


Single-step purification of full-length human androgen receptor

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The full-length human androgen receptor with an N-terminal biotin acceptor peptide tag was overexpressed in *Spodoptera frugiperda* cells in the presence of 1 μ M dihydrotestosterone. Site-specific biotinylation of BAP was achieved in vivo by co-expression of *E. coli* biotin holoenzyme synthetase. The androgen receptor was purified by single-step affinity chromatography using Streptavidin Mutein Matrix under native conditions. The resultant protein was active, stable, 95% homogeneous, and we obtained sufficient yield for use in functional and structural studies.

Received June 14th, 2005; Accepted October 10th, 2005; Published October 21st, 2005 | **Abbreviations:** AR: androgen receptor; BAP: biotin acceptor peptide; BirA: biotin holoenzyme synthetase; DBD: DNA binding domain; DHT: dihydrotestosterone; LBD: ligand-binding domain; SA-HRP: streptavidin-horseradish peroxidase conjugate; Sf9: *Spodoptera frugiperda* | Copyright © 2005, Juzumiene et al. This is an open-access article distributed under the terms of the Creative Commons Non-Commercial Attribution License, which permits unrestricted non-commercial use distribution and reproduction in any medium, provided the original work is properly cited.

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Introduction

The androgen receptor (AR) is a ligand-activated transcription factor and a member of the nuclear receptor superfamily [Mi and Rastinejad, 2001; Evans, 1988]. Consistent with other nuclear receptors, AR consists of four functionally and structurally distinct domains: the N-terminal transactivation domain, DNA binding domain (DBD), hinge region, and C-terminal ligand-binding domain (LBD) [McEwan, 2004]. Androgens, such as testosterone and dihydrotestosterone (DHT), bind with high affinity to AR, resulting in a distinct conformational change in the receptor LBD. After ligand binding, AR translocates to the nucleus, binds specific DNA response elements located in target promoters, and regulates genes that are essential for the development of the male phenotype [Wilson et al., 1991]. Several mutations in the AR gene are linked to human disease, including androgen insensitivity syndrome, prostate cancer, and spinal and bulbar muscular atrophy (SBMA), thus validating AR as a major therapeutic target [Heinlein and Chang, 2004; McPhaul, 2002; Poletti, 2004].

Functional and structural studies of AR require large amounts of homogeneous protein. Purification of AR, as with other nuclear receptors, is complicated due to insolubility, instability, low abundance, and problems with aggregation. Various approaches have been taken to solve these problems, including the use of different affinity tags for the overexpression of recombinant full-length AR or AR domains in bacterial [Roehrborn et al., 1992], insect [Liao and Wilson, 2001; Chang et al., 1992; Zhu et al., 2001], or mammalian cell [Quarmby et al., 1990] expression systems as well as purification under native or denaturing conditions. Most of these expression and purification protocols have many disadvantages including low yield and purity, solubility and stability issues, and lack of post-translational modifications. In addition, denaturing conditions may interfere with protein folding, thereby affecting receptor activity.

In this article we describe a rapid, single-step purification protocol that yields 95% homogeneous full-length AR protein from Sf9 cells. We employ technology that allows the biotin-labeling of recombinant hAR in Sf9 cells [Duffy et al., 1998]. Affinity chromatography is then used to purify AR under native conditions based on the interaction of biotin and streptavidin. Streptavidin Mutein Matrix (Roche Applied Science, Indianapolis, IN, USA) is used for the purification of AR. This high-performance resin demonstrates low nonspecific binding, high protein purity, and efficient recovery. This purification protocol is likely to be applicable to a wide range of proteins, including other full-length nuclear receptors.

