Biochimica et Biophysica Acta 1783 (2008) 1536–1543

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamcr

Direct protein–protein interaction of 11β-hydroxysteroid dehydrogenase type 1 and hexose-6-phosphate dehydrogenase in the endoplasmic reticulum lumen

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A R T I C L E I N F O

Article history:
Received 4 February 2008
Received in revised form 1 March 2008
Accepted 3 March 2008
Available online 12 March 2008

Keywords:
11beta-hydroxysteroid dehydrogenase
Hexose-6-phosphate dehydrogenase
Glucocorticoid
Endoplasmic reticulum
Protein interaction
Reductase

A B S T R A C T

Hexose-6-phosphate dehydrogenase (H6PDH) has been shown to stimulate 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1)-dependent local regeneration of active glucocorticoids. Here, we show that coexpression with H6PDH results in a dramatic shift from 11β-HSD1 oxidase to reductase activity without affecting the activity of the endoplasmic reticular enzyme 17β-HSD2. Immunoprecipitation experiments revealed coprecipitation of H6PDH with 11β-HSD1 but not with the related enzymes 11β-HSD2 and 17β-HSD2, suggesting a specific interaction between H6PDH and 11β-HSD1. The use of the 11β-HSD1/11β-HSD2 chimera indicates that the N-terminal 39 residues of 11β-HSD1 are sufficient for interaction with H6PDH. An important role of the N-terminus was indicated further by the significantly stronger interaction of 11β-HSD1 mutant Y18-21A with H6PDH compared to wild-type 11β-HSD1. The protein–protein interaction and the involvement of the N-terminus of 11β-HSD1 were confirmed by Far-Western blotting. Finally, fluorescence resonance energy transfer (FRET) measurements of HEK-293 cells expressing fluorescently labeled proteins provided evidence for an interaction between 11β-HSD1 and H6PDH in intact cells. Thus, using three different methods, we provide strong evidence that the functional coupling between 11β-HSD1 and H6PDH involves a direct physical interaction of the two proteins.

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1. Introduction

The 11β-HSD1-dependent local activation of glucocorticoids recently attracted much attention because of its implications in the pathogenesis of metabolic diseases including obesity, insulin resistance and type 2 diabetes, atherosclerosis and hypertension [1–3]. Inhibition of the production of active 11β-hydroxyglucocorticoids (cortisol, corticosterone) from inactive 11-ketoglucocorticoids (cortisone, 11-dehydrocorticosterone) by 11β-HSD1 is currently considered as a novel promising therapeutic approach for these disorders. This strategy implies that 11β-HSD1 functions as a reductase.

In tissue homogenates or upon purification 11β-HSD1 is a bidirectional enzyme catalyzing both the oxidation of 11β-hydroxyglucocorticoids and the reduction of 11-ketoglucocorticoids [4,5]. Although 11β-HSD1 acts as a dehydrogenase in some cells, such as preadipocytes and testicular Leydig cells, it predominantly functions as a reductase in most cell types including metabolically relevant hepatocytes and mature adipocytes [6–10]. 11β-HSD1 is an endoplasmic reticulum (ER) membrane protein with a single N-terminal transmembrane helix and its catalytic moiety facing the lumenal compartment [11–13]. It preferentially utilizes NADP(H) as cofactor [14,15], whereby both the topology and the cofactor availability can be considered as important determinants for the reaction direction of 11β-HSD1 [13,16]. Because the ER membrane is almost impermeable for NADP(H), the reaction direction of 11β-HSD1 depends on the intralumenal availability of the cofactor [17].

In the ER lumen, cofactor NADPH is generated by the enzyme H6PDH [18,19], which is an isoform of the well known and extensively studied glucose-6-phosphate dehydrogenase (G6PDH) that catalyzes the first and rate-limiting step of the pentose phosphate cycle in the cytoplasm. Distinct from G6PDH, H6PDH not only utilizes glucose-6-phosphate as a substrate but also other hexose-6-phosphates and sugars. It was demonstrated that H6PDH, depending on the substrate, exhibits different preference for $NADP^+$ and NAD^+, respectively [20,21]. At physiological pH and with the major substrate glucose-6-phosphate, the generation of NADH by purified H6PDH is approximately 30% that of NADPH [21]. Compared with the activity of G6PDH and the production of NADPH in the cytoplasm, the estimated ER-lumenal NADPH production by H6PDH is relatively low and contributes only a few percent to the total cellular NADPH formation [22].

The functional role of H6PDH remained obscure until recently when a number of studies indicated that NADPH generation by

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doi:10.1016/j.bbamcr.2008.03.001

H6PDH leads to the stimulation of the 11β-HSD1-dependent formation of active glucocorticoids [23–27]. In a previous study, using the HEK-293 cell system, we demonstrated that coexpression of 11β-HSD1 with H6PDH resulted in a more than 20-fold increase in the ratio of reductase/dehydrogenase activity of 11β-HSD1 [24]. However, the molecular mechanism underlying the H6PDH-dependent stimulation of 11β-HSD1 reductase activity remained unclear, and the question arose whether H6PDH enhanced 11β-HSD1 reductase activity by increasing the overall concentration of NADPH and NADH in the ER lumen or whether it stimulates 11β-HSD1 by physical interaction and direct delivery of cofactor NADPH for cortisone reductase activity [16].

To investigate the mechanism by which H6PDH stimulates 11β-HSD1 reductase activity, we employed the HEK-293 cell system, which lacks the endogenous expression of 11β-HSD1 and H6PDH, thus allowing the expression of recombinant wild-type and mutant 11β-HSD1 and other short-chain dehydrogenase/reductase enzymes in the presence or absence of H6PDH, followed by analyses of enzymatic activity, intracellular localization and protein–protein interactions.

2. Experimental procedures

2.1. Chemicals and reagents

Cell culture reagents were purchased from Invitrogen (Carlsbad, CA), [1,2,6,7-^{3}H]-cortisol, [2,4,6,7-^{3}H]-estrone and [2,4,6,7-^{3}H]-estradiol were from Amersham Health AG (Wädenswil, Switzerland) and [1,2,6,7-^{3}H]-cortisone from American Radiolabeled Chemicals (St. Louis, MO). The unlabeled steroid hormones were from Steraloids (Wilton, NH). All other chemicals were from Fluka AG (Buchs, Switzerland) and were of the highest grade available.

