NAD dependent deacetylase implicated in ageing, cell protection, and energy metabolism in mammalian cells. How the endogenous activity of SIRT 1 is modulated is not known. The enzyme CD38 is a multifunctional enzyme capable of synthesis of the second messenger, cADPR, NAADP, and ADPR. However, the major enzymatic activity of CD38 is the hydrolysis of NAD. Of particular interest is the fact that CD38 is present on the inner nuclear membrane. Here, we investigate the modulation of the SIRT 1 activity by CD38. We propose that by modulating availability of NAD to the SIRT1 enzyme, CD38 may regulate SIRT1 enzymatic activity. We observed that in CD38 knockout mice, tissue levels of NAD are significantly increased. We also observed that incubation of purified recombinant SIRT1 enzyme with CD38 or nuclear extracts of wild-type mice led to a significant inhibition of its activity. In contrast, incubation of SIRT1 with cellular extract from CD38 knockout mice was without effect. Furthermore, the endogenous activity of SIRT1 was several time higher in nuclear extracts from CD38 knockout mice when compared to wild-type nuclear extracts. Finally, the *in vivo* deacetylation of the SIRT1 substrate P53 is increased in CD38 knockout mice tissue. Our data support the novel concept that nuclear CD38 is a major regulator of cellular/nuclear NAD level, and SIRT1 activity. These findings have strong implications for understanding the basic mechanisms that modulate intracellular NAD levels, energy homeostasis, as well as ageing and cellular protection modulated by the SIRT enzymes.

Keywords: SIRT1; CD38; Knockout mice; cADPR; NAD; Longevity; Liver

Regulation of intracellular nicotinamide adenine dinucleotide (NAD) levels has been implicated in several cellular functions. NAD is the substrate for the Sir2 homologue, SIRT1—which modulates ageing and energy metabolism in mammalian cells [1–3]. SIRT enzymes are NAD dependent deacetylases that are located in different organelles [1–9]. In particular SIRT1 is located in the nuclei and has the capacity to deacetylate histones and P53 [3,5,9]. SIRT1 has been implicated in longevity, regulation of metabolism, and protection of neuronal and cardiac cells against injury [1–9]. However, the mechanisms that regulate intracellular activation of SIRT enzymes have not been completely elucidated.

The enzyme CD38 is a multifunctional enzyme capable of synthesis of the second messenger cADPR, NAADP, and ADPR [10–24]. However, the major enzymatic activity of CD38 is the hydrolysis of NAD. Of particular interest is the fact that CD38 is present on the inner nuclear membrane [11,14,16,25]. The exact function of nuclear CD38 has not been elucidated [24–26]. In fact, to date the role of CD38 as a modulator of the SIRT enzymes has not been explored. Both CD38 and the SIRT enzymes are located in

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the nuclei of mammalian cells [1,24–26]. We have previously shown that CD38 is a major NADase present in mammalian cells and is involved in the regulation of intracellular NAD levels [27]. Here we propose that by modulating availability of NAD to the SIRT1 enzyme, CD38 may regulate SIRT1 enzymatic activity. We observed that in CD38 knockout mice, tissue levels of NAD are significantly increased. We also observed that incubation of purified recombinant SIRT1 enzyme with CD38 or nuclear extracts of wild-type mice led to a significant inhibition of its activity. In contrast, incubation of SIRT1 with cellular extract from CD38 knockout mice was without effect. Furthermore, the endogenous activity of SIRT1 was several times higher in nuclear extracts from CD38 knockout mice when compared to wild-type nuclear extracts. Finally, the *in vivo* deacetylation of the SIRT1 substrate P53 is increased in CD38 knockout mice tissue. Our data support the novel concept that nuclear CD38 is a major regulator of cellular/nuclear NAD levels and SIRT1 activity. These findings have strong implications for understanding the basic mechanisms that modulate intracellular NAD levels, energy homeostasis, as well as ageing and cellular protection modulated by the SIRT enzymes.

Material and methods

CD38 wild-type and knockout mice. CD38 knockout mice (C57BL/6J.129 *CD38*$^{-/-}$, N12 backcross) were produced as described previously [18] and were maintained in the Mayo Clinic and Trudeau Institute Animal Breeding facility in accordance with all Institutional Animal Care and Use Committee Guidelines.

