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Expression and purification of full-length mouse CARM1 from transiently transfected HEK293T cells using HaloTag technology

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Abstract

Coactivator-associated arginine methyl transferase 1 (CARM1) is a protein arginine methyltransferase (PRMT) family member that functions as a coactivator in androgen and estrogen signaling pathways and plays a role in the progression of prostate and breast cancer. CARM1 catalyzes methylation of diverse protein substrates. Prior attempts to purify the full-length mouse CARM1 protein have proven unsatisfactory. The full-length protein expressed in *E. coli* forms insoluble inclusion bodies that are difficult to denature and to refold. The presented results demonstrate the use of a novel HaloTagTM technology to purify full-length CARM1 from both *E. coli* and mammalian HEK293T cells. A small amount of CARM1 was purified from *E. coli*; however, the protein was truncated on the N-terminus by 10–50 amino acids, most likely due to endogenous proteolytic activity. In contrast, substantial quantities of soluble full-length CARM1 were purified from transiently transfected HEK293T cells. The CARM1 from HEK293T cells was isolated alongside a number of co-purifying interacting proteins. The covalent bond formed between the HaloTag and the HaloLink resin allowed the use of stringent wash conditions without risk of eluting the CARM1 protein. The results also illustrate a highly effective approach for purifying and enriching both CARM1-associated proteins as well as substrates for CARM1's methyltransferase activity.

Keywords

CARM1; PRMT family; methyl transferase; HaloTag; HaloLink resin; affinity purification

Introduction

Protein arginine methyl transferases (PRMT) are a diverse family of proteins with at least 10 mammalian representatives [1, 2]. These proteins catalyze the transfer of a methyl group from S-adenosylmethionine (SAM) to diverse protein targets [3]. In turn, substrate methylation affects many different cellular processes such as RNA splicing, DNA damage repair, and transcriptional regulation [3]. Most PRMT family members methylate the glycine-arginine rich repeats (GAR) found in substrate proteins [4]. During the methylation reaction, the PRMT enzyme transfers one or more methyl groups from SAM to a terminal amino group(s) in the arginine sidechain on the substrate protein [4].

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PRMT4, also known as coactivator - associated arginine methyltransferase 1 (CARM1), was identified for its function as a coactivator of transcriptional activation [5]. It is the largest known PRMT enzyme (608 amino acids); CARM1 forms homodimers necessary for catalytic activity [6]. CARM1 is unique among PRMT family members in that it does not methylate the GAR motif on its substrates [7], but methylates a diverse number of dissimilar sequences. Its catalytic core, comprised of amino acids 150–470, is highly conserved among all of the other PRMTs and is essential for the methyltransferase enzymatic activity [8]. The structure of CARM1 core, spanning amino acids 28–508, has been solved [9]. Although not necessary for catalytic activity, amino acids 1–130 and 480–608 of CARM1 interact with coactivators and are important for transcriptional activation [10]. CARM1 is known to interact with p160 family members and assemble into large complexes composed of diverse proteins that affect transcriptional activation [11, 12]. It is localized primarily in the nucleus where it methylates a large number of various substrates involved in chromatin remodeling and RNA processing [11, 13, 14]. Intracellular CARM1 levels are important for estrogen receptor and androgen receptor signaling; CARM1 expression levels are altered in breast cancer and prostate cancer tissue [15, 16].

Several PRMT family members, including CARM1, are able to transfer the methyl group from SAM onto themselves; however, the mechanism or the physiological role of automethylation is not well understood [17–19]. In order to identify and characterize the automethylation activity of CARM1 it is necessary to express and purify the full-length protein. Previous attempts to effectively characterize the post-translational modifications on CARM1 have been hampered by the lack of effective purification methodologies for full-length protein. Additionally, tags such as GST [17] and Flag tag [our unpublished results] have been shown to be methylatable – an artifactual finding that complicates additional analysis. The full-length CARM1 protein expressed in *E. coli* forms mostly insoluble inclusion bodies that are very difficult to refold. The C-terminus, comprising the last 100 amino acids, is largely unstructured and prone to proteolysis during protein purification [9]. This region of CARM1 is likely the cause of the difficulty in purifying the full-length protein. The C-terminus has been implicated in the transcriptional coactivation of different hormone signaling pathways [10, 20], and contains the putative site of automethylation [21]. Therefore it is important to purify full-length CARM1 protein in order to identify post-translational modifications and potential binding partners.

HaloTag™ is a proprietary 34 kDa protein tag developed by the Promega Corporation (Madison, WI) for use in protein purification. This tag is based on the dehalogenase enzyme present in *Rhodococcus* bacteria [22]. The HaloTag functions by forming a covalent bond between itself and a chloroalkane substrate. The high-affinity, irreversible, covalent interaction between HaloTag and chloroalkane-derivatized resin allows for rapid purification of HaloTag-tagged proteins. HaloTag has also been shown to increase the soluble expression of tagged proteins in *E. coli* [23]. The presented results show the successful purification of full-length CARM1 protein from mammalian cells using the HaloTag technology. We show that HaloTag enables the covalent attachment of CARM1 protein to a solid support, thereby creating a protein-affinity resin that can be used to effectively capture CARM1-interacting proteins. Additionally, this work describes the use of an active site inhibitor, sinefungin, to enhance the protein-protein interactions between CARM1 and its protein substrates – an effective approach that is readily generalizable to other protein-protein interactions.

