Protein kinase C phosphorylates and regulates UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase

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Abstract UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase) is the key enzyme in the de novo synthesis pathway of neuraminic acid, which is widely expressed as a terminal carbohydrate residue on glycoconjugates. UDP-GlcNAc 2-epimerase is a bifunctional enzyme and catalyzes the first two steps of neuraminic acid synthesis in the cytosol, the conversion of UDP-*N*-acetylglucosamine to ManAc and the phosphorylation to ManAc-6-phosphate. So far, regulation of this essential enzyme by posttranslational modification has not been shown. Since UDP-*N*-acetylglucosamine is a cytosolic protein containing eight conserved motifs for protein kinase C (PKC), we investigated whether its enzymatic activity might be regulated by phosphorylation by PKC. We showed that UDP-GlcNAc 2-epimerase interacts with several isoforms of PKC in mouse liver and is phosphorylated in vivo. Furthermore, PKC phosphorylates UDP-GlcNAc 2-epimerase and this phosphorylation results in an upregulation of the UDP-GlcNAc 2-epimerase enzyme activity.

Key words: Neuraminic acid synthesis; Protein kinase C; UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase

1. Introduction

Sialylation of glycoproteins and glycolipids plays an important role during development and regeneration, and in the pathogenesis of diseases. Terminal sialic acids are involved in a variety of cellular interactions, such as cell–cell adhesion or cell migration [1,2]. The most abundant sialic acid is *N*-acetylneuraminic acid (Neu5Ac) [3]. Neu5Ac is synthesized in the cytosol from UDP-*N*-acetylglucosamine by four consecutive steps. The final activation of Neu5Ac to CMP-Neu5Ac prior to its addition to glycoconjugates is catalyzed in the nucleus. The first two steps in Neu5Ac biosynthesis are catalyzed by one bifunctional enzyme, UDP-GlcNAc 2-epimerase [4]. Recently, rat, mouse and human UDP-GlcNAc 2-epimerase have been sequenced and characterized [5–7] and the two enzymatic activities have been mapped to different regions of the polypeptide [8]. As a monomer, the polypeptide has no enzymatic activity, but its dimer displays *N*-acetylmannosamine kinase activity. When the monomers are assembled to a hexamer, both UDP-*N*-acetylglucosamine-2-epimerase and *N*-acetylmannosamine kinase (UDP-GlcNAc 2-epimerase) activity are detected [4]. It has been shown that UDP-GlcNAc 2-epimerase is the key enzyme of Neu5Ac synthesis, since its activity is feedback-inhibited by CMP-Neu5Ac [9]. The biological significance of the enzyme is illustrated by the observation that in hepatoma, the low expression of sialic acid is correlated with a dramatically reduced activity of UDP-GlcNAc 2-epimerase [10]. In sialuria, a Neu5Ac storage disease, free sialic acid accumulates in the cytoplasm, and gram quantities of neuraminic acid are secreted in the urine [11,12]. It has been demonstrated that the metabolic defect involves lack of feedback inhibition of UDP-GlcNAc 2-epimerase by CMP-Neu5Ac, resulting in constitutive overproduction of free Neu5Ac [13]. Furthermore, UDP-GlcNAc 2-epimerase was found to be a major determinant of cell surface sialylation in human hematopoietic cell lines and a critical regulator of the function of specific cell surface adhesion molecules [14]. Since the primary structure of UDP-GlcNAc 2-epimerase contains eight potential phosphorylation sites for protein kinase C (PKC) (for review, see [15]), an involvement of PKC in the regulation of the UDP-GlcNAc 2-epimerase enzyme activity has been suggested. The aim of this study was to illustrate a possible role of PKC in the regulation of the biosynthesis of neuraminic acids.

2. Materials and methods

2.1. Cell culture and expression of UDP-GlcNAc 2-epimerase

Spodoptera frugiperda cells (Sf9 cells) were cultured and infected with recombinant baculovirus containing the coding sequence of the UDP-GlcNAc 2-epimerase as described [8].

In brief, Sf9 cells were maintained as monolayer cultures in plastic flasks (Greiner, Frickhausen, Germany) or in suspension by using Erlenmeyer flasks (100 ml) on orbital shakers (100–120 rpm) at 27°C. Cells were grown in TC-100 medium supplemented with 2 mM L-glutamine and 10% fetal calf serum (all from Biochrom, Berlin, Germany) or in serum-free SF9oo II medium (Gibco BRL). Antibiotics (100 U/ml penicillin, 40 U/ml streptomycin) were added to both culture media.