Western blot for P53. Wild-type and CD38 knockout livers were surgically removed, washed three times in ice-cold Hanks' balanced salt solution (HBSS), and homogenized in 40 mM Tris–HCl (pH 7.4) containing 0.25 M sucrose using a Dounce homogenizer. The homogenates were centrifuged at 10,000*g* for 10 min and the resultant supernatant assayed for protein using the *DC* protein assay (Bio-Rad, Hercules, CA). The lysates (1000 μl) were adjusted to contain 1 mg protein and 10 μg mouse monoclonal anti-p53 antibody conjugated to agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) added overnight at 4 °C with gentle rocking. The antibody–protein complex was centrifuged at 1000*g* for 2 min, the pellet washed 4× in sucrose buffer, and resuspended in 30 μl of sucrose buffer and 30 μl Laemmli buffer. The supernatants were denatured at 100 °C for 3–5 min and 50 μl of sample subjected to SDS–PAGE using the Criterion Gel System (Bio-Rad) and a 4–15% gradient gel. The gels were run at a constant current of 200 V for 70 min followed by transfer to PVDF membranes (Bio-Rad). The membranes were blocked for 1 h in 5% nonfat dried milk in TBS containing 0.1% Tween 20 followed by incubation with anti-acetylated p53 rabbit polyclonal antibody (1:1000) (Abcam, Inc., Cambridge, MA) overnight at 4 °C with gentle rocking. The membrane was then probed with an HRP-conjugated donkey anti-rabbit antibody (1:10,000) (Santa Cruz Biotechnology) for 1 h at room temperature. Blots were visualized by exposing them to BioMax MR film (Eastman Kodak Co, Rochester, NY) using Supersignal Substrate (Pierce Biotechnology, Inc., Rockford, IL). SIRT 1 Western blot was performed using a SIRT1 specific antibody from Upstate Biotechnology (Charlottesville, VA).

SIRT 1 activity. SIRT1 activity was determined using the SIRT1 Fluorimetric Kit (Biomol International, LP, Plymouth Meeting, PA) according to the manufacturer's instructions. Briefly, nuclei prepared from wild-type or CD38 knockout mice (1 μg protein/well) were incubated in 40 mM Tris–HCl (pH 7.4) containing human recombinant SIRT1 (1 U/assay), 500 μM NAD^+, and 100 μM *Flour de Lys*-SIRT1 substrate for 30 min at 37 °C. Following incubation, the reaction was terminated by addition of a solution containing *Flour de Lys* Developer and 2 mM nicotinamide. Values were determined by reading fluorescence on a fluorimetric plate reader (Spectramax Gemini XPS, Molecular Devices, Sunnyvale, CA) with an excitation wavelength of 360 nm and emitted wavelength of 460 nm. Calculation of net fluorescence included the subtraction of a blank consisting of buffer containing no NAD^+ and expressed as percent of control. Endogenous SIRT activity was determined as described above with the exception that no recombinant SIRT enzyme was added to the reaction mixture and that the nuclear preparations were sonicated before the assay. The data are expressed as NAD stimulated deacetylation. No deacetylation was observed in the absence of NAD in both wild-type and CD38 knockout mice nuclei.

Nuclei isolation. Mouse liver nuclei were isolated according to Benech et al. [28] with minor modifications. All the steps of the preparation were performed at 4 °C. The livers were excised and washed five times with 20 ml ice-cold TKM solution (50 mM Tris–HCl, pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$) to remove blood cells. Livers were then cut into small pieces and thoroughly homogenized (10 strokes) in 5.0 ml TKM solution supplemented with 0.25 M sucrose (homogenizing medium) using a Dounce homogenizer. The homogenate was filtered through three layers of cheesecloth and centrifuged at 800*g* for 10 min. The pellet was homogenized in the same volume of medium (five strokes) and centrifuged again at 800*g* for 10 min. The resulting pellet was re-suspended in 1.0 ml of medium (five strokes) and added to the top of a sucrose gradient containing (from top to bottom) 0.5 ml each of TKM solution with the following concentrations of sucrose, respectively: 1.0, 1.5, and 2.1 M. The tubes were centrifuged in an SW 55 Beckman rotor at 70,000*g* for 60 min. The resulting pellet was re-suspended in homogenizing medium and centrifuged at 800*g* for 10 min. The final pellet containing the purified nuclei was re-suspended in homogenizing medium (five strokes) at a protein concentration of 2.5 mg/ml and stored at −70 °C until used. Protein concentration was determined using the *Dc* protein assay (Bio-Rad, Hercules, CA). The purity of nuclear preparations was determined as described before [23,27,28], nuclear preps were found to be nearly 100% pure.