Materials and Methods

Expression and purification of HaloTag-CARM1 from *E. coli*

Full-length mouse CARM1 was subcloned from CMX-Flag-CARM1 [18] into pFN18A HaloTag T7 Flexi vector (Promega, Madison, WI) using PCR amplification. The CARM1

construct contained an N-terminal HaloTag tag. The transformed Rosetta2(DE3)pLysS *E. coli* expression strain was used for HaloTag-CARM1 expression. Induction of expression, cell lysis, and inclusion body isolation was performed as previously described [24]. Briefly, Luria-Bertani media (500 ml) was seeded with 10 ml of overnight starter culture and grown at 37 °C until OD_{600 nm} reached 0.45; the culture was then shifted to an 18 °C - shaking incubator and grown until OD_{600nm} reached 0.6. Protein expression was then induced with the addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 24 hrs. Cells were lysed by sonication in lysis buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 0.005% Igepal CA-630, pH 7.5) added at a ratio of 10 ml of buffer per 1 g of wet weight *E. coli* pellet. The extent of sonication was tightly controlled: sonication in 4 cycles (each at 20 seconds on at 35 % power, 60 seconds off, at 0 °C) on a Digital Sonifier S-450D (Branson, Danbury, CT). The lysate was centrifuged at 26,000 g for 30 min at 4 °C. The inclusion bodies in the pellet were washed twice by resuspension in lysis buffer supplemented with 1 % Triton X-100, followed by centrifugation as above. A final wash with lysis buffer was performed to remove residual detergent. Purified HaloTag-CARM1 inclusion bodies were dissolved in lysis buffer supplemented with 6 M guanidine hydrochloride (GdnHCl) and centrifuged at 26,000 g for 30 min. The inclusion bodies were analyzed by SDS-PAGE after methanol/chloroform precipitation [25].

A 20 µl sample of cleared lysate was incubated with HaloTag TMR Ligand (Promega) for 15 min at 22 °C to allow the coupling of the TMR fluorophore to the HaloTag (onput sample). The cleared lysate was then incubated overnight with the HaloLink resin (Promega) at 4 °C on a rotator platform. The HaloLink resin was added at a ratio of 100 µl of resin (25 % slurry) per 10 ml of cleared lysate. The resin was then centrifuged at 2,000 g for 5 min; 20 µl of the supernatant was saved and combined with HaloTag TMR ligand as above (flowthrough). The HaloLink resin was then washed twice with 50–100 resin volumes of lysis buffer. Washes were removed by centrifuging the resin at 2,000 g for 5 min, and aspirating the wash. The resin was then washed with lysis buffer supplemented with 1 M urea for 20 min at 22 °C on a rotating platform. Lastly, the HaloLink resin was washed two more times with lysis buffer without urea. The resin was then resuspended in cleavage buffer (50 mM HEPES, 150 mM NaCl, 0.5 mM EDTA, 0.1 mM dithiothreitol (DTT), pH 7.5) supplemented with 15 µg/ml of TEV protease. The cleavage buffer volume was the same as the volume of the HaloLink resin slurry used for the purification. The resuspended resin was cleaved for 2 hrs at 22 °C on a rotating platform. The resin was centrifuged at 2,000 g for 5 min, and the supernatant containing the cleaved CARM1 was flash frozen in liquid nitrogen and stored at –80 °C. Protein concentration was determined using the Bradford assay (Thermo Scientific, Rockford, IL), using BSA as a protein standard. Protein purity was determined by analyzing the scanned Coomassie-stained gel with ImageQuant v5.2 software (GE Healthcare, Piscataway, NJ).

Expression and purification of CARM1 from transiently transfected HEK-293T cells

The CARM1 sequence in pFN18A HaloTag T7 Flexi vector, created as described above, was subcloned into pFC14K HaloTag CMV Flexi and pFN21K HaloTag CMV Flexi mammalian expression vectors (Promega) using restriction enzymes. The pFC14K and pFN21K vectors contain a C- and N-terminal HaloTag, respectively. Adherent HEK293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (10 % FBS-DMEM) and penicillin/streptomycin antibiotics at 37 °C in a 5 % CO₂ atmosphere. The HEK293T cells were transfected using a polyethyleneimine (PEI) method, loosely based on previously published work [26]. Briefly, 25 kDa PEI (50 % w/v) (Sigma-Aldrich, St. Louis, MO) was diluted in H₂O to 1 mg/ml and filter-sterilized. The ratios of DNA to PEI were optimized for cell viability and transfection efficiency; the following ratio was used: 2.5 µg of DNA was

combined with 6.7 μ l of diluted PEI per 10 cm² of 70 – 90 % confluent HEK293T cells. The plasmid DNA and PEI were diluted separately in serum-free DMEM and incubated for 5 min at 22 °C. The diluted DNA and PEI were then combined and incubated for 25 min at 22 °C; the final volume of the complex constituted 20 % of the final plating volume. The solution was then combined with serum-free DMEM (80 % of final plating volume) and added to phosphate buffered saline (PBS, Invitrogen) – washed cells. Cells were incubated as above for 3.5 hrs and then supplemented with the same volume of 20 % FBS-DMEM, for a final serum concentration of 10 %. The cells were then cultured for 48 hrs prior to harvest. Growth media was aspirated, cells were washed with PBS, scraped off the dish, counted on a hemocytometer, and centrifuged at 2,000 g for 10 min. The HEK293T cell pellets containing overexpressed HaloTag-CARM1 were stored at –80 °C.

