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

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ABSTRACT

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.

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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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.