2.2. Cell culture and transient transfection

HEK-293 (human embryonic kidney) cells were grown at 37 °C under 5% carbon dioxide in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, 4.5 g/L glucose, 50 U/mL penicillin/streptomycin, and 2 mM glutamine. Cells were grown to 90% confluence and split 1:5 every third day for propagation or 1:2 24 h prior to transfection according to the calcium phosphate precipitation method. For transient expression human 11β-HSD1 and 11β-HSD2 containing a C-terminal FLAG-epitope [11], human 17β-HSD1 and 17β-HSD2 with a C-terminal histidine-tag [28] or human H6PDH and G6PDH with a C-terminal myc epitope [24] cloned into pcDNA3 were used. The construction of the chimera 12F (residues 1–39 of 11β-HSD1 and 88–405 of 11β-HSD2) and 21F (amino acids 1–87 of 11β-HSD2 and 40–292 of 11β-HSD1) and mutant Y18-21A (substitutions of tyrosine residues 18–21 to alanine) was described previously [11]. HEK-293 cells grown in 10 cm dishes were transfected with 8 μg of SDR expression plasmid and 8 μg of plasmid for H6PDH, G6PDH or pcDNA3 control according to the calcium phosphate precipitation method.

2.3. Activity assays

Enzyme activities were measured essentially as described earlier [28]. Briefly, cells were transferred 24 h post-transfection in 96 well plates followed by incubation for another 24 h. The rates of conversion of cortisol to cortisone, estradiol to estrone and the reverse reactions were determined by incubation of the cells at 37 °C and 5% CO_2 for different time intervals ranging from 0.5 to 4 h (to reach a final conversion between 10–30%) in the presence of 10 nM of the corresponding radiolabeled steroid ([1,2,6,7-^{3}H]-cortisol, [1,2,6,7-^{3}H]-cortisone, [2,4,6,7-^{3}H]-estrone or [2,4,6,7-^{3}H]-estradiol) and various concentrations of unlabeled substrate (10–1990 nM). The reactions were stopped by adding an excess of unlabeled steroids in methanol, followed by separation of the steroids using thin-layer chromatography and scintillation counting. The activities were compared by calculating K_{cat} (V_{max}/K_m) and normalizing K_{cat} values to the values obtained for the oxidase reaction of the corresponding SDR enzyme in the absence of H6PDH.

2.4. Coimmunoprecipitation and immunoblotting

For coimmunoprecipitation experiments HEK-293 cells were split in 10 cm dishes and transfected 24 h later with the constructs indicated. The cells were then incubated for 48 h at 37 °C to achieve sufficient protein expression, followed by washing twice with PBS and lysis for 1 h at 4 °C in a buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Triton X-100. Cell lysates (1 mg of total proteins) were then incubated for 3 h with 40 μl mouse monoclonal anti-FLAG antibody M2-coupled agarose beads (A2220, Sigma-Aldrich, Buchs, Switzerland) to bind FLAG-tagged 11β-HSD1, 11β-HSD2 or chimeric proteins. Alternatively, HIS-Select™ Nickel Affinity Beads (E3528, Sigma-Aldrich) were used to bind histidine-tagged 17β-HSD1 and 17β-HSD2. The beads were then washed four times with TBS, and the precipitated protein was eluted with SDS-PAGE sample buffer without dithiothreitol. After separation on SDS-PAGE, the proteins were transferred to a nitrocellulose membrane followed by immunodetection with primary antibodies against the corresponding tag (mouse anti-FLAG M2 antibody, Sigma-Aldrich; mouse Tetra-His antibody, Qiagen; or rabbit anti-myc antibody, Abcam, Cambridge, UK) and secondary horse-radish peroxidase (HRP)-conjugated antibodies (HRP-goat anti-mouse IgG (BioRad) and HRP-goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). The secondary antibodies were visualized by enhanced chemiluminescence (ECL Plus™) Western blotting detection reagents (Amersham Health AG).

2.5. Affinity purification and enzyme activity of H6PDH

HEK-293 cells stably transfected with the myc-tagged H6PDH construct were rinsed twice with PBS, and the protein was immunopurified with anti-myc antibody-coupled agarose beads (A7470, Sigma-Aldrich) according to the protocol of the manufacturer. Bound protein was eluted from the beads by incubation with 100 μg/ml c-myc peptide (M 2435, Sigma-Aldrich) in 50 mM Tris–HCl pH 7.4 for 30 min at 25 °C. Analysis by SDS-PAGE and Coomassie-staining revealed a single protein band of approximately 90 kDa, corresponding to H6PDH. The purified protein was supplemented with 1 mg/ml BSA and 15% glycerol, snap-frozen in dry ice/ethanol and stored at −70 °C. The activity of H6PDH was measured by fluorometric detection of NADPH formation in the presence of 100 μM glucose-6-phosphate and 250 μM $NADP^+$ as described previously [25].

2.6. Far-Western blotting

Far-Western blotting was performed essentially as described [29]. A total amount of 1 mg of total protein from HEK-293 cells transfected with wild-type or mutant 11β-HSD1 or with pcDNA3 control was subjected to immunoprecipitation followed by SDS-PAGE and electrotransfer to a nitrocellulose membrane. The membrane was then incubated for 2 h in blocking buffer 1 (0.05% Tween-20 in PBS) and for another 2 h in blocking buffer 2 (1% BSA in PBS) to allow partial renaturation of the proteins on the nitrocellulose membrane. The membrane was briefly washed with PBS followed with incubation for 2 h with the affinity-purified H6PDH diluted in 50 mM Tris–HCl, pH 7.4. The membrane was then washed 4 times with PBS and blocked for 1 h in TBS supplemented with 2% milk. Binding of myc-tagged H6PDH was detected with rabbit anti-myc antibody (Abcam) and secondary HRP-conjugated goat anti-rabbit IgG (Santa Cruz) as described above.