After growing to a density of 2×10^6cells/ml, Sf9 cells were infected with recombinant baculovirus containing the coding sequence of the UDP-GlcNAc 2-epimerase [8]. During infection, cells are grown in suspension culture in an orbital shaker at 120 rpm and at 27°C. After an optimal infection period of 60 h, the cells were harvested.

2.2. Analytical procedures and antibodies

Protein determination was performed in 96-well enzyme-linked immunosorbent assay (ELISA) plates using 100 μl bicinchionic acid protein reagent (Pierce) and 25 μl sample. Plates were evaluated in a 96-well ELISA reader (Spectra) at 570 nm. Rabbit polyclonal anti-

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; UDP-GlcNAc 2-epimerase, UDP-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase

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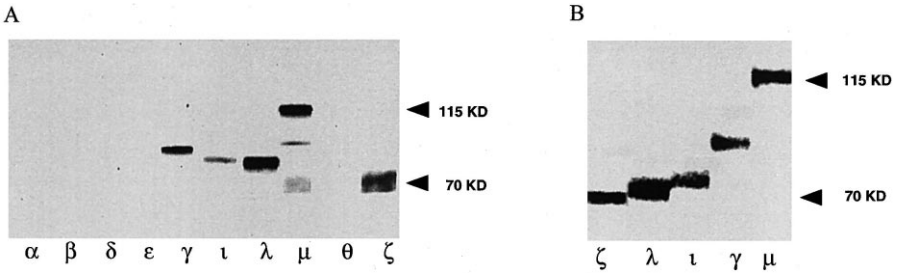

Fig. 1. A: Western blot analysis of PKC isoforms in mouse liver. Cytosol of mouse liver was separated on a 7.5% SDS–PAGE gel and analyzed with isoform-specific PKC antibodies. B: Co-immunoprecipitation of PKC isoforms with UDP-GlcNAc 2-epimerase. UDP-GlcNAc 2-epimerase was immunoprecipitated from cytosol of mouse liver. Precipitates were separated on a 7.5% SDS–PAGE gel and analyzed for co-immunoprecipitated proteins with isoform-specific PKC antibodies.

bodies against purified UDP-GlcNAc 2-epimerase from rat liver were obtained and purified as described elsewhere [6]. Antibodies to PKC isoforms were obtained from Transduction Laboratories and all secondary antibodies were from Dianova (Hamburg, Germany).

2.3. Preparation of cytosol from liver or Sf9 cells

Livers from NMRI mice were removed and transferred to two volumes ice-cold homogenization buffer containing 10 mM NaH_2PO_4, 1 mM EDTA and a protease inhibitor cocktail (Sigma), pH 7.5. The tissue was homogenized with an Ultraturrax (Braun Melsungen, Germany) for 1 min at 10 000 rpm. The homogenates were centrifuged for 1 h at 100 000 × *g* and the supernatants representing the cytosols were collected. Sf9 cells were homogenized in homogenization buffer by passing them 10 times through a syringe fitted with a G22 × 1.25 needle. Homogenates were then centrifuged for 1 h at 100 000 × *g* and the supernatants representing the cytosols were collected.

2.4. Immunoprecipitation

Five mg protein A-agarose (Pharmacia) was precoated with 12 μg anti-UDP-GlcNAc 2-epimerase antibodies [6] in phosphate-buffered saline (PBS) for 2 h at room temperature and washed twice with PBS. Cytosol was precleared with 5 mg protein A-agarose for 1 h, then incubated for 2 h at 4°C with precoated protein A-agarose. Immunoprecipitates were pelleted by centrifugation, washed three times with PBS and subjected to sodium dodecyl sulfate (SDS) gel electrophoresis and immunoblotting.

2.5. Immunoblotting

Samples were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose filters. Blots were blocked with 10% fat-free dry milk powder in PBS, incubated with the primary antibodies, washed with PBS and incubated with the appropriate secondary antibodies (Sigma, Dianova). After washing, proteins were detected by enhanced chemiluminescence (Amersham Buchler) according to the manufacturer's instructions, and visualized by exposing the blots to Kodak BioMax films for time periods between 10 and 120 s. The antigen detection limit was 50 pg. The coefficient of variation in amounts of detected antigens was 5–10% in different experiments.