ADP-ribosyl cyclase and NADase activity. ADP-ribosyl cyclase activity was measured using the NGD technique as previously described [20,29], and NADase activity was determined using etheno-NAD as described before [27]. Enzyme preparations were incubated in a medium containing 0.2 mM NGD, 0.25 M sucrose, and 40 mM Tris–HCl (pH 7.2) at 37 °C. Activity was determined by measuring the change in fluorescence over time at 300 nm excitation and 410 nm emission.

Immuno-staining for CD38 and Confocal microscopy. Mice liver nuclei were obtained as described previously [28]. Nuclei were fixed in suspension in PBS plus 2% paraformaldehyde for 20 min at room temperature with constant agitation. Fixative was removed by 10 min centrifugation at 800*g* and nuclei were washed three times for 10 min with TBS–Triton X-100 0.1%. After that, nuclei were incubated in blocking buffer (TBS, 30 mM glycine, and 5% BSA) for 60 min. Then incubated with anti-CD38 primary antibody (sc-7049 Santa Cruz Biotechnology) overnight at 4 °C with constant agitation. Primary antibody was removed by centrifugation at 800*g* and nuclei were washed three times for 10 min with TBS-T. Finally, Nuclei were incubated for 1 h with secondary antibody (donkey anti-goat Alexa 546, Molecular Probes) in blocking buffer at room temperature.

Laser confocal images were obtained using the Olympus Fluoview laser scanning confocal microscopy, with objective Olympus UplanApo oil, 100×, 1.35 numerical aperture. Nuclei were excited at 543 nm and their emission recorded at 570 nm.

Detection of NAD and cADPR levels in liver by cycling assay. Mouse liver was frozen in liquid N_2, pulverized into a powder, and extracted with 10% trichloroacetic acid (TCA) at 4 °C. TCA was removed with water–saturated ether. The aqueous layer containing the NAD and cADPR was removed and adjusted to pH 8 with 1 M Tris. To remove nucleotides except cADPR, a mixture containing hydrolytic enzymes was added to the samples with the following final concentrations: 0.44 U/ml pyrophosphatase, 12.5 U/ml alkaline phosphatase, 0.0625 U/ml NADase, 2.5 mM

$MgCl_2$, and 20 mM sodium phosphate, pH 8.0. The detection of cADPR was performed by some modification of the cycling method described recently [30]. Detection of NAD was determined as described before [27].

Materials. All other reagents, of the highest purity grade available, were supplied from Sigma Chemical (St. Louis, MO), except when stated otherwise.

The reported experiments were repeated three to six times and data are expressed as means ± SE. Student's *t*-test was used to evaluate statistical significance; p values <0.05 were considered significant.

Results and discussion

CD38 is the major NADase and controls NAD levels in mouse liver

To date the mechanisms that modulate intracellular levels of NAD have not been completely elucidated. We have recently shown that CD38 controls intracellular NAD levels [27]. Using CD38 wild-type and knockout mice we found that CD38 is the main NADase in liver [27]. Neither ADP-ribosyl cyclase activity nor cADPR levels, markers of CD38 activity, can be detected in CD38 knockout mice [27]. Furthermore, liver from wild-type mice contains CD38 and NADase activity [27]. In contrast, NADase activity in liver from CD38 knockout mice is nearly absent [27]. We also have shown the role of CD38 on controlling intracellular levels of NAD. We found that NAD levels in wild-type liver are maintained at levels between 0.2 and 0.4 nmol/mg of protein [27]. In contrast, NAD levels in CD38 knockout mice liver are 10–20 times higher than levels in wild-type animals [27]. These data clearly indicate a major role for CD38 as a regulator of intracellular NAD levels in liver. Several nuclear processes have been shown to be regulated by NAD, in particular SIRT 1 mediated deacetylation is a nuclear NAD dependent process [1–9]. Here, we explored the role of CD38 on the SIRT 1 activity. In particular we determined the role of endogenous nuclear CD38 on the activity of SIRT.