2.7. Fluorescence resonance energy transfer (FRET)

Fusion proteins of H6PDH with EYFP were generated by cloning human H6PDH into *Eco*RI and *Xba*I endonuclease restriction sites of pEYFP-C1 or into *Eco*RI and *Age*I sites of pEYFP-N1 (Clontech Laboratories, Mountain View, CA) to obtain H6PDH–EYFP. To generate 11β-HSD1–ECFP, human 11β-HSD1 was inserted into the *Nhe*I endonuclease restriction site of pECFP-C1 (Clontech). The ECFP–11β-HSD1 chimera was not constructed because the ECFP moiety would be oriented toward the cytosol whereas the catalytic domain of 11β-HSD1 would face the ER lumen. HEK-293 cells were transfected with different combinations of expression plasmids, grown for 24 h and fixed in a buffer containing 150 mM sodium phosphate, pH 7.4, 120 mM sucrose and 4% paraformaldehyde. Acceptor photobleaching was used to assess FRET efficiency on a Zeiss LSM510 confocal microscope equipped with a 63×/1.4 oil immersion objective. ECFP was excited with a 458 nm laser line, emission measured with a BP470-500 filter. EYFP was excited with a 514 nm laser line, emission measured with a LP530 filter. For each measure we made sure that EYFP bleaching was over 90%, and FRET efficiency (E) was calculated after background subtraction and CFP bleaching correction as follows: $E=(I_D-I_{DA})/I_D$, where I_D and I_{DA} are ECFP intensities in the bleached region after and before photobleaching of EYFP, respectively. For each condition, 4 to 10 cells were assessed.

2.8. Determination of the parameters of H6PDH peptides for quantification by LC-multiple reaction monitoring (MRM)

The band corresponding to overexpressed H6PDH after separation of proteins by SDS-PAGE was sliced, washed and in-gel digested with trypsin overnight at 37 °C. Tryptic peptides were separated by nano-HPLC (Agilent 1100 nanoLC system, Agilent Technologies, Santa Clara, CA) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems, Foster City, CA). The LC system was equipped with a capillary column with an integrated nanospray tip (75 μm i.d.×100 mm, Spectronex, Basel, Switzerland) filled with MagicC18 (Michrom Bioresources, Inc., Auburn, CA). Solvent A consisted of 0.1% formic acid/2% acetonitrile, solvent B was composed of 0.1% formic acid/80% acetonitrile. Elution was performed with a gradient of 0 to 45% solvent B in 30 min at a flow rate of 300 nL/min. Samples were loaded on a Peptide CapTrap (Michrom BioResources). In the IDA mode the mass spectrometer cycled through six analyses, one full-scan MS experiment, and one enhanced resolution experiment for the four most intense peaks followed by four enhanced product ion experiments. H6PDH peptides were determined searching UniProt data base using Mascot (Matrix Science).

2.9. Quantification of H6PDH in cells by LC-MRM

MRM-relevant data as transition values, charge state and retention time of H6PDH and the six most abundant proteins were extracted from the Mascot result file using MRM Buddy, a software developed at the FMI (Novartis Research Foundation). Whole cells were lyzed in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, Complete protease inhibitors (Roche Diagnostics, Rotkreuz, Switzerland), 1% w/v dithio-threitol), and the proteins were precipitated using chloroform/methanol [30] and resuspended in 100 μl 47 mM Tris–HCl, pH9.0. The cysteines were reduced with dithiothreitol, alkylated with iodoacetamide and digested with trypsin overnight at 37 °C. The digest was 40-times diluted with solvent A and analyzed by LC-MRM keeping the HPLC settings constant (see above). Quantitative data were evaluated using Analyst 1.4.1.

2.10. Statistical analysis

To estimate apparent K_{cat}, apparent V_{max} and K_m values of the enzymatic reactions were calculated by nonlinear regression using Data Analysis Toolbox (MDL Information Systems Inc., Nashville, TN, USA) assuming first-order rate kinetics. Data represent mean±SD of at least four independent experiments.

3. Results

3.1. Effect of cofactor regenerating enzymes on the reaction direction of 11β-HSD1 and related SDR enzymes

In a previous study, we have shown that H6PDH causes 11β-HSD1 to function as a reductase [24]. In line with the previous findings, Fig. 1A shows a significant stimulation of 11β-HSD1 reductase activity and a concomitant loss of oxidase activity upon coexpression with H6PDH in intact HEK-293 cells, resulting in an approximately 20-fold increased ratio of 11β-HSD1 reductase/oxidase activity. In contrast, coexpression with H6PDH had no significant effect on the activity of the endoplasmic reticular enzyme 17β-HSD2 [31,32], which utilizes NADH and predominantly catalyzes the oxidation of estradiol to estrone (Fig. 1B). 17β-HSD2 reductase activity even tended to decrease in the presence of H6PDH. As expected, coexpression with H6PDH neither altered the activity of 11β-HSD2 (Fig. 1C) nor the reduction of estrone to estradiol by 17β-HSD1 (Fig. 1D), two enzymes facing the cytoplasm. The reason for the decreased 17β-HSD1-dependent oxidase activity remains unclear.

Since the ER membrane is considered to be impermeable for pyridine nucleotides, we investigated a potential effect of G6PDH on the selected SDR enzymes. Neither the two ER-lumenally oriented enzymes 11β-HSD1 and 17β-HSD2 nor 11β-HSD2 and 17β-HSD1,