2.6. In vitro phosphorylation assay

Cytosol of UDP-GlcNAc 2-epimerase-overexpressing Sf9 cells was purified by FPLC using a Superdex™ 200 column as described by Hinderlich et al. [4]. Twenty-five μl enriched aliquots of UDP-GlcNAc 2-epimerase were incubated with [^{32}P]ATP (Hartmann, Braunschweig, Germany), PKC (Biomol, Hamburg, Germany) in the presence and absence of phorbol 12-myristate 13-acetate (PMA) or staurosporine (Biomol, Hamburg, Germany) for 10, 20 or 60 min at 37°C followed by SDS gel electrophoresis and phosphoimaging (Molecular Dynamics).

2.7. In vivo phosphorylation assay

Sf9 cells were infected with recombinant baculovirus containing the coding sequence of the UDP-GlcNAc 2-epimerase. Twelve hours after infection, 1 mCi [^{32}P]H_3PO_4 (Hartmann, Braunschweig, Germany) was added to the cultures. Fourty-eight hours after infection, cells were homogenized and UDP-GlcNAc 2-epimerase was immunoprecipitated and subjected to SDS gel electrophoresis and phosphoimaging.

2.8. Quantification of UDP-GlcNAc 2-epimerase activity

UDP-GlcNAc 2-epimerase activity was measured by a previously published modified method [16]. In brief, the final volume of incubation mixtures was 225 μl, incubations were carried out at 37°C for 30 min, and reactions were stopped by addition of 350 μl ethanol. UDP-GlcNAc 2-epimerase assay: 45 mM Na_2HPO_4, pH 7.5, 10 mM $MgCl_2$, 1 mM UDP-GlcNAc, 25 nCi of UDP-(^{14}C)-GlcNAc (NEN, Dreieich, Germany). Five μl of radiolabeled compounds was separated by thin layer chromatography using HPTLC plates (Merck, Darmstadt, Germany) and the following eluent: (*n*-propanol:1 M sodium acetate pH 5:water = 7:1:2, vol.). Radioactivity was determined using a phosphoimager.

3. Results

3.1. Several PKC isoforms are associated with UDP-GlcNAc 2-epimerase in mouse liver

In a first series of experiments, we analyzed the isoform expression of PKC in rat liver. Freshly prepared cytosol from mouse liver was analyzed by Western blotting and probed with PKC isoform-specific antibodies. Fig. 1A illustrates that in mouse liver, PKC isoforms γ, ι, λ, μ and ζ are expressed and that PKC isoforms α, β, δ, ε and θ are not detectable. Since the primary structure of the murine UDP-GlcNAc 2-epimerase contains eight consensus sites for PKC phosphorylation, we analyzed immunoprecipitates of UDP-GlcNAc 2-epimerase from mouse liver cytosol with PKC isoform-specific antibodies. All PKC isoforms expressed in mouse liver (γ, ι, λ, μ and ζ) were detected by co-immunoprecipitation with UDP-GlcNAc 2-epimerase antibodies (Fig. 1B). For control, the experiments were also performed in the absence of UDP-GlcNAc 2-epimerase-specific antibodies where no isoform of PKC could be detected (data not shown).

3.2. In vivo phosphorylation of UDP-GlcNAc 2-epimerase

Since UDP-GlcNAc 2-epimerase contains eight potential phosphorylation sites for PKC, we addressed the question

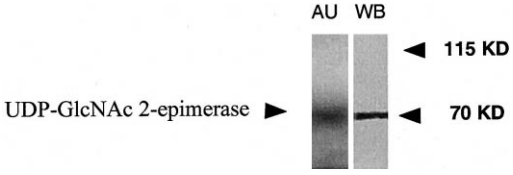

Fig. 2. In vivo phosphorylation of UDP-GlcNAc 2-epimerase. UDP-GlcNAc 2-epimerase-overexpressing cells were allowed to grow in medium containing radiolabeled phosphate. After 48 h, cells were lysed and UDP-GlcNAc 2-epimerase was immunoprecipitated and subjected to Western blotting (WB) and autoradiography (AU).