Reagents and Instruments

pDW464 plasmid was purchased from ScienceReagents, Inc. (El Cajon, CA). Bac-to-Bac[®] Baculovirus Expression System, PBS, Sf-900 II Serum-Free Medium (SFM), NuPage[®] Novex[®] Bis-Tris gels, BL21(DE3)pLysS cells, pDEST-15 vector, MagicMark[™] Western standards were purchased from Invitrogen Corporation (Carlsbad, CA). Dihydrotestosterone (DHT) was bought from Sigma-Aldrich (St. Louis, MO). Protease Inhibitor Cocktail Set III came from Calbiochem (San Diego, CA). Streptavidin Mutein Matrix was purchased from Roche Applied Science (Indianapolis, IN). Chromatography was performed on an ÄKTA[™] purifier Amersham Biosciences Corp. (Piscataway, NJ) using a Tricorn[™] 5/50 column (Amersham Biosciences Corp). Criterion[™] XT gels, Precision Plus protein standards were from Bio-Rad Laboratories (Hercules, CA). Streptavidin-horseradish peroxidase conjugate, Glutathione Sepharose[™] 4 Fast Flow, pGEX-5X-1 plasmid came from Amersham Biosciences. D-biotin, NeutrAvidin, SuperSignal[®] West Pico kit, MemCode[™] Reversible Protein Stain kit were purchased from Pierce Biotechnology, Inc (Rockford, IL). Costar[®] 96 well plates were from Corning, Inc (Corning, NY). Microson XL 2000 sonicator was bought from Misonix (Farmingdale, NY).

Methods

Construction of recombinant shuttle vector pDW464/hAR and recombinant baculovirus

Full-length (1-924) human androgen receptor cDNA was subcloned into the pDW464 plasmid which is designed to produce biotinylated proteins in *S. frugiperda* cells [Duffy et al., 1998]. The plasmid encodes *E. coli* biotin holoenzyme synthetase (BirA), which site-specifically biotinylates the biotin acceptor peptide (BAP, 23 amino acids). Recombinant baculoviruses were generated by recombination of pDW464/hAR with baculovirus DNA in vivo using the Bac-to-Bac® baculovirus expression system.

Expression of hAR in Sf9 cells

Sf9 cells were cultivated in 200 ml of Sf-900 II Serum-free medium (SFM) as a suspension culture at 27°C. The cells were infected with recombinant baculovirus at a density of 2×10^6 cells/ml. Androgen receptor ligand DHT (1 μ M final concentration) was added after 24 hours and cells were incubated for an additional 24 hours. Cells were harvested by centrifugation and stored at -80°C.

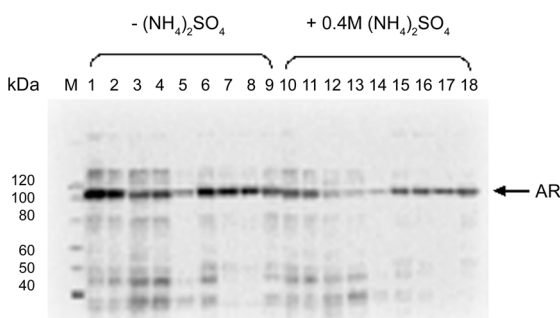


Figure 1. Western blot of AR batch purification using Streptavidin Mutein Matrix under different binding conditions. M: Markers; lanes 1-9: binding in the absence of $(\text{NH}_4)_2\text{SO}_4$; lanes 10-18: binding in the presence of 0.4M $(\text{NH}_4)_2\text{SO}_4$; lanes 1 and 10: nuclear-extract fraction before purification; lanes 2 and 11: flow-through; lanes 3-5 and 12-14: washes; lanes 6-8 and 15-17: elutions; lanes 9 and 18: beads. 2.5 μ l of each fraction was loaded in lanes 1, 2, 10, and 11, and 15 μ l was loaded in all other lanes.

Preparation of nuclear extract from Sf9 cells

Sf9 cells derived from one liter of culture were thawed and resuspended on ice in 100 ml hypotonic buffer: 10 mM Tris-HCl, pH 8.0, 10 mM NaCl, 1.5 mM MgCl_2 , 10 mM β -mercaptoethanol, 1 μ M DHT, and 1/200 ml of Protease Inhibitor Cocktail Set III. The suspension was transferred to an ice-cold Dounce homogenizer, incubated for 15 minutes on ice, and lysed with 25 strokes. After centrifugation at 7,000X g for 10 minutes at 4°C, the supernatant containing the cytoplasmic fraction was discarded and the pellet was resuspended in 30 ml nuclear-extract buffer: 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 1.5 mM MgCl_2 , 10 mM β -mercaptoethanol, 50 mM β -glycerophosphate, 50 mM NaF, 1 μ M DHT, and 1/200 ml protease inhibitor cocktail set III. The nuclear-extract fraction was incubated for 30 minutes at 4°C with rotation, then clarified by centrifugation at 50,000X g for 30 minutes

at 4°C. The supernatant was transferred to a clean chilled tube after filtration through a 0.45 μ m filter.

