UDP-glucurionate decarboxylase, a Key Enzyme in Proteoglycan Synthesis

CLONING, CHARACTERIZATION, AND LOCALIZATION*

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UDP-glucurionate decarboxylase (UGD) catalyzes the formation of UDP-xyllose from UDP-glucurionate. UDP-xyllose is then used to initiate glycosaminoglycan biosynthesis on the core protein of proteoglycans. In a yeast two-hybrid screen with the protein kinase Akt (protein kinase B), we detected interactions with a novel sequence, which we cloned and expressed. The expressed protein displayed UGD activity but did not display the activities of homologous nucleotide sugar epimerases or dehydratases. We did not detect phosphorylation of UGD by Akt nor did we detect any influence of Akt on UGD activity. Effects of UGD on Akt kinase activity were also absent. Northern blot and Western blot analyses revealed the presence of UGD in multiple tissues and brain regions. Subcellular studies and histochemistry localized UGD protein to the perinuclear Golgi where xylosylation of proteoglycan core proteins is known to occur.

Once thought to function only as structural proteins, proteoglycans are now known to be crucially involved in a number of signaling pathways in animals, especially during development (1, 2). Glycosaminoglycan (GAG)1 moieties of proteoglycans are polymeric, unbranched polysaccharides that can serve as coreceptors by binding a variety of secreted growth factors such as fibroblast growth factor, transforming growth factor-beta, and once again selected for histidine prototrophy and evaluated for expression and enzymatic activity by GST- or HA-tagged fusion protein plasmid expression in HEK293T cells. The activity characterization agreed with previously published data (18, 19). AktKD was subcloned as bait into the PJ69 yeast strain. A total of 107 independent clones were introduced sequentially by LiAc-mediated transformation (21) into the FY69 yeast strain. A total of 1.0 × 106 independent clones were screened. Positive interactions were identified by selecting for histidine and adenine prototrophy. Positive clones were further evaluated for β-galactosidase expression on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmids rescued from positive clones were retransformed into the HTP7 yeast strain (CLONTech) and once again selected for histidine prototrophy and evaluated for β-galactosidase expression by nitrocellulose filter lift assays (21).

MATERIALS AND METHODS

Yeast Two-hybrid Screen—Full-length rat Akt was cloned by reverse transcriptase-PCR from adult rat brain total RNA. Utilizing PCR-based mutagenesis we constructed a triple mutant activated, kinase-dead Akt (AktΔKD, T308D, S473D, and K179A) (18, 19). Wild type Akt, activated Akt (AktΔ, T308D, S473D), kinase-dead Akt (AktΔKD, K179A), and activated kinase-dead Akt (AktΔKD) were evaluated for expression and enzymatic activity by GST- or HA-tagged fusion protein plasmid expression in HEK293T cells. The activity characterization agreed with previously published data (18, 19). AktKD was subcloned as bait into yeast expression vector pPC97 containing the GAL4 binding domain and used to screen a rat hippocampal and cortical cDNA library in pPC86 expressing the GAL4 transactivation domain (20). The plasmids were introduced sequentially by LiAc-mediated transformation (21) into the FY69 yeast strain. A total of 1.0 × 106 independent clones were screened. Positive interactions were identified by selecting for histidine and adenine prototrophy. Positive clones were further evaluated for β-galactosidase expression on plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal). Plasmids rescued from positive clones were retransformed into the HTP7 yeast strain (CLONTech) and once again selected for histidine prototrophy and evaluated for β-galactosidase expression by nitrocellulose filter lift assays (21).

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1 The abbreviations used are: GAG, glycosaminoglycan; DeRed, Discosoma sp. Red; EYFP, enhanced yellow fluorescent protein; GST, glutathione S-transferase; HA, hemagglutinin; HPLC, high performance liquid chromatography; TDP, thymidine 5'-diphosphate; UDP, uridine 5'-diphosphate; UGD, UDP-glucurionate decarboxylase; ER, endoplasmic reticulum.

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was subsequently subcloned into pCMV-HA and pCMV-GST vectors.

Epimerase Activity Assay—UDP-galactose-4-epimerase activity was assayed essentially as described previously (22). Modifications included the use of 100 μg of lysates from GST-UGD transformed bacteria and 0.011 units of UDP-glucose dehydrogenase (Sigma) per a final reaction volume of 500 μl.

Dehydratase Activity Assay—The assay described by Vara et al. (23) was modified by the use of unlabeled TDP-glucose as substrate and NaB₃H₄ for the reduction of a 4-keto reaction product. Reaction products were separated by thin layer chromatography on aluminum-backed silica plates with a solvent mixture of pyridine, ethyl acetate, and water 26:66:8 (v/v/v). Dried plates were visualized by autoradiography and stained by spraying with a solution of 10% H₂SO₄ in ethanol followed by heating for 20 min at 120 °C. Alternatively, the plates were scraped and assayed by scintillation counting.