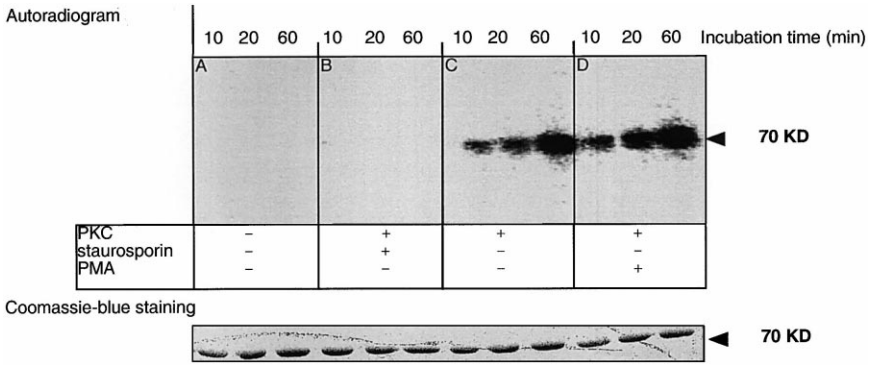

Fig. 3. In vitro phosphorylation of UDP-GlcNAc 2-epimerase. Autoradiogram: purified UDP-GlcNAc 2-epimerase was incubated in the presence of [^{32}P]ATP with PMA, staurosporine or PKC according to the underlying scheme. After incubation times of 10, 20 or 60 min, the mixture of molecules was subjected to electrophoresis and phosphorylated UDP-GlcNAc 2-epimerase was visualized by autoradiography. Coomassie blue staining: for control, the gel was stained with Coomassie blue to confirm equal loading of all lanes. All lanes contain a similar amount of UDP-GlcNAc 2-epimerase protein indicated by equal staining of the 70 kDa band.

whether UDP-GlcNAc 2-epimerase is phosphorylated in vivo. Therefore, we incubated Sf9 cells with radiolabeled H_3PO_4 12 h after infection with recombinant baculovirus containing the coding sequence of the UDP-GlcNAc 2-epimerase. Fourty-eight hours after infection, cells were homogenized and UDP-GlcNAc 2-epimerase was immunoprecipitated from the cytosol and subjected to SDS gel electrophoresis, Western blotting and autoradiography. The immunoprecipitated UDP-GlcNAc 2-epimerase was detected with UDP-GlcNAc 2-epimerase-specific antibodies (Fig. 2, WB), and incorporation of phosphate was shown by autoradiography (Fig. 2, AU), indicating that UDP-GlcNAc 2-epimerase is phosphorylated in vivo.

3.3. In vitro phosphorylation of UDP-GlcNAc 2-epimerase

UDP-GlcNAc 2-epimerase is not only associated with PKC isoforms; as shown by in vitro phosphorylation experiments, it is also a substrate of PKC. Purified UDP-GlcNAc 2-epimerase from the cytosol of overexpressing Sf9 cells was incubated with radiolabeled ATP in the absence and presence of PKC. Additionally, these experiments were performed in the absence and presence of PMA, an activator of PKC, and staurosporine, an inhibitor of serine/threonine kinases. After incubation times between 10 and 60 min, UDP-GlcNAc 2-epimerase was analyzed by autoradiography after SDS–polyacrylamide gel electrophoresis (PAGE). PKC phosphorylated UDP-GlcNAc 2-epimerase within 10 min, and an increased incubation time led to an increased phosphorylation of UDP-GlcNAc 2-epimerase (Fig. 3C). Addition of the PKC stimulating agent, PMA, did not lead to increased phosphorylation (Fig. 3D), whereas addition of staurosporine completely abolished phosphorylation of UDP-GlcNAc 2-epimerase (Fig. 3B). For control, the experiment was also performed in the absence of PKC (Fig. 3A). In all experiments, gels were stained with Coomassie blue to confirm equal loading of all lanes.

3.4. Modulation of UDP-GlcNAc 2-epimerase enzyme activity by phosphorylation

We not only demonstrated that UDP-GlcNAc 2-epimerase is phosphorylated by PKC, but also that its enzyme activity is upregulated after PKC-dependent phosphorylation. In vitro, UDP-GlcNAc 2-epimerase enzyme activity was determined after incubation of the enzyme with PKC in the absence and presence of PMA and staurosporine. Incubation of the UDP-GlcNAc 2-epimerase with PKC alone resulted in an increase of the enzyme activity by 30%. In the presence of the PKC and the PKC activator PMA, this enzyme activity increased again between 25% and 48%, resulting in a total increase between 50% and 100% compared to the control conditions in the absence of PKC. In the presence of the serine/threonine kinase inhibitor, staurosporine, the enzyme activity of the UDP-GlcNAc 2-epimerase remained un-