Batch purification of hAR using Streptavidin Mutein Matrix

Batch purification was performed according to the manufacturer's instructions. Briefly, 100 μ l of Streptavidin Mutein Matrix gel slurry, corresponding to 50 μ l packed gel, was suspended in equilibration buffer: 100 mM potassium phosphate, pH 7.2, 0.15 M NaCl in the presence or absence of 0.4 M $(\text{NH}_4)_2\text{SO}_4$. After equilibration, 50 μ l of Streptavidin Mutein Matrix (gel bed volume) was incubated with 400 μ l of nuclear-extract fraction containing expressed hAR for 30 minutes at 4°C in the presence of 1 μ M DHT and 0.4 M $(\text{NH}_4)_2\text{SO}_4$ final concentration. Another set of samples was processed without ammonium sulfate during the binding step. After incubation, samples were centrifuged for 30 seconds at 2000X g and supernatant (flow-through fraction) was removed. The gel was washed twice with one bed volume (50 μ l) of wash buffer (100mM potassium phosphate pH 7.2, 0.15M NaCl, 1 μ M of DHT, 10 mM β -mercaptoethanol), followed by a third wash with 400 μ l. Elution was performed in three steps by adding 150 μ l (50 μ l per step) of elution buffer: 100 mM potassium phosphate, pH 7.2, 0.15 M NaCl, 1 μ M DHT, 10 mM β -mercaptoethanol, 2 mM D-biotin, incubating for 10 minutes at 4°C, spinning, then collecting the supernatant.

Purification of biotinylated hAR with the Streptavidin Mutein Matrix using an ÄKTA™ purifier

Chromatography was performed on an ÄKTA™ purifier using a Tricorn™ 5/50 column (Amersham Biosciences) packed with 1 ml of Streptavidin Mutein Matrix. The column was prepared according to the manufacturer's instructions, except that the equilibration buffer was modified to 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl. The purification protocol included equilibration of the column with 5 column volumes (CV) of equilibration buffer, loading the nuclear-extract fraction containing biotinylated AR in the nuclear-extract buffer, and washing the column with 10 CV of washing buffer: 20 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 10% glycerol, 10 mM β -mercaptoethanol, 1 μ M DHT. Elution was performed with 10 CV of elution buffer: 50 mM Tris-HCl, pH 8.0, 0.2 M NaCl, 10% glycerol, 4 mM DTT, 1 μ M DHT, 5 mM D-biotin. All buffers were filtered through 0.45 μ m filters before use and chromatography was performed at 0.2 ml/min flow rate at 4°C. One-milliliter fractions were collected during the washing and elution steps and analyzed by SDS-PAGE.

SDS-PAGE analysis of purified hAR

Expression and purification of hAR was monitored using precast NuPage® Novex® Bis-Tris gels (Invitrogen) or Criterion™ XT gels (Bio-Rad Laboratories) followed by staining with SimplyBlue™ (Invitrogen) or blotting on PVDF membranes (70V for 1.5 hours). Blotted membranes were blocked with 5% low-fat milk in phosphate-buffered saline (PBS) followed by washes with PBST (PBS with 0.1% Tween®). The blots were

developed using a streptavidin-horseradish peroxidase conjugate and SuperSignal® West Pico kit.

in 50 μ l NuPage 2X sample loading buffer, heated for 5 min at 95°C, and analyzed by SDS-PAGE and western blot.

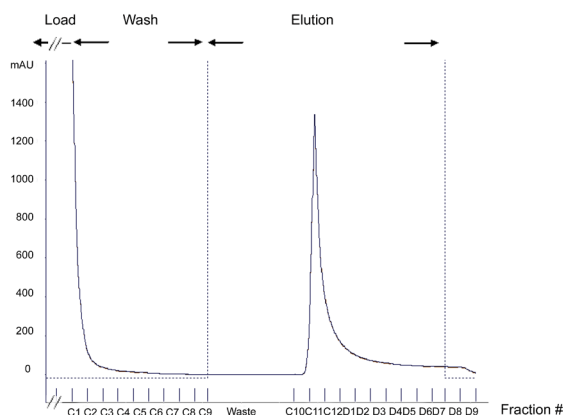


Figure 2. Chromatogram of AR purification on an ÄKTA™ purifier showing loading, washing, and elution steps. See text for details.