CD38 is present in the nuclei

It has been previously shown that CD38 is expressed in rat liver and is localized to its inner membrane [24]. Furthermore, nuclear CD38 has been implicated in the control of nuclear Ca^{2+} fluxes [24]. Here, we confirm that CD38 is present in isolated nuclei from wild-type mice. In Fig. 1 we show the immuno-staining for CD38 in isolated nuclei. Furthermore, both ADP-ribosyl cyclase and NADase activities can be detected in isolated nuclei (Fig. 1). Immunoprecipitation studies were performed to confirm the role of CD38 as the enzyme responsible for the ADP-ribosyl cyclase and NADase activities. We observed that immunoprecipitation of CD38 in nuclear extracts leads to nearly complete immunoprecipitation of the cyclase and NADase activity (data not shown). Furthermore, as shown in Fig. 1 neither the cyclase nor the NADase activities can be detected in nuclei prepared from CD38 knockout mice.

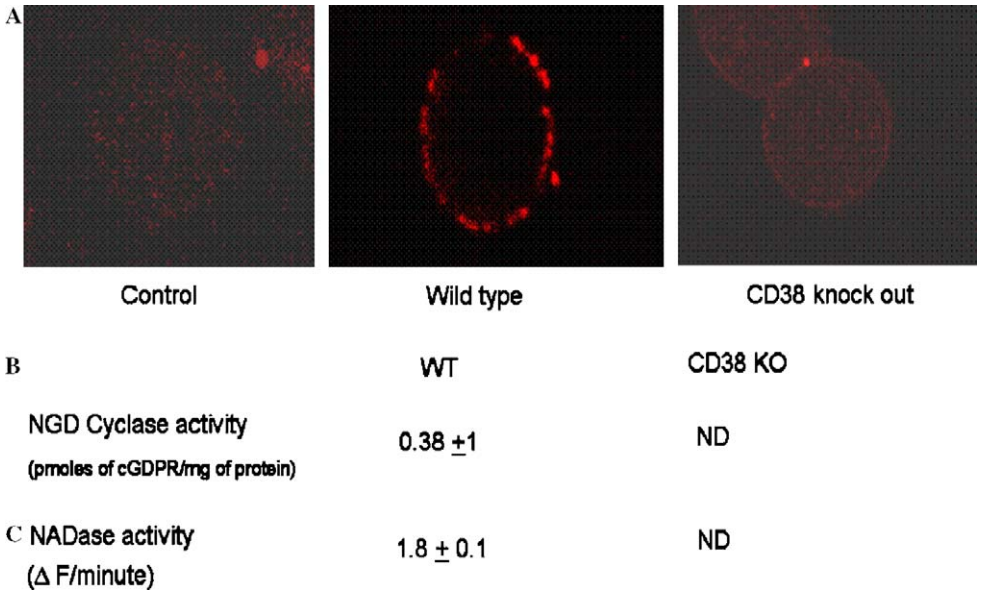

Fig. 1. Characterization of CD38, ADP-ribosyl cyclase, and NADase activity in wild-type (WT) and CD38 deficient (KO) mouse liver nuclei. (A) Confocal immunostaining for CD38 in liver nuclei. The nuclear extracts were probed with a goat polyclonal anti-CD38 antibody using standard immunohistochemistry techniques. Control shows the image of nuclei exposed to secondary antibody but no CD38 specific antibody, Wild-type and CD38 knockout shows the staining of the nuclei with both the specific CD38 antibody and the secondary antibody as described in material and methods. (B) Measurement of ADP-ribosyl cyclase activity in liver nuclear. Nuclear extracts were assayed for cyclase activity using NGD as the substrate as described in Materials and methods. Wild-type extracts produced 0.38 ± 1 pmol cGDPR/mg protein and KO extracts had non-detectable activity (ND). (C) NADase activity was determined by addition of 0.1 mM 1-N^6-etheno-NAD and measuring the amount of its flurogenic product, 1-N^6-ADPR. Wild-type homogenates yielded a change in fluorescence of 1.8 ± 0.1 AFU/min. NADase activity was not detectable (ND) in the CD38 KO extracts. Experiments were repeated 3–4 times.