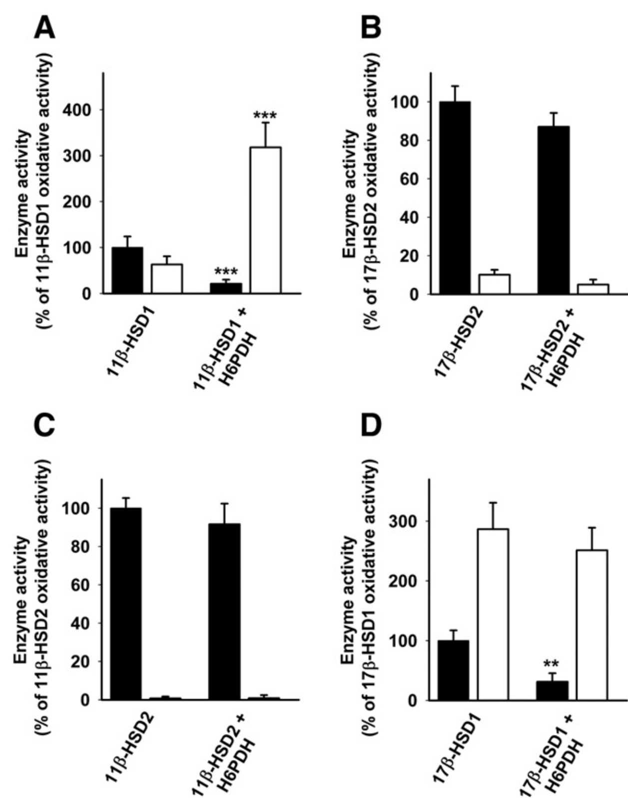

Fig. 1. Effect of H6PDH on oxidase and reductase activities of 11β-HSD1 and related SDRs. HEK-293 cells were transfected with plasmids for 11β-HSD1 (A), 17β-HSD2 (B), 11β-HSD2 (C) or 17β-HSD1 (D) together with pcDNA3 control plasmid or H6PDH expression vector. Oxidase activities (black bars) were measured by determining the conversion of cortisol to cortisone (A,C) or estradiol to estrone (B,D) in the presence of various concentrations of substrate. For reductase activities (white bars), the reverse reactions were measured. The y-axis displays calculated, apparent K_{cat} (V_{max}/K_m) values as a percentage of apparent K_{cat} of the oxidase reaction in the absence of H6PDH. Data represent mean±SD from four independent experiments. $^{**}p<0.01$; $^{***}p<0.001$.

facing the cytoplasm, were influenced by coexpression with G6PDH (data not shown). The fact that G6PDH did not stimulate the NADPH-dependent conversion of estrone to estradiol suggests that cofactor availability under the conditions used was not a limiting factor for 17β-HSD1 function.

These observations supported our hypothesis that a more specific mechanism might be responsible for the regulation of 11β-HSD1 function by H6PDH than simply an increase in intralumenal NADPH concentration.

3.2. Assessment of endogenous H6PDH expression in HEK-293 cells

Because H6PDH-deficient mice were reported to have lost the ability to convert 11-dehydrocorticosterone to corticosterone [26], and we found that HEK-293 cells transfected only with 11β-HSD1 catalyzed the reductase and oxidase reactions with similar efficiencies (Fig. 1A), we investigated whether the observed reductase activity in HEK-293 cells might be due to endogenous H6PDH expression. Real-time RT-PCR experiments revealed a very low H6PDH mRNA expression level in untransfected HEK-293 cells with less than 100 copies/cell (data not shown, [24]). Western blot analysis, using a rabbit polyclonal antiserum against the lactonase domain (residues 539–791) of human H6PDH [33], yielded a band at approximately 90 kDa in cells overexpressing H6PDH, whereas no signal could be detected in untransfected HEK-293 cells (data not shown).

As an additional, sensitive and antibody-independent method for protein detection and quantification, we applied multiple reaction monitoring (MRM) analysis. The MRM analysis of peptide transitions specific for H6PDH resulted in strong signals in H6PDH overexpressing cells and no peaks for untransfected HEK-293 cells (Fig. 2). The signal to noise ratio of the most intense H6PDH signals is in the range of 1000, indicating an at least 1000 times lower H6PDH expression level in untransfected HEK-293 cells. These results suggest that in the HEK-293 cells used in the present study, H6PDH is either absent or expressed at very low levels that are unlikely to be biologically relevant. The HEK-293 cells expressing recombinant 11β-HSD1 and H6PDH had enzymatic activities comparable with those of freshly isolated rat hepatocytes and approximately two-fold higher activities compared with fully differentiated 3T3-L1 adipocytes, suggesting that the enzymes were not aberrantly overexpressed.

3.3. Coimmunoprecipitation of 11β-HSD1 and H6PDH

To investigate whether a direct protein–protein interaction might be responsible for the functional coupling of 11β-HSD1 and H6PDH, we performed coimmunoprecipitation experiments. FLAG-tagged 11β-HSD1 and myc-tagged H6PDH were coexpressed in HEK-293 cells, followed by immunoprecipitation of 11β-HSD1 with agarose beads that were coupled with anti-FLAG antibody M2 (Sigma-Aldrich). H6PDH coprecipitated with 11β-HSD1 but not with 11β-HSD2 or 17β-HSD2 (Fig. 3A). As controls, we incubated anti-FLAG coupled agarose beads with lysates of untransfected cells or of cells transfected with myc-tagged H6PDH only. The proteins bound to the beads were eluted, separated by SDS-PAGE and analyzed by immunoblotting using an anti-myc antibody. As shown in lane 1 and 2, no band was detected at 90 kDa in these control experiments, indicating that H6PDH did not bind unspecifically to the beads and that the band identified in lane 3 corresponds to H6PDH that was coprecipitated with 11β-HSD1.