We also assayed for a dehydratase product spectrophotometrically as described previously (24). Bacterially expressed RmlB (24) was used as a positive control for dehydratase activity.

UDP-glucuronate Decarboxylase Assay—Decarboxylase activity was assayed as described previously (25). Briefly, lysates from GST-UGD-transformed bacteria or alternatively from transfected HEK293T cells, 2 mM NAD⁺, and 750 μM unlabeled UDP-glucuronate (Sigma) were incubated at 37 °C for 1 hour. Reaction products were separated by HPLC and quantified using a mass spectrometer.

FIG. 1. Schematic representation of UDP-xylose synthesis from UDP-glucuronic acid by UGD (*). Enzymatic attachment of UDP-xylose to the core protein (via a serine hydroxyl) then initiates proteoglycan synthesis by the formation of the linker tetrasaccharide and subsequent GAG attachment and elongation.

FIG. 2. Full-length nucleotide and amino acid sequence for rat UGD. Underlined sequences represent hydroxyl-containing amino acids and an XXKK motif potentially involved in substrate and NAD⁺ binding, respectively (see text for details).
Northern Analysis—A multiple tissue Northern blot membrane (CLONTECH) containing 2 μg of mRNA/lane was hybridized using Expresshyb (CLONTECH) according to manufacturer’s instructions with a probe generated from the yeast two-hybrid fragment by random priming (Invitrogen) in the presence of [γ-32P]dATP and dCTP (PerkinElmer Life Sciences). Multiple cell line blots were prepared from cell lysates using Trizol (Invitrogen) as per the manufacturer’s protocol. Each lane contained 20 μg of total RNA. Equal amounts of ribosomal RNA and -actin were present in each lane.

Transient Transfections and Bacterial Transformations for UGD Activity Assay—HEK293T cells were transiently transfected using Lipo-jectAMINE 2000 (Invitrogen) as per the manufacturer’s protocol. Cells were lysed (after 48 h) in the following buffer: 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 10 mM -glycerol phosphate, 5 mM pyrophosphate, 1 mM NaOV₄, 0.1% -mercaptoethanol, 1 μM microcystin. Lysates were briefly sonicated, centrifuged at 16,000 × g for 15 min, and the supernatant used in activity assays.

DH5α subcloning efficiency Escherichia coli (Invitrogen) were transformed with a GST-UGD plasmid, and fusion protein expression was induced as per the manufacturer’s protocol. Cell lysates were sonicated, centrifuged at 16,000 × g for 15 min, and the supernatant used in activity assays.

**FIG. 3.** CLUSTAL-W alignment of UGD (top) and putative orthologs in *M. musculus* (GenBankTM accession number AAK55410), *C. elegans* (GenBankTM accession number T15892), *D. melanogaster* (GenBankTM accession number AAF50474), *F. neoformans* (GenBankTM accession number AAK59981), and *P. sativum* (GenBankTM accession number BAB40967).
activity assays or snap-frozen and kept at −80 °C for up to 3 months without loss of activity.

**In Vitro Binding Assays**—HEK293T cells were co-transfected using LipofectAMINE 2000 (Invitrogen) with GST-UGD and HA-AKT, HA-AKTKD, HA-AKT*, or an unrelated fusion protein designated HA-X. Similar experiments were performed with HA-UGD and the corresponding GST-Akt plasmids (data not shown). After 48 h, cells were lysed in the following buffer: 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 2 mM EGTA, 0.1% Triton X-100, 0.1% Nonidet P-40, 50 mM β-glycerol phosphate, 1 mM dithiothreitol, 100 μg/liter phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma). Lysates were briefly sonicated and centrifuged at 16,000 g for 15 min. After centrifugation, 1.0 mg of the supernatants was incubated with a 50% slurry of prepared glutathione-Sepharose (Amersham Biosciences) for 1.5 h at 4.0 °C with slow rotation. Samples were washed five times with phosphate-buffered saline (Invitrogen). The agarose was resuspended in sample loading buffer, separated by SDS-PAGE, and immunoblotted using the indicated antibodies. Anti-GST (Sigma) and anti-HA (Covance) primary antibodies were both used at dilutions of 1:2000.

**Antibody Generation**—A rabbit polyclonal antibody was raised against GST-tagged UGD yeast two-hybrid fragment (Covance). The antibody was affinity-purified as described previously (28). Immunoblots were performed with a primary antibody dilution of 1:1000.