DNA binding assay

Costar 96-well plates were coated with 10 μ g/well neutravidin in 100 μ l of 0.1M NaHCO₃, pH 8.5 for 4 hr at room temperature. Wells were blocked with 150 μ l/well of 2% milk in 0.1M NaHCO₃, pH 8.5 for 1 hr at room temperature and washed 5 times with 300 μ l/well PBST (PBS + 0.1% Tween 20). Biotinylated DNA (2pmol) containing AR response element (ARE) in 100 μ l of PBST was added per well and incubated 1 hr at room temperature. Remaining neutravidin sites were blocked by adding 0.1mM biotin for 1 hr at room temperature. Wells were washed 5 times with 300 μ l PBST and either 100 μ l of AR expressing Sf9 cell lysate or 1-2 μ g of purified AR in PBST was added in the presence of 1 μ M DHT. After overnight incubation at 4°C by gentle rotation, the plate was washed 5 times with PBST and 20 μ l of NuPage 1X sample loading mix was added per well. The plate was heated for 10 min at 95°C and samples were analyzed by SDS-PAGE.

GST pull-down assay

AR cofactor ARA24 was cloned in the pDEST-15 vector and expressed in BL21(DE3)pLysS cells as a GST-fusion protein. pGEX-5X-1 vector, coding GST protein alone (negative control), was also transformed and expressed in BL21(DE3)pLysS cells. GST-fusion proteins were then purified according to manufacturer's instructions using Glutathione Sepharose 4 Fast Flow beads. Purified proteins were analyzed by SDS-PAGE to estimate protein expression. GST protein was found to be expressed 5 times greater than GST-ARA24 (data not shown). For GST pull-down assays, either 50 μ l of GST-ARA24 or 10 μ l of GST (with an additional 40 μ l of bead slurry for 50 μ l total) was used for each pull-down sample. GST-proteins were incubated overnight at 4°C by gentle rotation with AR expressing Sf9 cell lysate (100 μ l) or with 2 μ g purified AR in a total volume of 500 μ l (adjusted with cell lysis buffer) in the presence of 5 μ M DHT. Following incubation, beads were pelleted and washed 5 times with cell lysis buffer (500 μ l). The beads were resuspended

kDa M F C7 C10 C11 C12 D1 D2 D3 D4

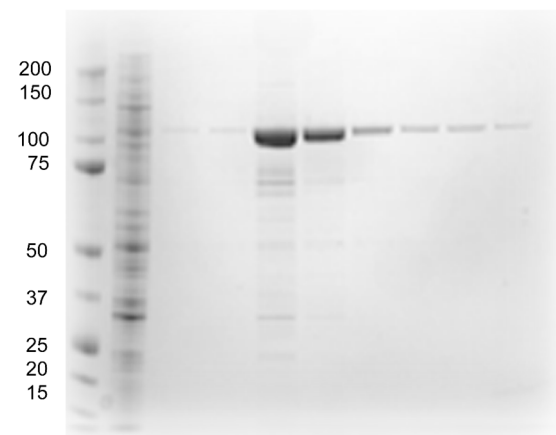


Figure 3. Analysis of AR purification on an ÄKTA™ purifier by SDS electrophoresis on a NuPage® NOVEX® Bis-Tris 4-12% gel (SimplyBlue™ staining). M: Markers; F: flow-through fraction; lanes C7 and C10: wash-step fractions; lanes C11 – D4: elution-step fractions. A 15- μ l aliquot from each fraction was loaded on the gel, with the exception of the flow-through fraction lane, in which only 8 μ l was loaded.

Results

Expression levels of hAR in Sf9 cells and the soluble AR fraction were determined by western blot using streptavidin-HRP to detect the biotinylated N-terminal tag of AR. Full-length AR with a BAP tag (~ 26 amino acids) migrated as a ~107 kDa band on SDS-PAGE and almost all protein was retained in the soluble fraction (data not shown).