The HEK293T cell pellets were lysed in lysis buffer (see above) supplemented with BaculoGold protease inhibitor (BD Biosciences, San Jose, CA). Cells were lysed at a ratio of 1 ml of lysis buffer per 2×10^7 cells. The cells were lysed by freeze/thaw and sonication with 4 cycles (5 seconds on at 35 % power, 30 seconds off, at 0 °C). The crude lysate was centrifuged at 27,000 g for 30 min. The cleared lysate was then mixed with HaloLink resin and incubated, washed and cleaved as described above. HaloLink resin was added at a ratio of 75 μ l of resin (25 % slurry) per 1 ml of cleared lysate. Onput and flowthrough fractions were reacted with the HaloTag TMR ligand as above. The purified CARM1 was cleaved off the HaloLink resin with TEV protease in cleavage buffer as described in the previous section. Purified CARM1 was assayed using SDS-PAGE and Coomassie blue staining. In addition, a Western blot was performed using a polyclonal anti-CARM1 antibody generated against CARM1 C-terminus [6].

Fluorescent staining of enzymatically active HaloTag

Samples containing HaloTag-CARM1 from either *E. coli* or HEK293T cells reacted with HaloTag TMR ligand (chloroalkane conjugated to tetramethylrhodamine) were mixed with SDS sample buffer and loaded on an SDS-PAGE gel. After electrophoresis, the gel was scanned on the Typhoon Laser Scanner (GE Healthcare) using appropriate filter sets for TAMRA fluorophore (excitation, 550 nm; emission, 573 nm). The scanned gel was analyzed using ImageQuant v5.2 software.

In order to visualize the localization of overexpressed Halo-CARM, mouse embryonic fibroblasts - MEF20 (CARM1 null) cell line was used [6]. MEF20 cells were cultured on glass coverslips and transfected with either N- or C- terminally tagged HaloTag-CARM1 or with N-terminally Flag-tagged CARM1 using FugeneHD transfection reagent (Roche, Indianapolis, IN) as per manufacturer's instructions. In all cases, protein overexpression was driven by the CMV promoter. After 48 hrs, the cells transfected with HaloTag-CARM1 constructs were incubated for 15 min with 5 μ M HaloTag TMR ligand diluted in growth media. After incubation, the cells were washed 3 times with DMEM, and then incubated with fresh growth media for 15 min to allow covalent coupling of TMR ligand to the HaloTag. Both the Flag-CARM1 and HaloTag-CARM1 expressing cells were then fixed in 3 % paraformaldehyde and washed with phosphate buffered saline (PBS) (Invitrogen). Cells were then permeabilized for 10 min with 0.1% Triton X-100 in PBS-GC (PBS supplemented with 0.02 % gelatin) followed by washes with PBS-GC. The MEF20 cells transfected with Flag-CARM1 were then incubated with C-terminus – specific anti-CARM1 polyclonal antibody, followed by Alexa 546 – labeled goat anti-rabbit antibody (Invitrogen) and by 4', 6-diamidino-2-phenylindole (DAPI, Invitrogen). The HaloTag-CARM1 expressing cells were stained only with DAPI. The glass coverslips were mounted and imaged with a Leica DM6000 microscope system (Leica Microsystems, Wetzlar, Germany) using filters appropriate for TAMRA and Alexa 546 fluorophores.

Nuclear lysate preparation

HEK293T cells were cultured in the presence of 20 μ M of adenosine dialdehyde (AdoX, EMD), an indirect methyltransferase inhibitor, for 48 hrs in order to inhibit endogenous methylation. A 90 % confluent 15-cm dish containing adherent HEK293T cells was washed in 10 ml PBS. The PBS was removed and a second 10-ml volume of PBS was added. Cells were lifted from the plate using a rubber policeman and centrifuged for 5 min at 180 g to pellet cells. Pellets were gently resuspended in 2 ml PBS and centrifuged for 3 min at 405 g. The pellet was then resuspended in 1 ml of buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors) and swelled on ice for 15 min. 62 μ l of 10 % NP-40 was added and the tube was vortexed for 10 seconds. Immediately thereafter the tubes were centrifuged for 10 sec at 2,300 g. The pellet (containing nuclei) was then resuspended in buffer B (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 15 % glycerol, 1 mM DTT, protease inhibitors) and rocked for 15 min at 4 °C. The tubes were then centrifuged at 18,000 g for 10 minutes at 4 °C. The supernatant (nuclear lysate) was reserved for incubation with immobilized CARM1 affinity column.

In vitro methylation experiments

The relative methyltransferase activity of purified CARM1 proteins was determined as previously described [19]. Briefly, 1 μ g of purified GST-PABP1 protein [27] was combined with CARM1 and incubated in 15 μ l of reaction buffer (5 mM $MgCl_2$, 20 mM HEPES pH 7.9, 1 mM EDTA, 1 mM DTT, 10 % glycerol) containing 1 μ l of 3H -S-adenosylmethionine (1 μ Ci/ μ l) (GE Healthcare) for 15 min at 30 °C. The reaction was stopped by the addition of SDS sample buffer. The reactions were assayed by electrophoresis on SDS-PAGE gel followed by Coomassie staining. The stained gel was then incubated in “Amplify” (GE Healthcare) scintillation fluid for 30 min followed by vacuum drying at 80 °C for 2 hrs. Dried gels were exposed to film at –80 °C until suitable autoradiography exposure intensity was reached.