**Subcellular Fractionation**—Brains from five male adult rats were homogenized in 100 ml of 0.32 M sucrose with a glass/Teflon homogenizer. Homogenate was centrifuged for 10 min at 800 x g to give pellet (P1) and supernatant (S1). S1 was centrifuged for 15 min at 9200 x g to give pellet (P2) and supernatant (S2). S2 was centrifuged for 90 min at 100,000 x g to give pellet (P3) and supernatant (S3). The P2 fraction was resuspended in 3 ml of 0.32 M sucrose and hypotonically lysed in 27 ml of ice-cold water. Lysate was homogenized with a glass/Teflon homogenizer. Hepes (2M; pH 7.4) was added to a final concentration of 50 mM, and the sample was centrifuged for 20 min at 25,000 x g to give pellet (LP1) and supernatant (LS1). LS1 was centrifuged for 90 min at 165,000 x g to give pellet (LP2) and supernatant (LS2). Protein concentration of the fractions was determined, and 12 μg of protein from each fraction was separated by SDS-PAGE followed by immunoblot.

**In Vivo Enzyme Localization**—HEK293T cells were transiently co-transfected with a total of 2 μg of pDsRed-UGD and either pEYFP-Golgi or pEYFP-ER (CLONTECH) using LipofectAMINE 2000 according to the manufacturer’s directions (Invitrogen). pEYFP-Golgi encodes a fusion protein consisting of enhanced yellow fluorescent protein (EYFP) and a sequence encoding the NH2-terminal 81 amino acids of human β-1,4-galactosyltransferase; pEYFP-ER encodes a fusion protein consisting of EYFP, the ER targeting sequence of calreticulin, and the ER retrieval sequence KDEL. Cells were grown on four-well glass microscope slides and were processed for enzyme localization studies 48 h after transfection. Photomicrographs shown in Fig. 10 were obtained with a Nikon Eclipse TE300 inverted microscope and captured with Openlab (Improvement).

**RESULTS**

**Molecular Cloning of UGD, an Akt Yeast Two-hybrid Interactor**—In an effort to identify protein-binding partners of the signaling protein kinase Akt (protein kinase B), we utilized a yeast two-hybrid analysis with an activated, but kinase-dead, form of Akt as bait. We identified two interactors, one of which

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**FIG. 4.** Augmented evolution of 14CO2 from [glucuronyl-U-14C]-labeled UDP-glucuronate with increasing amounts of UGD. Each of the four reactions utilized a total of 1 mg of cell lysate with varying proportions of transfected (GST-UGD) and untransfected (293T) lysates. Evolved 14CO2 was determined in a liquid scintillation counter. These results were reproduced in nine similar experiments with both mammalian and bacterial cell lysates.

**FIG. 5.** HPLC analysis of UGD reaction products. A, enzyme reaction with native UGD utilizing uniformly labeled [glucuronyl-U-14C]UDP-glucuronate. B, HPLC elution of uniformly labeled [xylose-U-14C]UDP-xylose standard. C, heat-inactivated enzyme reaction utilizing uniformly labeled [glucuronyl-U-14C]UDP-glucuronate. HPLC elution fractions (0.5 ml) were collected and counted in a liquid scintillation counter. These results were reproduced in six separate experiments.
remains uncharacterized. The other interactor, which we determined to be UGD, was identified six times in the two-hybrid screen. A rat brain cDNA library was screened utilizing the UGD yeast two-hybrid fragment as probe. Four interacting library fragments were recovered. A full-length cDNA sequence of UGD was assembled from two of the rat brain cDNA fragments, the yeast two-hybrid fragment (encompassing amino acids 32–420), and database analysis (Fig. 2). The starting methionine was assigned to the first in-frame AUG codon preceded by an upstream stop codon. The full-length open reading frame of UGD contains 1260 nucleotides coding for a protein of 420 amino acids with a predicted molecular mass of 47 kDa (Fig. 2). Data base analysis revealed varying homology with putative and established UDP-glucuronate decarboxylases, nucleotide sugar epimerases, and nucleotide sugar dehydratases. Based on homology with UDP-galactose-4-epimerase, regions of UGD were identified that may be important in substrate and NAD+ binding. Specifically, UDP-galactose-4-epimerase and UGD contain a critical threonine or serine required for binding to the 4-hydroxyl group of the sugar substrates. Also, a conserved YXXX motif for binding to NAD+ is present in both UGD and UDP-galactose-4-epimerase (29) (Fig. 2). Data base analysis suggested that UGD is highly conserved across a wide evolutionary range from plants to mammals (Fig. 3). Hydropathy plot analysis (30) of the UGD protein indicated a single putative transmembrane domain at residues 20–38. Consistent with a type II transmembrane protein topology, the analysis predicted a short NH2-terminal segment most likely protruding into cytoplasm and a longer luminal carboxyl-terminal domain. Such a topology also exists in the putative homologs of UGD in Mus musculus, C. elegans, and Drosophila melanogaster but not in Filobasidiella neoformans or Pisum sativum.