These data indicates that CD38 is the primary NADase in isolated nuclei.

Effect of CD38 and metabolites of NAD on the activity of purified recombinant SIRT-1

Since CD38 can modulate intracellular NAD levels and is present in the nuclei we postulated that it may regulate the activity of the NAD dependent deacetylases such as SIRT-1. Using purified recombinant SIRT-1 enzyme we determined the effect of purified CD38 upon SIRT-1 deacetylase activity. Addition of 1 U of purified CD38 to the 1 U of purified SIRT1 leads to a complete inhibition of the SIRT activity.

To further understand the mechanism of CD38 inhibition of SIRT1 activity we determined the role of NAD metabolites generated by CD38, namely NAD, nicotinamide, ADPR, and cADPR, on SIRT1 activity. We postulate that the mechanism of inhibition of the SIRT activity induced by CD38 is mediated by decreased availability of the substrate NAD and increased availability of the SIRT inhibitor nicotinamide. In fact, we observed that of all the NAD metabolites tested only NAD could activate SIRT1 (Fig. 2). Furthermore, only nicotinamide provided significant inhibition of the NAD dependent deacetylating activity of SIRT1 (Fig. 2). Neither cADPR nor ADPR were able to stimulate or inhibit the SIRT1 activity (Fig. 2). In contrast, as previously described, the SIRT1