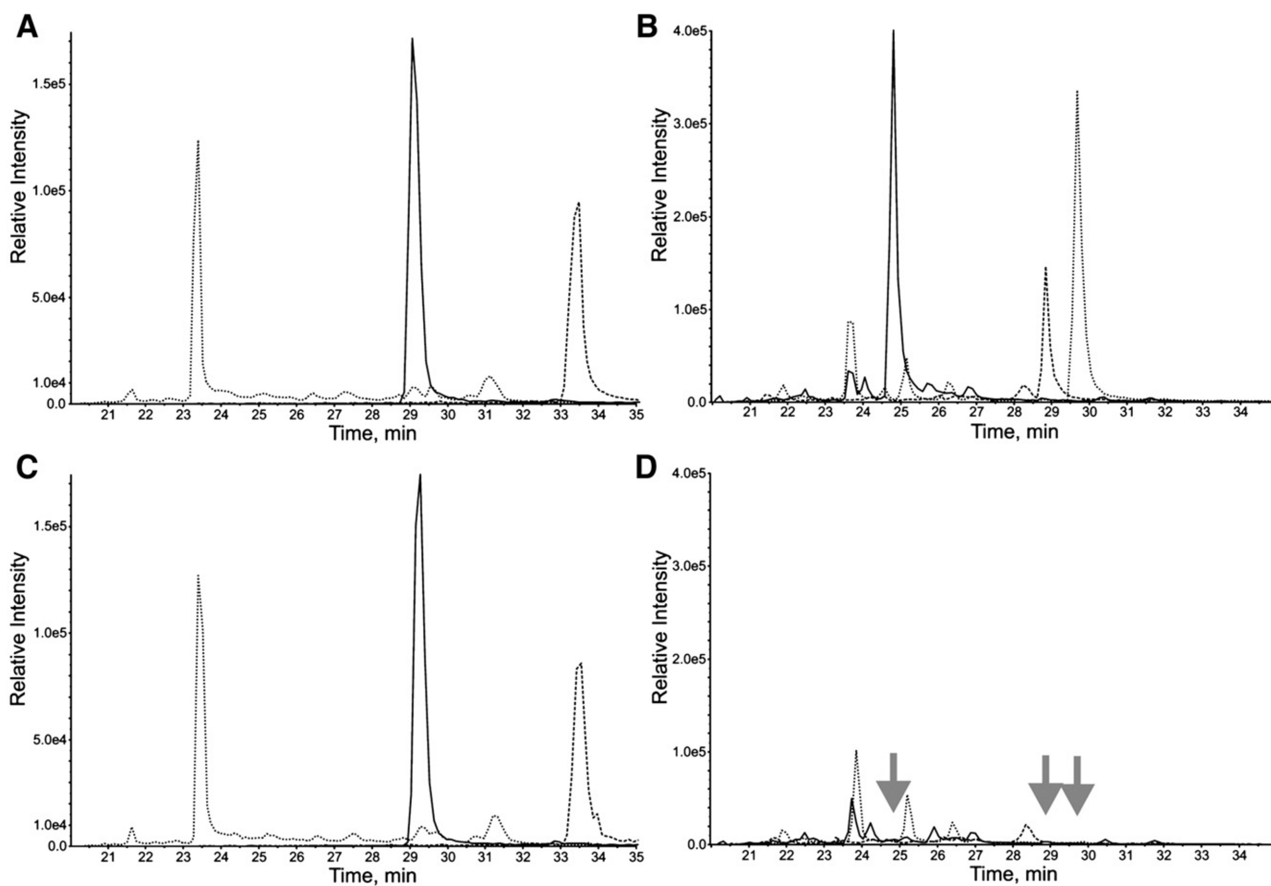

Fig. 2. Analysis of H6PDH protein expression in HEK-293 cells. An amount of 2 µg of tryptic digested total cell protein extracts of cells overexpressing H6PDH (A and B) and of untransfected HEK-293 cells (C and D) were analyzed using MRM. (A) and (C) show signals recorded for the background protein actin, demonstrating the good reproducibility of the method (same line style belongs to the same peptide transition). (B) and (D) represent the signals of the three H6PDH-specific peptides that were recorded in the same run generating the signals for actin (same peptide transitions share the same curve style). Arrows indicate the expected positions of the H6PDH-specific signals of endogenously expressed protein in HEK-293 cells.

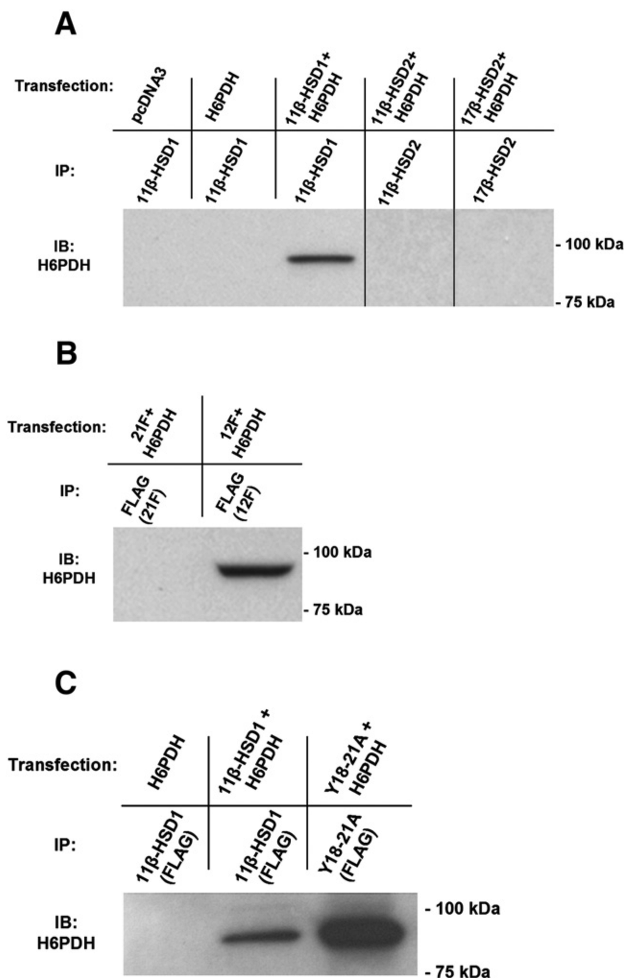

Fig. 3. Coimmunoprecipitation of H6PDH with 11β-HSD1. HEK-293 cells were transfected with the plasmids indicated, followed by immunoprecipitation (IP) of FLAG-tagged 11β-HSD1 and 11β-HSD2 with anti-FLAG antibody-coupled agarose beads or histidine-tagged 17β-HSD2 with histidine nickel affinity beads. Bound proteins were eluted from the beads, subjected to Western blotting, and coprecipitated H6PDH was visualized with anti-myc antibody. (A) Interaction of H6PDH with 11β-HSD1. The experiments with 11β-HSD1, 11β-HSD2 and 17β-HSD2 were performed independently. (B) The N-terminal 39 amino acids of 11β-HSD1 are sufficient for interaction with H6PDH. (C) H6PDH shows stronger interaction with mutant Y18-21A than with 11β-HSD1. One out of three comparable and independently performed experiments is shown.