**Identification of UDP-glucuronate Decarboxylase Activity**

Based on sequence similarities, we assayed our cloned, expressed protein for UDP-galactose-4-epimerase activity, dTDP-glucose-4,6-dehydratase activity, and UGD activity. We assayed for epimerase activity by spectrophotometrically monitoring the production of UDP-galactose from UDP-galactose via coupling to NAD+ in the presence of UDP-galactose dehydrogenase (22). Our clone failed to demonstrate any epimerase activity in this assay (data not shown).

We examined dehydratase activity (conversion of dTDP-glucose to dTTP-4-keto-6-deoxyglucose) in two ways. First, we reduced the 4-keto product of the reaction with tritiated NaBH4 and determined that a single sharp peak appeared with an elution time identical to that of authentic uniformly labeled [\(^{14}\)C]UDP-xylose (Fig. 5B). To obtain definitive evidence that UDP-xylose was the product of our assay, we performed electrospray mass spectrometry. Negative ion spectra of reaction mixtures in the presence of heat-inactivated enzyme (Fig. 6B) only showed peaks corresponding to UDP-glucuronate ([M+H]− = 579.3), NAD+ ([M+H]− = 662.3), and a NAD+ degradation product ([M+H]− = 540.4) (reflecting the loss of nicotinamide from NAD+). Analysis of reactions containing active UGD (A) revealed additional peaks corresponding to UDP-xylose ([M+H]− = 535.2) and monosodiated UDP-xylose ([M+Na+2H]− = 557.1). y axis scale is in millions.

**Molecular and Functional Analysis of Akt/UGD Interactions**

Since UGD was cloned based on its interactions with Akt, we wondered whether such interactions are physiologic. In HEK293T cells transfected with GST-UGD and HA-Akt, pull-down of GST-Akt brought down HA-UGD (data not shown) and pull-down of GST-UGD brought down HA-Akt, while an unrelated protein did not co-precipitate with UGD (Fig. 7).

We wondered whether there are any functional interactions...
between Akt and UGD. We have been unable to demonstrate alterations in Akt activity with UGD transfection in basal, serum-starved, or insulin-like growth factor-stimulated HEK293T cells (data not shown). Although UGD does not contain the canonical Akt phosphorylation motif (RXRXXS/TX) present in many known Akt substrates (32), we examined the possibility of Akt phosphorylation of UGD. We were unable to detect phosphorylation of GST-UGD by activated HA-Akt in an in vitro kinase reaction nor did we detect any influence of activated HA-Akt on UGD activity (data not shown).

**Tissue Localization of UGD**—Northern analysis revealed the highest densities of UGD mRNA in heart, brain, and testes (Fig. 8A). Substantial levels were also evident in kidney, liver, and lung with much lower densities in spleen and skeletal muscle. Northern analysis of transformed cell lines revealed substantial levels of UGD mRNA in 3T3, RBE7, and PC12 cell lines but little to no transcript in 293T, HeLa, COS-1, Jurkat, or 9L glioma cell lines (Fig. 8B). In all of these tissues and cell lines we detected a single transcript of about 2.1 kb, suggesting that there is no significant alternative splicing.

For immunochemical studies we developed a rabbit polyclonal antibody to GST-UGD. The antibody recognized a band of 47 kDa in transiently transfected cells (data not shown) as well as in native rat tissues (Fig. 9). Additionally, in all tissues an additional band of about 70 kDa was evident (data not shown). Kidney samples contained a second 38-kDa cross-reactive band recognized by the antibody. Preabsorption of purified antibody with GST-UGD eliminated the 47-kDa band in all tissues. Preabsorption of purified antibody with GST alone or agarose beads did not alter the 47-kDa band (Fig. 9A).

UGD protein was most enriched in kidney, liver, and brain with negligible staining in Western blots of heart, spleen, skeletal muscle, lung, and testes (Fig. 9B). This pattern differed from the distribution of UGD mRNA, which was highly concentrated in testes and heart (Fig. 8A). Within the central nervous system, UGD protein distribution varied with the highest amounts in the cerebellum, thalamus, and spinal cord and much lower levels in cerebral cortex, hippocampus, corpus striatum, and olfactory bulb (Fig. 9B).