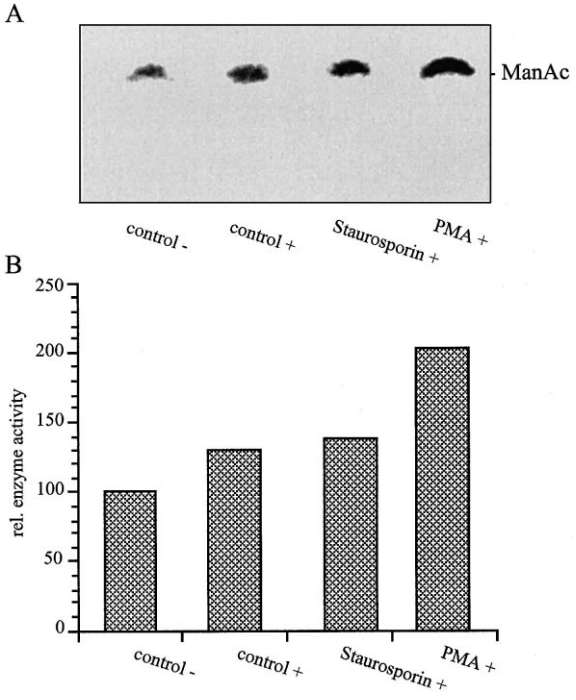

Fig. 4. Modulation of the enzymatic activity of the UDP-GlcNAc 2-epimerase. A: UDP-GlcNAc 2-epimerase was incubated with (+) and without PKC (−) in the absence (control) or presence of PMA (PMA) or staurosporine (staurosporine). One representative autoradiogram of an HPTLC plate showing the reaction product (ManAc) is shown. B: Enzymatic activity of UDP-GlcNAc 2-epimerase was determined by quantification of the reaction product on HPTLC plates and computer analysis. One representative out of four independent experiments is shown.

changed compared to the situation in the presence of PKC. Fig. 4 illustrates the results of one representative experiment. Fig. 4A shows the autoradiogram of an HPTLC plate of the reaction product, ManAc, after the epimerization of UDP-GlcNAc in the absence and presence of PMA and staurosporine. In this particular experiment, the presence of PKC and PMA results in an increase of UDP-GlcNAc 2-epimerase enzyme activity by 100% (Fig. 4B).

4. Discussion

Neu5Ac is the most common naturally occurring sialic acid and terminal carbohydrate of a variety of glycoconjugates [2]. UDP-GlcNAc 2-epimerase has been shown to be the key enzyme of Neu5Ac biosynthesis in rat liver, and it is a regulator of cell surface sialylation [4,14]. In this study, we demonstrated that UDP-GlcNAc 2-epimerase is associated with PKC isoforms in liver and is phosphorylated in vivo. Furthermore, UDP-GlcNAc 2-epimerase is a substrate for PKC in vitro and we provide evidence that its basal enzyme activity is upregulated after phosphorylation by PKC. To our knowledge, this is the first report on a direct involvement of PKC in the biosynthesis of carbohydrate structures. PKC activity is known to be upregulated during hyperglycemia [17] but there is no published evidence that PKC is directly involved in glycan biosynthesis.

UDP-GlcNAc 2-epimerase is a bifunctional enzyme and it catalyzes not only the epimerization of UDP-GlcNAc to ManAc but also the phosphorylation of ManAc to ManAc-phosphate [4]. The enzyme activity of the UDP-GlcNAc 2-epimerase is regulated by feedback inhibition by CMP-Neu5Ac. CMP-Neu5Ac is the activated endproduct of neuraminic biosynthesis and is a substrate of several sialyltransferases in the Golgi apparatus. Furthermore, the oligomeric state is a possible regulator of the enzyme activity. In vitro, the oligomerization of UDP-GlcNAc 2-epimerase is dependent on UDP-GlcNAc, since UDP-GlcNAc stabilizes the hexameric state [4]. The UDP-GlcNAc 2-epimerase is only active when assembled as a hexamer. Until now, no additional regulatory mechanism has been described. Since phosphorylation of ManAc to ManAc-phosphate can also be catalyzed by GlcNAc kinase [18] and epimerization is the initial step in neuraminic acid synthesis, we concentrated our study on the regulation of the UDP-GlcNAc 2-epimerase activity. The primary sequence of rat, mouse and human UDP-GlcNAc 2-epimerase contains eight potential PKC consensus sites. Therefore, we focused our experiments on UDP-GlcNAc 2-epimerase interactions with PKC in liver, since this is the organ with the highest UDP-GlcNAc 2-epimerase expression. We propose that PKC, a key element in various signal transduction pathways, phosphorylates and regulates the enzymatic activity of the UDP-GlcNAc 2-epimerase and might therefore be involved in cell surface sialylation. The exact mechanism of PKC-mediated phosphorylation of UDP-GlcNAc 2-epimerase in vivo will be the subject of future investigations.

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