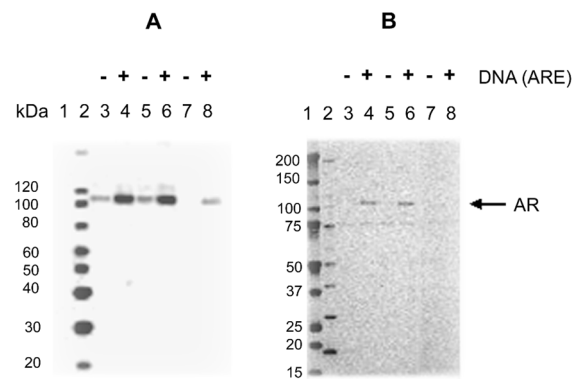


Figure 4. Purified AR binds to DNA containing an androgen receptor response element. (A) Western blot. 1: Precision Plus protein standards; 2: MagicMark™ Western standards; lanes 3,5,7 contain no DNA (ARE), lanes 4,6,8 have 2 pmol ARE; 1mg of purified AR were used in lanes 3,4; 2mg of purified AR were used in lanes 5,6; AR expressing Sf9 cell lysate was used in lanes 7,8. (B) same PVDF membrane used in (A) was stained with MemCode™.

To explore the best binding conditions of AR to the Streptavidin Mutein Matrix, we performed batch-method purification in accordance with manufacturer's instructions, and analyzed protein purity by Western blot

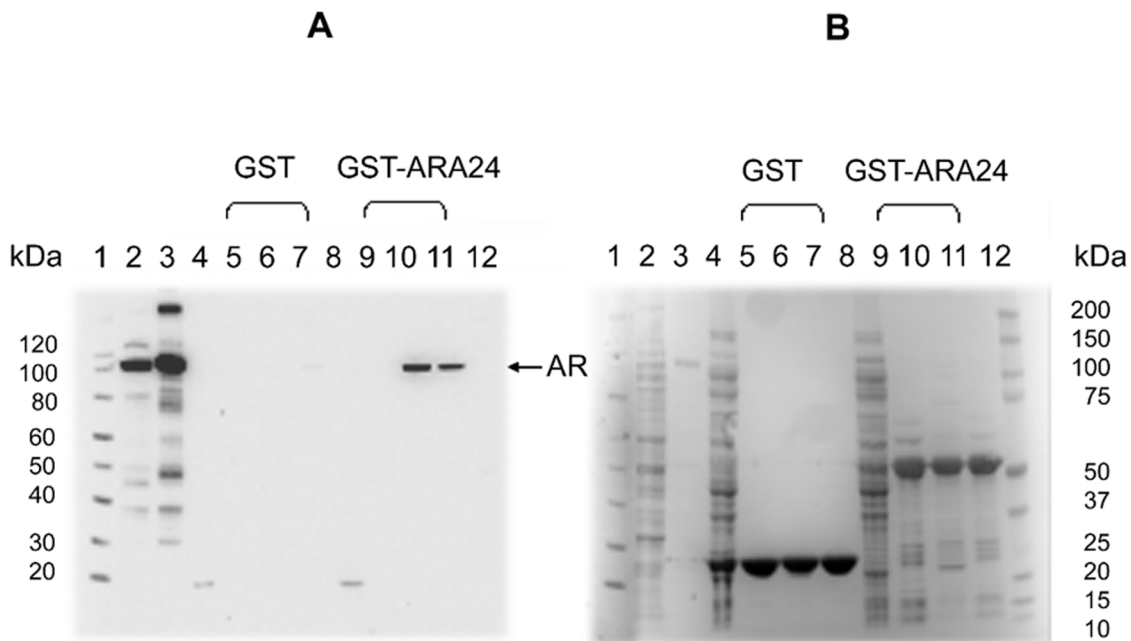


Figure 5. Recombinant AR interacts with ARA24 *in vitro*. (A) Western blot. 1: MagicMark™ Western standards; 2: Sf9 cell lysate with expressed AR (1/40th of the input); 3: purified AR (1/4th of the input); 4 and 8: E.coli lysates with the over expressed GST and GST-ARA24 proteins respectively; lanes 5-7 contains GST protein incubated in the absence of AR (lane 5), with AR expressing Sf9 lysate (lane 6) or purified AR (lane 7); lanes 9-11 contains GST-ARA24 protein incubated in the absence of AR (lane 9), with AR expressing Sf9 lysate (lane 10) or purified AR (lane 11); 12: Precision Plus protein standards. (B) same PVDF membrane used in (A) was stained with MemCode™.