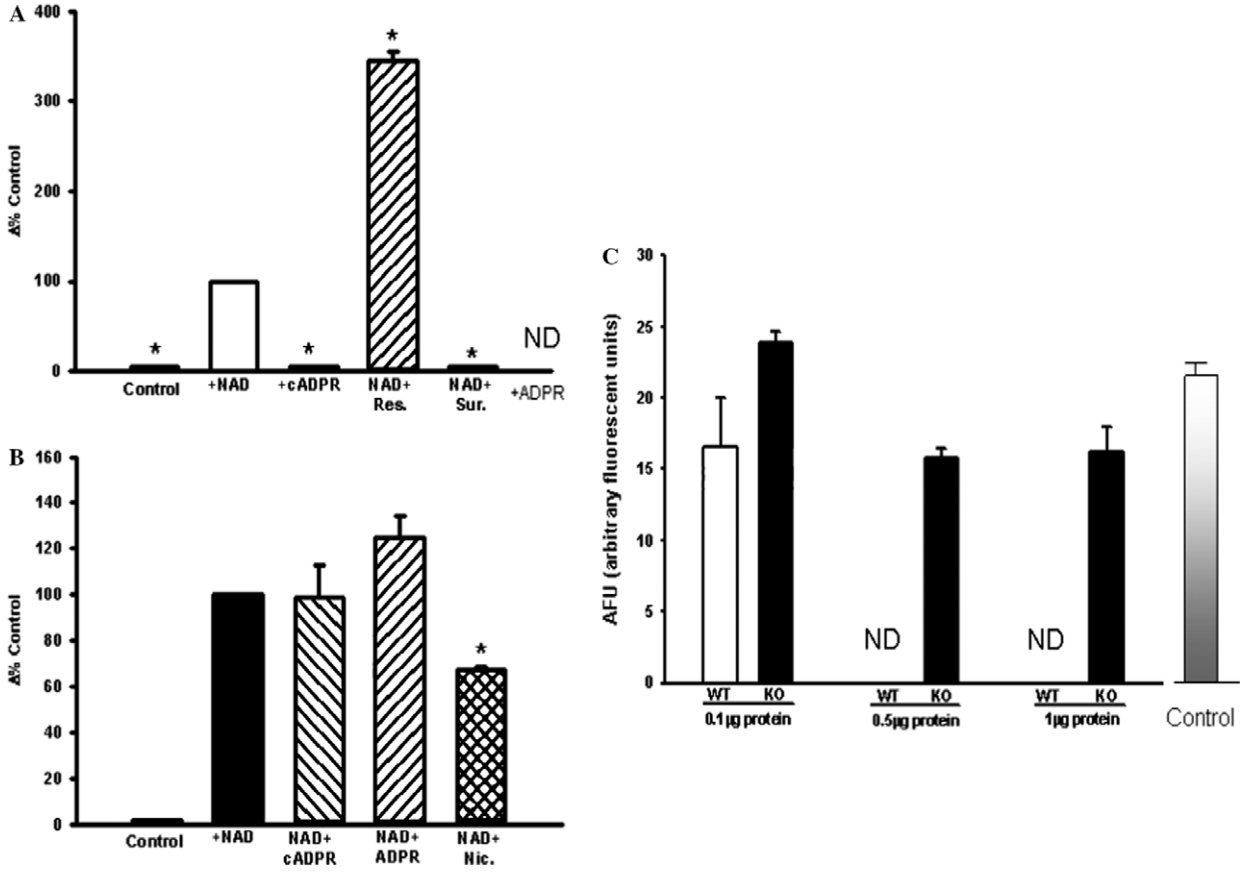

Fig. 2. Effect of NAD metabolites and nuclear extracts upon the activity of recombinant SIRT-1. Recombinant SIRT1 (1 U/ml) were incubated with different metabolites of NAD or extracts of liver nuclei from wild-type and CD38 knockout mice for 3 min before the addition of the SIRT-1 substrate Fluor de Lys-SIRT 1 substrate, and the activity of the enzyme was carried for different times, activity was determined during linear portion of the curve (initial SIRT1 activity rate). (A) The effect of agents upon the activity of SIRT 1 was determined after incubation of the enzyme with no addition (control), 100 μM NAD (+NAD), 100 μM cADPR (+cADPR), 100 μM NAD, and 100 Reveratrol μM (NAD + Res), 100 μM NAD and 100 μM suramin (NAD + Sur). One hundred micromolars ADPR (+ADPR). The activity was expressed as % of control in the presence of 100 μM NAD. The activity of 1 U/ml SIRT 1 in the presence of 100 μM NAD was about 1.2 ± 0.3 pmol/min of hydrolysis of Fluor de Lys-SIRT 1 substrate. (B) We tested the effect of metabolites of CD38 upon the recombinant SIRT 1 activity. SIRT 1 was pre-incubated with no-addition (control), 100 μM NAD (+NAD), 100 μM NAD, and 100 μM cADPR (NAD + cADPR), 100 μM NAD and 100 μM ADPR (NAD + ADPR), or 100 μM NAD and 100 μM nicotinamide (NAD + Nic.). (C) The activity of the recombinant SIRT-1 activity was determined after pre-incubation with 100 μM NAD and different concentrations of nuclear extracts from wild-type (WT) and CD38 knockout mice (KO) for 3 min. The activity of the control was determined in the presence of after 100 μM NAD. ND indicates not detected. The endogenous activity of the SIRT enzymes in wild-type and CD38 knockout nuclear extracts was subtracted from the total activity to provide only the recombinant SIRT 1 activity the endogenous activity of SIRT 1 on these assays was at least 10 times lower than the recombinant SIRT 1 activity. The NADase activity of 0.5 μg of Wt nuclei is equivalent to 1 U of purified CD38. Experiments were repeated 3–4 times. *Significantly different from control ($p < 0.05$).

activity was inhibited by suramin and stimulated by reverostrol [1].