3.4. Involvement of the N-terminal region of 11β-HSD1 in the interaction with H6PDH

In an attempt to define the region of 11β-HSD1 that is responsible for the interaction with H6PDH, we applied a previously described chimera between 11β-HSD1 and 11β-HSD2, where the N-terminal membrane anchor sequences, that are located upstream of the conserved pyridine nucleotide binding sites, were exchanged [11]. The chimera containing the N-terminal 39 amino acids of 11β-HSD1 and residues 88–405 of 11β-HSD2 (12F) retained its ability to co-precipitate H6PDH (Fig. 3B). In contrast, the chimera consisting of the N-terminal region of 11β-HSD2 and residues 40–292 of 11β-HSD1 (21F) did not interact with H6PDH. Wild-type and chimeric proteins were equally well expressed (data not shown, see [11]). This indicates that the N-terminal part of 11β-HSD1 is involved in the interaction with H6PDH.

In addition, we studied the impact of mutations in the tyrosine motif of the N-terminal transmembrane helix on the interaction with H6PDH. Mutant Y18-21A, where the four tyrosine residues at positions 18–21 were substituted by alanine, showed substantially stronger coprecipitation of H6PDH (Fig. 3C), further indicating a crucial role of the N-terminus of 11β-HSD1 for the interaction with H6PDH. Mutant Y18-21A and wild-type 11β-HSD1 showed comparable protein expression levels and intracellular localization as determined by semi-quantitative immunoblotting analysis and fluorescence microscopy (data not shown, see [11]).

Analysis of the mutant Y18-21A enzyme activity revealed an approximately 5-times lower ability to convert cortisone compared with wild-type 11β-HSD1. Unlike the wild-type enzyme, mutant Y18-21A almost exclusively catalyzed the reduction of cortisone in the absence of H6PDH, and coexpression with H6PDH did not significantly stimulate its reductase activity, despite the stronger interaction (Fig. 4).

3.5. Immunopurification of H6PDH and Far-Western blotting

To further confirm the interaction between 11β-HSD1 and H6PDH, we performed Far-Western blotting experiments. Myc-tagged H6PDH was immunopurified by a single-step purification using anti-myc antibody-coupled agarose beads (Sigma-Aldrich), followed by elution with 100 μg/ml c-myc peptide. The eluate contained a single protein band of approximately 90 kDa, corresponding to H6PDH, that was absent when cells were transfected with pcDNA3 control vector instead of H6PDH expression plasmid (Fig. 5A). The purified H6PDH was catalytically active (Fig. 5B), indicating that it retained its native conformation. Purified H6PDH was then incubated with nitrocellulose membranes on which different wild-type and chimeric 11β-HSD1 proteins were transferred after SDS-PAGE. After removal of unspecific binding by washing, bound H6PDH was detected by anti-myc antibody. A band corresponding to the expected size of 11β-HSD1 was detected in the lane where wild-type 11β-HSD1 was loaded and a stronger signal was obtained for mutant Y18-21A (Fig. 5C), in line with the immunoprecipitation experiments. H6PDH did not bind to chimeric 21F protein but yielded a signal with chimeric 12F protein, although the intensity was weaker than expected from the immunoprecipitation experiment.

3.6. Evidence for the interaction between 11β-HSD1 and H6PDH in intact cells by fluorescence resonance energy transfer (FRET)

Previously, we showed that both 11β-HSD1 and H6PDH are located at the lumenal surface of the ER membrane [24]. However, localization in the same cellular compartment did not answer the question whether the two proteins also physically interact with each other. Therefore, we constructed expression plasmids for 11β-HSD1 fused C-terminally to

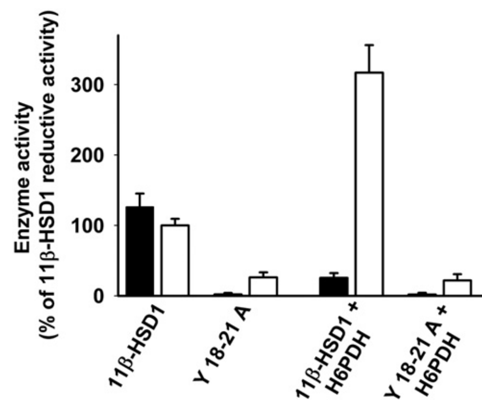

Fig. 4. Comparison of oxidase and reductase activities of wild-type 11β-HSD1 and mutant Y18-21A. HEK-293 cells were transfected with plasmids for 11β-HSD1 or mutant Y18-21A and either pcDNA3 control plasmid or H6PDH expression vector. Oxidase activities (black bars) were measured by determining the conversion of cortisol to cortisone at various concentrations of substrate. For reductase activities (white bars), the reverse reaction was measured. The y-axis displays calculated apparent K_{cat} (V_{max}/K_m) values as a percentage of apparent K_{cat} of the reductase reaction of wild-type enzyme in the absence of H6PDH. Data represent mean ± SD from four independent experiments.

ECFP and H6PDH fused either N- or C-terminally to EYFP and coexpressed 11β-HSD1–ECFP with the corresponding fusion protein of H6PDH and EYFP. As a positive FRET control, a direct fusion of ECFP to EYFP [34] was applied. The ECFP–EYFP fusion protein yielded a FRET signal of 19.9±1.3%, whereas the coexpression of 11β-HSD1–ECFP with EYFP as a negative control was 2.5±0.3% (Fig. 6). Significant FRET signals of 5.8±1% and 8.0±2.0% were obtained for 11β-HSD1–ECFP with H6PDH–EYFP and 11β-HSD1–ECFP with EYFP–H6PDH, respectively, whereby the presence or absence of cortisone did not alter the FRET signal (data not shown). These results strongly support an interaction of 11β-HSD1 and H6PDH in intact cells.