using streptavidin-HRP. The manufacturer's recommendation to perform binding in the presence of 0.4 M $(\text{NH}_4)_2\text{SO}_4$ did not enhance AR binding to the Streptavidin Mutein Matrix (Figure 1, lanes 2 versus 1 and 11 versus 10), and significantly reduced soluble AR in the nuclear-extract fraction (lane 1 versus 10). Small amounts of AR were detected in the wash fractions (lanes 3-5 and 12-14) and retained on the beads (lanes 9 and 19). However, more extensive elution resulted in the removal of almost all AR from the matrix (data not shown).

We did not optimize the batch method for large scale purification of AR; instead we optimized for use with an automated FPLC method. We believe that these conditions can be used for the batch method, if FPLC is not available. This information is important as it bears on the use of this system for other receptors.

For large-scale protein purification, we developed an automated procedure using the ÄKTA™ purifier chromatography system. The purification protocol included three steps: (1) loading nuclear extract in nuclear-extract buffer without the addition of ammonium sulfate; (2) washing, and (3) elution with 10 CV of appropriate buffers. A chromatogram of the purification process showed a single peak representing AR in fractions C11-D2 (Figure 2). Purified AR was approximately 95% homogeneous as determined by an SDS-PAGE gel stained with SimplyBlue™ (Figure 3) and the yield was 2 mg of protein from 1 liter of infected Sf9 cell culture. Approximately 30% of the AR protein was lost in the flow-through and wash-step fractions as determined by western blot (data not shown).

We also performed several experiments to test the functional activity of the purified AR protein. Using a DNA binding assay developed in our laboratory [Norris et al., 1999], we demonstrated that purified AR binds to DNA containing an androgen response element (ARE) (Figure 4A, lanes 3 - 6). For comparison, we also tested the ability of AR expressed in the Sf9 cell lysate to bind DNA (Figure 4A, lanes 7,8). In both cases, AR was found to be significantly enriched in wells containing DNA thus confirming functional DNA binding activity of recombinant AR. Also shown is a membrane stained for protein to demonstrate the purity of the DNA bound AR (Figure 4B). Next, we demonstrated that recombinant AR can interact with ARA24, a known AR coactivator and interacting protein. As shown in Figure 5A, recombinant AR, either purified or from Sf9 cell extract, interacts specifically with GST-ARA24 and not with GST alone (compare lanes 6,7 with lanes 10,11). A membrane stained for protein is also shown to allow for visualization of the GST proteins used in this assay. These results demonstrate that recombinant AR is functional with regard to coactivator binding. Several other AR interacting proteins have been analyzed in this assay and yielded similar results (data not shown). Furthermore, purified AR remained stable through several freeze/thaw cycles. We did not observe any protein degradation by SDS-PAGE electrophoresis or any loss of functional activity by DNA binding and GST pull-down assays. We have not determined directly if the biotin tag interferes with N-C terminal interaction. However, we have demonstrated that biotinylated AR is active in transcriptional studies (data not shown). This would suggest that N-C terminal interaction is not disrupted by the biotin tag.

Discussion

We have successfully applied this purification technique to AR when bound to other ligands, AR domains (both N- and C- terminus), and other full-length nuclear receptors. We were not able to purify apo AR in significant quantities since its expression level is greatly reduced compared to ligand-bound AR (data not shown). Roche Applied Science's Streptavidin Mutein Matrix was found to be superior for the purification of nuclear receptors when compared to other resins that exploit biotin-streptavidin interaction (Promega SoftLink™ Soft Release Avidin and Pierce ImmunoPure Immobilized Monomeric Avidin). The superior performance of the Streptavidin Mutein Matrix is a result of low nonspecific binding to the beads, efficient elution, low backpressure, and ease of use with automated chromatography systems. This technique allows the purification of AR in a single step, yielding amounts sufficient for functional and structural studies.

Acknowledgements

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