In order to visualize the specific CARM1 substrates among the many CARM1 co-purifying proteins the following approach was used. 10 μ l of 1 M urea wash fractions from either HaloTag-CARM1 or from HaloTag-GST affinity columns were incubated with 1 μ g of purified CARM1 protein in the presence of 1 μ l of 3H -S-adenosylmethionine (GE Healthcare) for 2 hrs at 30 °C. The reactions were stopped and assayed as described above.

To quantify the methyl transferase activity, the Coomassie-stained band corresponding to the GST-PABP1 protein was excised, combined with scintillation fluid and counted on a scintillation counter Beckman LS6000SC (Beckman Coulter, Brea, CA).

Affinity purification of CARM1 co-factors and substrates

The C-terminal tagged HaloTag-CARM1 and the N-terminal tagged HaloTag-GST proteins were used for making the affinity resins. HaloTag-CARM1 in pFC14K vector was transfected into and overexpressed in HEK293T cells (2×10^8 cells) as previously described. HaloTag-CARM1 purification was performed using 400 μ l of HaloLink resin; the protein was bound to resin overnight and washed with lysis buffer and with 1 M urea. The HaloTag-GST affinity column was constructed by incubating 300 μ g of purified HaloTag-GST protein (Promega) with 400 μ l of HaloLink resin in 10 ml of lysis buffer overnight.

The HaloLink resin with bound HaloTag-CARM1 or HaloTag-GST was not cleaved with TEV protease, thereby retaining the CARM1 and GST proteins on the resin. Each resin (HaloTag-CARM1 and HaloTag-GST) was split into two portions, each representing an affinity resin containing approximately 150 μ g of bound protein. The affinity resins were then incubated for 1 hr at 22 °C with 1 ml of 200 μ g/ml HEK293T nuclear lysate (from

AdoX – treated cells) in the presence of 100 μ M sinefungin (EMD) or buffer control. The affinity resins were washed twice with 1 ml of lysis buffer followed by 20 min of incubation with lysis buffer containing 1 M urea (urea elution). The urea wash was saved and stored at -80°C . The affinity resins were washed two more times with lysis buffer and cleaved with TEV protease as previously described. The elutions and 1 M urea wash were analyzed using SDS-PAGE followed by Coomassie and silver staining. Prior to SDS-PAGE, the 1 M urea wash fraction was concentrated 20 fold using methanol/chloroform precipitation as above. The TEV elution and the 1 M urea wash samples (unconcentrated) were also assayed for their ability to be methylated by CARM1 as described in the previous section.

Results and Discussion

CARM1 expression and purification in *E. coli*

The full-length mouse CARM1 protein was fused to the HaloTag (via linker, 35 kDa) in order to take advantage of the HaloTag's reported tendency to increase soluble protein expression [23]. HaloTag technology lends itself to a very simple purification plan (Fig. 1A). The cleared cell lysate was incubated with HaloLink resin overnight in order to capture the majority of the expressed HaloTag-CARM1 protein. The covalent bond formed between HaloTag and its resin – conjugated chloroalkane ligand allows for irreversible binding of the expressed HaloTag-CARM1 to the resin. The bound HaloTag-CARM1 does not leach off the resin, allowing the use of stringent wash conditions (e.g., denaturing buffers) without concern that the bound protein would release from the HaloLink. In order to recover purified HaloTag-CARM1 from the resin, a TEV protease cleavage was performed. The TEV protease cleavage site is located in the linker between the HaloTag and CARM1 sequences (as indicated in the pFN18A product documentation). After the TEV cleavage, the HaloTag protein is retained on the HaloLink resin while the CARM1 protein is eluted. TEV protease contained a His₆ tag that allows easy removal of protease from the eluted CARM1. However, it was found that the small quantity of residual TEV did not interfere with downstream CARM1 assays and therefore it was not removed.

The coding sequence of CARM1 was inserted into the N-terminal HaloTag vector and expressed in the Rosetta2(DE3)pLysS *E. coli* strain. The Rosetta2 strain synthesizes higher levels of rare tRNA codons which contribute to increased expression level of induced proteins, especially if the given protein's sequence contains codons that are rarely used in *E. coli*. HaloTag-CARM1 (101 kDa) expression was induced at 18°C in order to slow down *E. coli* metabolism, thereby increasing expression of soluble protein. Both soluble and inclusion body fractions were analyzed for HaloTag-CARM1 expression (dotted arrow, Fig. 1B). The soluble fraction (ON) was incubated with HaloLink resin overnight and the flowthrough (FT) fraction was collected. No apparent Coomassie-stained band corresponding to soluble HaloTag-CARM1 was seen in the soluble fraction (ON, Fig. 1B). In contrast, the inclusion bodies contained large amounts of insoluble HaloTag-CARM1 (11 mg of protein from 1 g of wet weight *E. coli* pellet) (IBs, Fig. 1B). The inclusion bodies were not homogenous and instead were composed of several C-terminal truncations (4–10 kDa smaller than full-length) of HaloTag-CARM1 (data not shown).