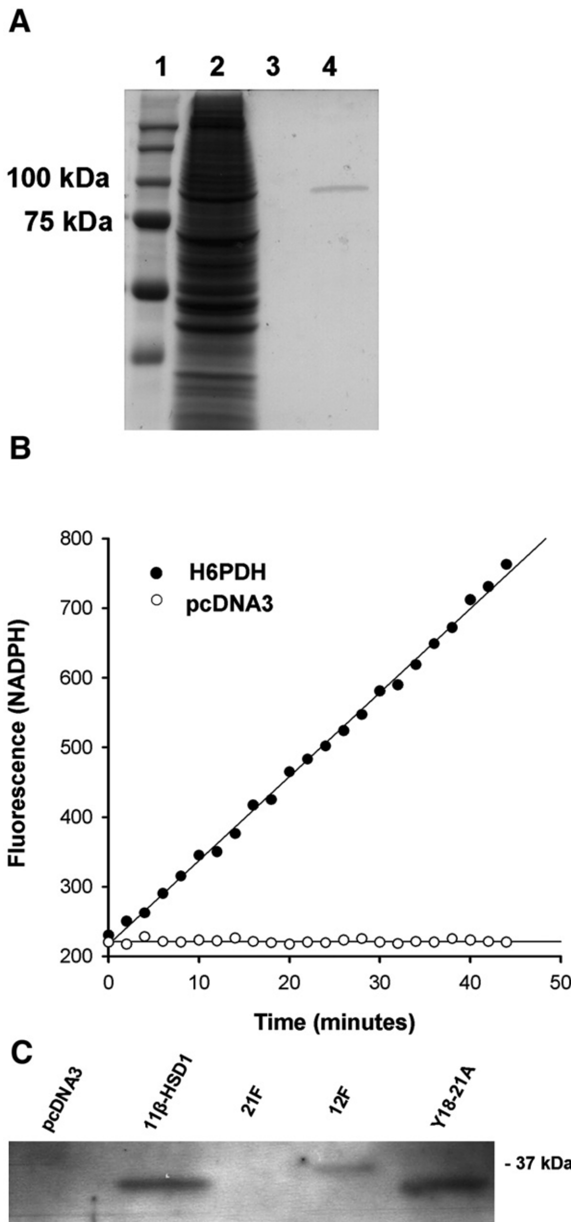

Fig. 5. Purification of H6PDH and Far-Western blot. (A) Coomassie-stained SDS-PAGE of purified H6PDH. Myc-tagged H6PDH was expressed in HEK-293 cells and subjected to a single-step purification using anti-myc antibody-coupled agarose beads. Lane 1, molecular weight marker; lane 2, crude lysate of HEK-293 cells transfected with H6PDH; lane 3, eluate of beads incubated with lysate of HEK-293 cells transfected with pcDNA3 control vector; lane 4, eluate of beads incubated with lysate of cells transfected with H6PDH. (B) Enzyme activity of the purified H6PDH. H6PDH activity was measured by spectrometric detection of NADPH formation in the presence of 100 μM glucose-6-phosphate and 250 μM $NADP^+$. The eluate from beads incubated with lysate of HEK-293 cells transfected with pcDNA3 control vector was inactive, whereas eluate derived from H6PDH expressing HEK-293 cells readily catalyzed the formation of NADPH. (C) Far-Western blotting. Lysates from HEK-293 cells transfected with the constructs indicated were subjected to SDS-PAGE and proteins transferred onto nitrocellulose membranes. The membrane was incubated with purified myc-tagged H6PDH, washed and bound protein detected with anti-myc antibody.

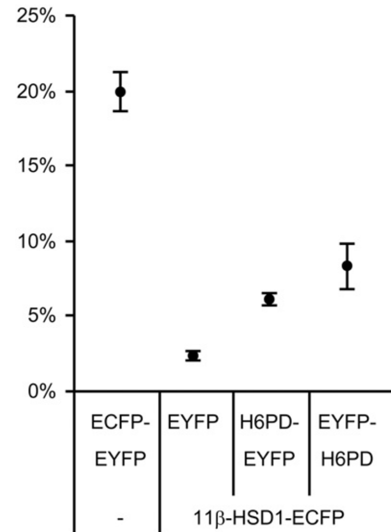

Fig. 6. Interaction of 11β-HSD1 and H6PDH in intact cells. HEK-293 cells were transfected with ECFP–EYFP, serving as a positive control for complete interaction, or co-transfected with N- or C-terminal fusions of H6PDH and EYFP together with 11β-HSD1–ECFP. 11β-HSD1 co-transfected with EYFP was used as negative control. Acceptor photobleaching was used to assess FRET efficiency. For each condition, 4 to 10 cells were assessed. Results are shown as mean FRET efficiency±S.E.M.

4. Discussion

The local control of active to inactive glucocorticoids by 11β-HSD enzymes allows a highly tissue- and cell-specific response to glucocorticoids and plays an important role in the regulation of energy metabolism, immune system, brain function as well as cell proliferation and differentiation. While 11β-HSD2 exclusively catalyzes the oxidation of active endogenous glucocorticoids, 11β-HSD1 can act both as a dehydrogenase or a reductase, depending on the absence or presence of H6PDH [3,35]. Coexpression with H6PDH adds another level of fine-tuning in the regulation of tissue-specific glucocorticoid action.

In the present study, using coimmunoprecipitation, Far-Western and FRET experiments, we demonstrate a direct protein–protein interaction between 11β-HSD1 and H6PDH both in cell homogenates and in intact cells. A direct interaction with H6PDH allows the supply of NADPH in close proximity to 11β-HSD1 for the efficient reduction of cortisone to cortisol despite a rather oxidative environment within the ER lumen. The interaction allows H6PDH to regulate 11β-HSD1 function without the need to change overall ER-lumenal NADPH concentrations. In contrast to its effect on 11β-HSD1, H6PDH did not stimulate the reductase activity of 17β-HSD2, which utilizes NADH and whose catalytic domain protrudes into the ER lumen. It was reported that purified H6PDH can generate both NADH and NADPH, although with an approximately three-fold preference for the formation of the latter [21]. If H6PDH increases overall intralumenal concentrations of both NADPH and NADH, one would expect to see a stimulation of the NADH-dependent reduction of estrone to estradiol by 17β-HSD2 and a decreased reverse reaction. However, it is unclear at present whether in vivo such a possible increase in NADH concentration in the ER lumen occurs and whether it could be effective on 17β-HSD2 activity. In analogy, in the cytoplasm, overexpression of G6PDH did not alter 11β-HSD2 or 17β-HSD1 activity, suggesting that overall cytoplasmic NADPH concentration did not significantly change. Although these observations do not rule out an effect of G6PDH and H6PDH on overall cofactor concentration in the

given compartment under some specific conditions, especially with respect to the high glucose medium used in the present study for culturing cells, they suggest that the direct interaction with H6PDH allows a more subtle regulation of 11β-HSD1 function.