**Subcellular Localization of UGD**—The addition of xylose in the biosynthesis of GAGs begins in the early endoplasmic reticulum and/or at the ER-to-Golgi interface and continues in the Golgi (33–35). Furthermore, UGD activity has been found in chick chondrocytes to co-localize with xylosyltransferase activity in subcellular fractions (35). To evaluate the intracellular localization of UGD, we conducted subcellular fractionations (Fig. 9C). UGD protein was predominantly particulate with highest densities in the crude microsomal fraction of whole brain (P3). To assess localization to synaptic vesicle fractions and other membranes within nerve terminals, we lysed the synaptosome-nerve terminal containing P2 fraction, which provides the LP1 (lysate) and LS1 (lysate supernatant fractions).
Centrifugation of LS1 yielded a supernatant LS2 fraction and a crude synaptic vesicle fraction, LP2. UGD was detected in LP2 and LS2 but not in LP1 fractions.

To assess the intracellular localization of UGD in intact cells, we conducted histochemistry. Because of the low levels of endogenous UGD, we were unable to conduct immunohistochemistry for native UGD. Accordingly, we transfected HEK293 cells with DsRed2-labeled UGD and compared its localization with co-transfected EYFP-ER and EYFP-Golgi, fusion protein markers for the endoplasmic reticulum and Golgi, respectively.

We observed intense staining for UGD in the perinuclear Golgi (Fig. 10).

DISCUSSION

In the present study, we present the molecular cloning and characterization of mammalian UGD. UGD is evolutionarily highly conserved with 75–80% amino acid sequence identity and 90% similarity between plants and mammals. Although we discovered UGD based on its binding to Akt, we have failed to thus far identify any major regulatory interactions between the two proteins.

Data base analysis revealed similarity of UGD to UDP-galactose-4-epimerase and TDP-glucose-4,6-dehydratase. These similarities may be based on properties shared by UGD and these other enzymes. These enzymes all employ NAD/H as a co-factor and bind UDP or TDP sugars. All of them are hypothesized to share an initial catalytic step involving oxidation of the 4-carbon of the sugar to a ketone. This is the sole function of the sugar dehydratase. The epimerase asymmetrically reduces the 4-ketone to stereospecifically produce the C-4 epimer, while UGD is thought to proceed through /H-decarboxylation of the 6-carbon followed by stereospecific reduction of the 4-carbon (28, 29).

Subcellular fractionation and histochemical studies localized UGD primarily to the Golgi. Consistent with this, hydropathy plot analysis of the protein sequence indicated a putative type II transmembrane topology common to Golgi-localized proteins (36). Previous studies of UGD activity in cultured chick chondrocytes as well as mouse mast cell tumors have localized the activity to the particulate cell fractions (35, 37, 38). Studies of xylose biosynthesis and xylosylation in intact cells, which monitor transformation of UDP-glucuronate to UDP-xylose in permeabilized chick chondrocytes, indicate predominant localizations to the Golgi and endoplasmic reticulum (34, 35).

Consistent with our hydropathy analysis, these studies evaluated the possible luminal or cytosolic orientation of membrane-associated enzyme activity by monitoring trypsin sensitivity and found UGD activity was lost in the presence of trypsin only when organelle membranes were disrupted (35). While UDP-
xylose transport into the Golgi has been described (33, 35), it has been suggested to be the result of nonspecific nucleotide sugar transport associated with other nucleotide sugar transporters (35). In contrast to our cloned sequence and its mammalian, C. elegans, and D. melanogaster homologs, hydropathy plot analysis of homologous sequences of plant and fungal origin showed no predicted membrane spanning domains; consistent with this, previous studies of plant and fungal UGD activity have found them to be associated with soluble cell fractions (39, 40). Indeed, the recent cloning of UGD from the pathogenic fungus Cryptococcus neoformans found the expressed enzyme to localize primarily to soluble cell fractions (17).

The enzymes crucial for GAG biosynthesis and their importance for proper growth and differentiation have been well characterized in C. elegans (13–15). In this species, epithelial invagination of the vulva is a major, developmentally regulated event. Screening for mutations that interfere with this process led to the identification of eight specific “squashed vulva” (SQV) genes (14). Seven of these eight genes have been cloned. Notably, all are involved in GAG biosynthesis or transport (13, 15, 41). SQV-1 is the only one of these not yet cloned. C. elegans genetic map data base analysis reveals a close proximity of a UGD ortholog to SQV-1 on chromosome IV. It is likely that UGD and SQV-1 are identical. If so, this would imply a critical role for UGD in embryonic development.

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