Coomassie staining in Fig. 1 shows that despite the apparent absence of HaloTag-CARM1 from the ON fraction, several proteins bound and eluted from HaloLink resin (elut, Fig. 1B). The band corresponding to CARM1 (solid arrow) comprised about 22 % of total eluted protein. The TMR ligand fluorescence revealed comparatively little full-length HaloTag-CARM1 protein (dotted arrow, Fig. 1C). Most of the TMR ligand – reactive proteins were much smaller (35–50 kDa). These proteins most likely represent the C-terminal fragments of HaloTag-CARM1 containing an enzymatically active HaloTag. These fragments have a higher affinity for HaloLink resin as evidenced by the enrichment of the fluorescent band

corresponding to full-length HaloTag-CARM1 in the flowthrough (FT, Fig. 1C). The binding kinetics to HaloLink resin may favor the lower molecular weight proteins, possibly due to their faster diffusion rates. The contaminating bands in the elution most likely represent partial CARM1 fragments (elut, Fig. 1B). HaloTag-CARM1 expression in other *E. coli* strains (BL21(DE3)pLysS [EMD] and KRX [Promega]) resulted in similar levels of insoluble protein and a significant number of C-terminal truncations in the elution fraction from HaloLink resin (data not shown).

In contrast to purified inclusion bodies (IBs), the eluted protein (N-term) did not blot with anti-CARM1, C-terminus – specific polyclonal antibody (*E. coli* panel, Fig. 2A). This indicates that the soluble *E. coli* – expressed protein is not full-length. The full-length CARM1 was present in inclusion bodies. The purified CARM1 was enzymatically active, and capable of dose-dependent methylation of PABP1 fragment (*E. coli* panel, Fig. 2B,C). The C-terminal truncation did not affect the enzymatic activity; this is consistent with published results describing that only the internal amino acids 150–470 comprise the catalytic core necessary for methyltransferase activity [9, 28].

In all, the yield for the purification was ~100 µg of eluted protein purified from 1 g wet weight of *E. coli*. The ability to recover small amounts of material with high specificity was due to the unique covalent linkage formed by the HaloTag. The fact that the HaloTag forms a covalent bond with the HaloLink resin allowed the HaloTag-CARM1 fusion to irreversibly bind to and to concentrate on the resin. However, the low yield, poor purity, and the presence of C-terminal truncations of soluble CARM1 expressed in *E. coli* necessitated the switch to expression in mammalian HEK293T cells.

CARM1 expression and purification in HEK293T cells

For mammalian overexpression, both N- and C-terminally tagged HaloTag-CARM1 constructs were created. The intracellular localization of HaloTag-CARM1 was not altered compared to Flag-CARM1 (compare panels I and III to V, Fig. 3A). Also, there were no noticeable localization differences between the N- and C-terminal tagged HaloTag-CARM1 proteins (compare I and III, Fig. 3A). In all cases, when tagged CARM1 was transiently transfected into CARM1-null MEF20 cells, most (80 %) of the overexpressed protein was cytoplasmic (Fig. 3A). A portion (20 %) of transfected MEF20 cells displayed either exclusively nuclear or both cytoplasmic and nuclear staining. This result is supported by the published report indicating that CARM1 can be localized in both the nucleus and the cytoplasm [29]. The protocol was designed for purification of CARM1 from the cytoplasmic fraction, the nuclear CARM1 protein was discarded in the pellet during the initial centrifugation after cell lysis.

The purification plan for HEK293T – overexpressed protein was identical to the approach used on HaloTag-CARM1 expressed in *E. coli* (Fig. 1A). Pilot expression and purification studies were performed using transient transfection mediated by Lipofectamine 2000 (data not shown). However, in order to increase the scale of purification and to substantially decrease the associated costs, adherent HEK293T cells were instead transfected with PEI. The resulting yield for the purification was 112 µg and 134 µg for the N- and C-terminal tagged HaloTag-CARM1 constructs starting with about 1×10^8 HEK293T transfected cells. The CARM1 protein was about 80 - 90 % pure, (solid arrow, elut lane, Fig. 3B). CARM1 that was purified using the C-terminal HaloTag was approximately 1 kDa larger than the N-terminally tagged material due to the residual amino acids from the TEV cleavage site. In order to track HaloTag-CARM1 expression and purification, both the HaloLink resin output (ON) and the flowthrough (FT) were assayed with the HaloTag TMR ligand (Fig. 3C). Notably a portion of HaloTag-CARM1 migrated poorly in the SDS-PAGE as evidenced by fluorescence at the top of the well (Fig. 3C). This is due to the difficulty of CARM1 to be

fully solubilized by SDS; this was similarly noted during previous attempts to express full-length CARM1 with an N-terminal His₆ tag (data not shown). Significant amounts of HaloTag-CARM1 failed to bind to the resin: about 47 % of the N-terminally tagged protein did not bind the HaloLink resin, while only 30 % of C-terminally tagged CARM1 failed to bind (ON and FT lanes, Fig. 3C). It is likely that the N-terminal HaloTag on CARM1 is sterically hindered in binding to HaloLink resin compared to the C-terminal tag. The HaloLink resin was added in excess, and it had more than enough capacity to bind all of the expressed HaloTag-CARM1 protein. The increase in incubation time of lysate with the resin, or the addition of fresh HaloLink resin to the flowthrough fraction both failed to facilitate complete recovery of HaloTag-CARM1 from the lysate (data not shown). Incomplete binding of the C-terminal tagged HaloTag-CARM1 to the resin was very reproducible but difficult to explain; it may be due to the intrinsic nature of the HaloTag-CARM1 chimera structure.