Measurements with hepatic tissue explants from H6PDH-deficient mice showed a 60% decreased generation of NADPH and significantly increased dehydrogenase activity [36]. The observation that 11β-HSD1 switches from a reductase to a dehydrogenase in H6PDH-deficient mice demonstrates the importance of H6PDH-dependent generation of ER-lumenal NADPH for cortisol production by 11β-HSD1. H6PDH-deficient mice show abnormal glucose homeostasis and disturbances in the hypothalamic–pituitary–adrenal axis [26,36,37]. In addition, H6PDH-deficient mice suffer from skeletal myopathy, characterized by a switch from type II to type I fibers and a disturbed regulation of the expression of several essential proteins such as sarco-endoplasmic reticulum calcium ATPase (SERCA), calsequestrin and calreticulin [38]. Whether these alterations in skeletal muscles are due to impaired gene expression as a result of a lack of glucocorticoids during differentiation or whether they are caused by ER stress due to a depletion of ER-lumenal NADPH, remains to be investigated.

Nevertheless, our results suggest that H6PDH is not the only enzyme able to generate NADPH in the ER lumen. Using Western blotting and MRM, we were not able to detect any endogenous expression of H6PDH protein in our HEK-293 cells. Despite the lack of endogenous H6PDH, 11β-HSD1 was able to catalyze both the oxidation of cortisol and the reduction of cortisone. These observations suggest that there are other sources for NADPH generation in the ER lumen, but that they play a minor role for 11β-HSD1 function under physiological conditions. In fact, isocitrate dehydrogenase was recently suggested to generate NADPH in the ER lumen [39]. The isocitrate-dependent generation of NADPH in microsomes prepared from rat liver and epididymal fat was almost completely latent, indicating ER-lumenal localization of the activity. The impact of isocitrate dehydrogenase activity on 11β-HSD1 function has not yet been studied.

Interestingly, mutant Y18-21A predominantly catalyzed the reductase reaction even in the absence of H6PDH, and coexpression with H6PDH was not able to stimulate the reductase activity of the mutant enzyme. As a possible explanation, NADPH available from other sources may be sufficient to saturate mutant Y18-21A, which has only about 20% of the activity of wild-type 11β-HSD1 in the absence of H6PDH, while NADPH becomes limited for the fully functional wild-type enzyme. In the presence of H6PDH, wild-type 11β-HSD1 has approximately 20–30 times higher activity than mutant Y18-21A. Another explanation could be that substitution of the four tyrosine residues by alanine in the transmembrane helix locks the mutant enzyme in a conformation that favors binding of NADPH. Recent kinetic analyses indicated an ordered sequential bi–bi mechanism with NADPH binding first to the active site in 11β-HSD1, followed by binding of cortisone [40,41]. If 11β-HSD1 exists in a complex with H6PDH at the ER membrane, interaction with H6PDH might induce a conformational change in 11β-HSD1 that favors binding of NADPH, whereby the mutant enzyme might be stabilized in this conformation even in the absence of H6PDH. Despite approximately ten-times stronger interaction with H6PDH compared with wild-type 11β-HSD1, the reductase activity of mutant Y18-21A was not stimulated by H6PDH. Thus, the additional NADPH produced locally by H6PDH did not further enhance reductase activity, indicating that the cofactor was not a limiting factor for the mutant enzyme under the conditions applied.

Unfortunately, the 11β-HSD1/11β-HSD2 chimeric enzyme that lost the interaction with H6PDH as well as several deletion mutants were catalytically inactive. The enzymatic activity of the Y18-21A mutant was also decreased, despite a stronger interaction with H6PDH, suggesting that 11β-HSD1 enzymatic activity is tightly regulated by or highly sensitive to conformational changes. In addition, a series of single point mutations in the N-terminal post-transmembrane region did not disrupt the interaction with H6PDH (data not shown), suggesting that several residues of 11β-HSD1 are involved in the interaction with H6PDH. Thus, in future experiments various combinations of amino acid substitutions have to be analyzed to pin point the exact interaction mechanism.

The protein–protein interaction allows a direct coupling of glucose-6-phosphate availability via H6PDH-dependent NADPH generation with the activation of glucocorticoids in the ER lumen. Glucocorticoids obtained their name because they are produced in the adrenal cortex and regulate glucose production [42]. Thus, it is not surprising that these glucose-regulating hormones themselves are controlled by the intracellular glucose status, allowing a fine-tuned control of energy metabolism and adding another level of tissue- and cell-specific regulation of glucocorticoid sensitivity. In a recent study, Marcolongo et al. demonstrated that inhibition of the glucose-6-phosphate transporter in the ER membrane decreased 11β-HSD1 reductase activity, probably due to a diminished supply of cofactor NADPH as a result of the limited availability of glucose-6-phosphate in the ER lumen [33]. Whether post-translational modification regulates the interaction between 11β-HSD1 and H6PDH, thereby controlling local glucocorticoid activation independent of H6PDH effects on other ER-lumenal enzymes, remains to be investigated.

Recently, the glucose-6-phosphate transporter and H6PDH have been suggested as potential pharmacological targets for modulating local glucocorticoid activation [33]. Based on the present study, we suggest that the development of therapeutics disrupting the protein–protein interaction between 11β-HSD1 and H6PDH offers an interesting alternative approach to decrease 11β-HSD1 reductase activity without affecting other H6PDH-dependent functions.

Acknowledgements

We thank Dr. J. M. Tavare, University of Bristol, UK, for the CFP-DEVD-YFP plasmid and Dr. A. Benedetti, Department of Pathophysiology, Siena, Italy, for anti-H6PDH antibody. This work was supported by grants from the Swiss National Science Foundation (No. 310000-112279) and the Roche Research Foundation (No. 2004-276). A.O. is a Novartis Research Foundation Professor. B.L. was supported by a fellowship from the Gesellschaft zu Ober-Gerwern.

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