Nuclear CD38 and the regulation of SIRT activity

We further determined the role of nuclear CD38 on the activity of the SIRT1 enzyme. Using isolated nuclei, we observed that the activity of the recombinant SIRT1 was inhibited by nuclear extracts from wild-type but not CD38 knockout mice (Fig. 2). This experiment confirms that nuclear CD38 can modulate the activity of SIRT. This effect appears to be mediated by the regulation of availability of the substrate NAD and the inhibitor nicotinamide by nuclear CD38 as discussed above. In fact, in co-immunoprecipitation studies no evidence of direct interaction between CD38 and SIRT was observed (data not shown). We characterized the purity of our nuclear preparation by the measurement of the activity of the endoplasmic reticulum marker glucose-6-phosphatase and the plasma membrane marker 5-nucleotidase. In both cases the nuclear preparation had less than 0.5% of contamination of either endoplasmic reticulum or plasma membrane. To further determine that the effect of the nuclear preparation was not due to contamination of the preparation with endoplasmic reticulum, we repeated the experiments from Fig. 2C with a preparation of microsomes (endoplasmic reticulum) from wild-type and CD38 knockout cells. In either way the activity of recombinant SIRT1 was not inhibited by the incubation with 1 μg of endoplasmic reticulum.

Lack of CD38 increases the endogenous activity of the nuclear NAD dependent deacetylation

To further confirm that CD38 is an endogenous regulator of the SIRT activity, we explored the endogenous activity of the NAD dependent deacetylation in isolated nuclei from wild-type and CD38 knockout mice. As shown in Fig. 3, we observed that the endogenous activity of the NAD dependent deacetylation was nearly absent when wild-type nuclei were used but was increased several times when using nuclei isolated from CD38 knockout mice. The activity of the SIRT in CD38 knockout nuclei was detected only in the presence of the addition of exogenous NAD indicating that the effect of the CD38 is not related to depletion of NAD during the preparation of the nuclei.

We also determined the *in vivo* role of CD38 on the endogenous activity of the SIRT enzyme. It has been previously shown that endogenous deacetylation of P53 is