Unlike the *E. coli* – expressed soluble CARM1, the protein purified from HEK293T cells was not truncated as indicated by the positive Western blot with a C-terminus – specific antibody (Fig. 2A). In both cases, the CARM1 purified with N- and C- terminal HaloTag was capable of concentration-dependent methyltransferase activity (Fig. 2B,C). The relative methyltransferase activity of CARM1 purified with either N- or C- terminal HaloTag placement was identical (Fig. 2D). In contrast, the relative activity of the CARM1 purified from *E. coli* seems about 3–4 times higher. The relative enzymatic activity was normalized to the percent of full-length CARM1 in the assayed sample. The analysis sidestepped the likely possibility that many truncations present in the elution fraction for the *E. coli* – sourced CARM1 protein were enzymatically active (Fig. 1B, Fig. 2B). It may be very difficult to dissect the relative methyltransferase activity for all of the CARM1 truncations co-purifying with the full-length CARM1 from *E. coli*. It is also possible that post-translational modifications on the CARM1 protein from HEK293T cells may affect its enzymatic activity.

Stringent wash conditions for removal of co-purifying proteins

In order to remove the co-purifying interacting proteins, several stringent wash conditions were tested. HaloLink resin containing covalently bound HaloTag-CARM1 (from HEK293T lysate) was washed with binding buffer followed by several concentrations of denaturants: either GdnHCl, urea, or Sarkosyl. The washed resin was then incubated with binding buffer prior to elution via TEV protease cleavage (TEV protease was present in all lanes, running slightly smaller than the 30 kDa marker (Fig. 4A,B)). All of the stringent wash conditions produced a purer CARM1 elution compared to buffer-only control (solid arrow, Fig. 4A). However, with increasing concentration of the denaturant, the recovery of CARM1 from HaloLink resin diminished. Notably, GdnHCl concentrations greater than 1 M, urea concentrations greater than 4 M, and all tested Sarkosyl concentrations resulted in lower yields (Fig. 4A). This decrease in recovery may be due to changes in the structure of bound HaloTag-CARM1 after exposure to denaturant. These changes may ultimately decrease the accessibility of TEV to the cleavage site, and therefore diminish the efficiency of the cleavage. TEV protease was not removed from the cleaved CARM1 sample; it is seen as the 27 kDa band present in all lanes in Figure 4A,B (dotted arrow). The Sarkosyl-washed HaloLink resin yielded by far the cleanest CARM1 sample, although at the same time the yield was 7-fold lower (Fig. 4B). Paradoxically, when the HaloLink resin was washed with higher (>1 M) concentrations of urea, the resulting CARM1 elution contained more contaminants (Fig. 4B). The methyltransferase activity of the eluted CARM1 was determined by incubating the same volume of elution from differentially washed resins with the fragment of PABP1. The eluted CARM1 methylated the PABP1 fragment reasonably well in all cases except when the resin was washed with GdnHCl concentrations greater than

1 M (due to low CARM1 yield) and when the resin was washed with high concentrations of Sarkosyl (> 0.1%) (Fig. 4C). These concentrations of denaturants may have irreversibly damaged CARM1's structure and affected its enzymatic activity. The 0.05% Sarkosyl – washed HaloLink resin resulted in the purest CARM1 that retained most of its activity. The 1 M urea wash was the most optimal condition in that significant increase in CARM1 purity was achieved (compared to buffer washed resin, Coomassie-stained lanes in Fig. 4A) without sacrificing total yield or activity.

Affinity purification of CARM1 – associated proteins

The many contaminating proteins present at a low level in the elution fraction in Figure 3B are likely CARM1 – interacting proteins found endogenously in HEK293T cells. During the purification process, HaloTag-CARM1 is covalently coupled to the HaloLink resin. This covalent attachment allows the resin to be used as an affinity resin to purify interacting proteins. The covalent bond prevents any leaching of CARM1 from the affinity resin. The TEV protease used for cleaving of CARM1 from the HaloLink resin ensures additional specificity during elution. Only the proteins that are bound to CARM1 are eluted by the cleavage; proteins that bind nonspecifically to the resin or to the HaloTag protein are retained on the resin. Additionally, the covalent attachment of CARM1 (bait protein) allows for testing of different wash conditions to elute the non-covalently interacting target proteins (prey proteins) without concern for the potentially interfering elution of CARM1.

In order to increase the affinity of the prey proteins for bound CARM1, an active site inhibitor, sinefungin, was tested. The sinefungin is a SAM analogue that interacts with the CARM1 active site and inhibits SAM binding; therefore interfering with the methyltransferase activity of CARM1. The affinity purification experiment was designed to test the hypothesis that when CARM1's active site is inhibited by sinefungin, the CARM1 protein will bind tighter to its protein substrates and cofactors. The binding of SAM analogue to the active site in CARM1 may induce a conformational change in CARM1, thereby increasing its affinity for binding partners.

The approach for the affinity purification of CARM1 binding proteins is closely related to the CARM1 purification strategy (subset of Fig. 1A). The HaloTag-CARM1 was bound to the HaloLink resin as previously described. A HaloTag-GST protein was also bound to the HaloLink resin in order to control for nonspecific interactions with HaloTag or with the resin itself. Then each resin (HaloTag-CARM1 and HaloTag-GST) was divided into two portions and incubated with nuclear lysate from HEK293T cells containing either 100 μ M sinefungin or a buffer control. After the incubation, the resin was washed with binding buffer, followed by a stringent wash with 1 M urea (urea elution). Then the resin was eluted by TEV protease cleavage that released the attached CARM1 (66 kDa) or GST (26 kDa) and the associated interacting proteins. The vast majority of interacting proteins bound CARM1 specifically, since very few HaloTag-GST interacting proteins were visible in either the urea elution or the TEV elution (lanes 3, 7, Fig. 5). This point is further emphasized by the small number of eluted proteins seen after very sensitive silver-staining (lanes 3, 7, Fig. 5B). It is noteworthy that even though that HaloTag-GST resin was loaded with more moles of bait than the HaloTag-CARM1 resin, the nonspecific binding was still low. The presence of 100 μ M sinefungin did not affect the number of retained proteins on the HaloTag-GST resin in either the urea elution nor in the TEV elution (Fig. 5A,B).

The 100 μ M sinefungin increased both the number and the amount of CARM1-associated proteins that eluted with either urea or TEV (Fig. 5A,B). This is most apparent in the silver staining in Figure 5B. The nuclear lysate containing the target (prey) proteins was derived from AdoX-treated HEK293T cells. The AdoX treatment significantly decreased the endogenous arginine methylation allowing the *in vitro* reaction with 3 H-SAM to detect

protein substrates for CARM1 methyltransferase activity. The addition of 100 μ M sinefungin to the HEK293T nuclear lysate greatly increased the number of CARM1 – bound substrates (compare lanes 2 and 4, 6 and 8, Fig. 5C).

These binding partners may be interacting with CARM1 directly or through other scaffolding proteins like GRIP1. The GRIP1 protein, a p160 family member, is a known interacting protein that binds a diverse number of cellular proteins including other methyltransferases and transcription factors [30]. Interaction with GRIP1 is important for CARM1's transcriptional coactivation activity [5]. Previously, relatively few CARM1 – interacting proteins have been identified; therefore, the described HaloTag-CARM1 affinity approach represents a robust method not only for capture of CARM1 binding proteins but also for characterizing which binding partners are methylated by CARM1. Since sinefungin can broadly inhibit arginine methyltransferases, this approach may be very useful in characterization of protein-protein interactions that are formed by other PRMT family members.

Conclusions

Full-length mouse CARM1 was purified from both *E. coli* and mammalian HEK293T cells. Although the vast majority of the *E. coli* – expressed HaloTag-CARM1 was in inclusion bodies, the covalent binding and the high affinity of the HaloTag permitted the scavenging of trace amounts of soluble CARM1. Unfortunately, the soluble CARM1 from *E. coli* was truncated on the C-terminus by about 2–5 kDa, arguably due to activity of endogenous proteases that may degrade the relatively unstructured C-terminal region of CARM1. Protein expression and purification from transiently transfected HEK293T cells yielded more than 130 μ g of 80 – 90 % pure CARM1 from 100 million cells containing 10.5 mg of total soluble protein. The recoverable yield corresponds to an expression level of 10 – 12 million CARM1 protein molecules per cell. This procedure has been repeated more than 10 times with perfectly reproducible yields and purity; we routinely obtained ~100 μ g of purified CARM1 from cells grown on a single 15 cm dish. This approach was significantly faster and cheaper than a comparable Flag-tag based approach using baculovirus-transduced Sf9 cells. The covalent bond formed by HaloTag facilitates the use of stringent wash conditions during purification as well as the use of fluorescent ligands that enable rapid in-gel imaging of expressed proteins. To the best of our knowledge, the HaloTag-based protein purification methodology is the only one that allows good purification and recovery of target proteins expressed at low levels in either *E. coli* or mammalian cells.

The abundance of proteins co-purifying with CARM1 underscores the utility of the HaloTag for use in discovery and characterization of interacting proteins. The likelihood that a novel protein-protein interaction identified using the HaloTag approach would be nonspecific and therefore artifactual may be substantially lower than when using tags that do not form covalent interactions. The covalent bond between the bait protein and the resin allows the selection of stringent binding and washing conditions that exclude nonspecific protein-protein interaction without concern that the bait protein will leach off the resin. The affinity purification approach employed with HaloTag-CARM1 is readily generalizable to other bait proteins, allowing for effective study and discovery of novel protein-protein interactions.

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Abbreviations

AdoX	adenosine dialdehyde
CARM1	coactivator-associated arginine methyl transferase 1
DAPI	4',6-diamidino-2-phenylindole
GdnHCl	guanidine hydrochloride
IB's	inclusion bodies
PABP1	poly(A) – binding protein 1
PEI	polyethyleneimine
PRMT	protein arginine methyl transferases
SAM	S-adenosylmethionine
TMR	tetramethylrhodamine (TAMRA)