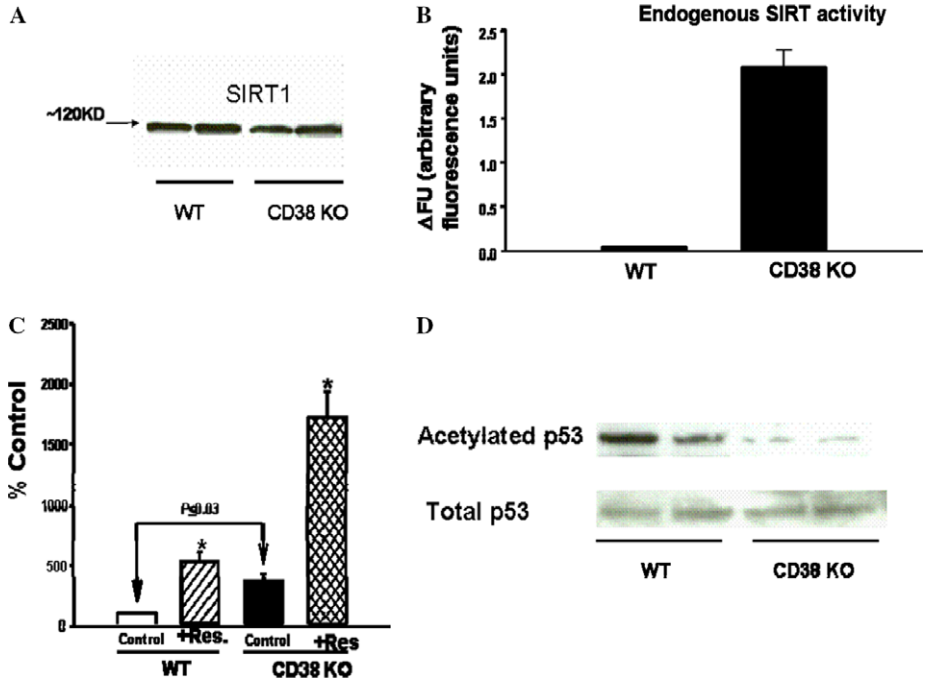

Fig. 3. SIRT 1 activity in wild-type and CD38 knockout nuclei. (A) Nuclear extracts from wild-type (WT) and CD38 knockout (CD38 KO) mice liver were assayed for the presence of SIRT 1 by Western blot analysis. (B) The endogenous activity of the SIRT enzyme was determined in the presence of 100 μM NAD as described in Materials and methods. The SIRT activity was absent in wild-type (WT) and CD38 deficient (KO) liver nuclei when NAD was not added to the reaction (these values were used as blanks and discounted from the total activity in the presence of NAD). Furthermore, the activity of the SIRT enzymes was nearly absent in wild-type nuclei and was 0.22 ± 0.03 pmol/min in CD38 knockout (CD38 KO) nuclei. (C) We show the activity of the endogenous SIRT 1 from wild-type and CD38 nuclei in the presence of 100 μM NAD (control) or 100 μM NAD plus 100 μM reveratrol. The activity is expressed as % of control (activity of the wild-type nuclei in the presence of NAD), no activity was detected in either preparation in the absence of exogenous NAD. In (D) the endogenous acetylation of P53 was determined in wild-type (WT) and CD38 knockout (KO) as described in Materials and methods. The total p53 was not altered in CD38 knockout liver. In contrast the endogenous (*in vivo*) acetylation of p53 was nearly abolished when CD38 was absent. Experiments were repeated 3–4 times. *Significantly different from control ($p < 0.05$).

mediated by the SIRT enzymes. Here we determined levels of acetylated P53 as a measure of the *in vivo* activity of SIRT enzymes. We observed that neither levels of SIRT1 nor total p53 were altered in CD38 knockout mice (Fig. 3). In contrast the *in vivo* levels of deacetylated p53 were increased several fold in CD38 knockout mice (Fig. 3). To avoid any possible confounding effects of the endogenous activity of CD38 and SIRT1 during the overnight incubation necessary for the immunoprecipitation, several controls were performed, including the addition of a combination of inhibitors of CD38 and SIRT1 during the immunoprecipitation such as 10 mM nicotinamide and 100 μM suramin, the addition of these inhibitors in isolation or in combination leading to no change of the results of the endogenous level of acetylated P53 (data not shown). These indicates that the level of acetylated P53 detected was not due to deacetylation of the P53 during the incubation necessary for the immunoprecipitation. Furthermore, we performed experiments where 100 μM *Flour de Lys*-SIRT1 substrate was added during the immunoprecipitation protocol, and after the 24 h incubation at 4 °C the content of acetylated Lys-SIRT1 substrate was determined, we observed that the amount of acetylated Lys-SIRT1 was not significantly changed after the incubation with either CD38 wild-type, CD38 knockout or blanks in the conditions of the endogenous acetylated P53 experiments. Taken together these data indicate that the endogenous activity of the SIRT enzyme and deacetylation of p53 are regulated by CD38.

Conclusion

CD38 is a multifunctional enzyme responsible for the synthesis of the second messengers cADPR, ADPR, and NAADP [10–26]. However, the primary activity of CD38 is the degradation of NAD [25,27]. The role of CD38 as a regulator of NAD dependent functions has been recently proposed [27]. In fact, we have recently shown that CD38 is one of the main regulators of intracellular NAD levels [27]. In this regard, and of particular interest is the recent discovery that NAD is a regulator of cellular response to injury and contributes to longevity [1–9]. These effects of NAD appear to be modulated by the SIRT enzymes. The SIRT enzymes are a group of NAD dependent deacetylases implicated in longevity, cellular protection, and regulation of energy metabolism [1–9]. SIRT1-7 are present in several intracellular organelles including nuclei and mitochondria and regulate the acetylation of histone and transcription factors including p53 [1–9]. Here we show that CD38 can regulate the activity of the SIRT enzymes via the regulation of the availability of the SIRT substrate, NAD, and the SIRT inhibitor, nicotinamide. In particular nuclear CD38 may be a regulator of SIRT1 activity. Our observation has major implications for the mechanism of action of CD38 and the regulation of the NAD dependent deacetylation *in vivo*. The results of this study may have implications for longevity, cellular protection, and regulation of metabolism. In particular, our data introduce a novel role for CD38 in the regulation of SIRT and may implicate CD38 as a therapeutic target in order to modulate the function of NAD dependent processes, in a cADPR and Ca^{2+} independent way.

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