References

1. Bedford MT. Arginine methylation at a glance. *J Cell Sci.* 2007; 120:4243–4246. [PubMed: 18057026]
2. Kuhn, P.; Xu, W.; Debabrata, C. *Progress in Molecular Biology and Translational Science.* Academic Press; 2009. Protein arginine methyltransferases: Nuclear receptor coregulators and beyond; p. 299-342.
3. Bedford MT, Richard S. Arginine methylation: An emerging regulator of protein function. *Mol Cell.* 2005; 18:263–272. [PubMed: 15866169]
4. Bachand F. Protein arginine methyltransferases: from unicellular eukaryotes to humans. *Eukaryotic Cell.* 2007; 6:889–898. [PubMed: 17468392]
5. Chen D, Ma H, Hong H, Koh SS, Huang SM, Schurter BT, Aswad DW, Stallcup MR. Regulation of transcription by a protein methyltransferase. *Science.* 1999; 284:2174–2177. [PubMed: 10381882]
6. Higashimoto K, Kuhn P, Desai D, Cheng X, Xu W. Phosphorylation-mediated inactivation of coactivator-associated arginine methyltransferase 1. *Proc Natl Acad Sci U S A.* 2007; 104:12318–12323. [PubMed: 17640894]
7. Schurter RT, Koh SS, Chen D, Bunick GJ, Harp JM, Hanson BL, Henschen-Edman A, Mackay DR, Stallcup MR, Aswad DW. Methylation of histone H3 by coactivator-associated arginine methyltransferase 1. *Biochemistry.* 2001; 40:5747–5756. [PubMed: 11341840]
8. Krause CD, Yang ZH, Kim YS, Lee JH, Cook JR, Pestka S. Protein arginine methyltransferases: Evolution and assessment of their pharmacological and therapeutic potential. *Pharmacol Ther.* 2007; 113:50. [PubMed: 17005254]
9. Troffer-Charlier N, Cura V, Hassenboehler P, Moras D, Cavarelli J. Functional insights from structures of coactivator-associated arginine methyltransferase 1 domains. *EMBO J.* 2007; 26:4391–4401. [PubMed: 17882262]
10. Teyssier C, Chen D, Stallcup MR. Requirement for multiple domains of the protein arginine methyltransferase CARM1 in its transcriptional coactivator function. *J Biol Chem.* 2002; 277:46066–46072. [PubMed: 12351636]
11. Xu W, Cho H, Kadam S, Banayo EM, Anderson S, Yates JR III, Emerson BM, Evans RM. A methylation-mediator complex in hormone signaling. *Genes Dev.* 2004; 18:144–156. [PubMed: 14729568]
12. Chen D, Huang SM, Stallcup MR. Synergistic, p160 coactivator-dependent enhancement of estrogen receptor function by CARM1 and p300. *J Biol Chem.* 2000; 275:40810–40816. [PubMed: 11010967]
13. Iberg AN, Espejo A, Cheng D, Kim D, Michaud-Levesque J, Richard S, Bedford MT. Arginine methylation of the histone H3 tail impedes effector binding. *J Biol Chem.* 2008; 283:3006–3010. [PubMed: 18077460]

14. Cheng D, Côté J, Shaaban S, Bedford MT. The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing. *Mol Cell*. 2007; 25:71. [PubMed: 17218272]
15. Messaoudi S, Fabbrizio E, Rodriguez C, Chuchana P, Fauquier L, Cheng D, Theillet C, Vandel L, Bedford MT, Sardet C. Coactivator-associated arginine methyltransferase 1 (CARM1) is a positive regulator of the cyclin E1 gene. *Proc Natl Acad Sci U S A*. 2006; 103:13351–13356. [PubMed: 16938873]
16. Majumder S, Liu Y, Ford OH, Mohler JL, Whang YE. Involvement of arginine methyltransferase CARM1 in androgen receptor function and prostate cancer cell viability. *The Prostate*. 2006; 66:1292–1301. [PubMed: 16705743]
17. Sayegh J, Webb K, Cheng D, Bedford MT, Clarke SG. Regulation of protein arginine methyltransferase 8 (PRMT8) activity by its N-terminal domain. *J Biol Chem*. 2007; 282:36444–36453. [PubMed: 17925405]
18. Xu W, Chen H, Du K, Asahara H, Tini M, Emerson BM, Montminy M, Evans RM. A transcriptional switch mediated by cofactor methylation. *Science*. 2001; 294:2507–2511. [PubMed: 11701890]
19. Kuhn P, Xu Q, Cline E, Zhang D, Ge Y, Xu W. Delineating *Anopheles gambiae* coactivator associated arginine methyltransferase 1 automethylation using top-down high resolution tandem mass spectrometry. *Protein Sci*. 2009; 18:1272–1280. [PubMed: 19472346]
20. Teyssier C, Ou CY, Khetchoumian K, Losson R, Stallcup MR. Transcriptional intermediary factor 1 α mediates physical interaction and functional synergy between the coactivator-associated arginine methyltransferase 1 and glucocorticoid receptor-interacting protein 1 nuclear receptor coactivators. *Mol Endocrinol*. 2006; 20:1276–1286. [PubMed: 16322096]
21. Kuhn, P.; Chumanov, R.; Wang, Y.; Burgess, R.; Xu, W. Automethylation of CARM1 allows coupling of transcription and mRNA splicing. 2010. Submitted for publication
22. Los GV, Encell LP, McDougall MG, Hartzell DD, Karassina N, Zimprich C, Wood MG, Learish R, Ohana RF, Urh M, Simpson D, Mendez J, Zimmerman K, Otto P, Vidugiris G, Zhu J, Darzins A, Klaubert DH, Bulleit RF, Wood KV. HaloTag: A novel protein labeling technology for cell imaging and protein analysis. *ACS Chem Biol*. 2008; 3:373–382. [PubMed: 18533659]
23. Ohana RF, Encell LP, Zhao K, Simpson D, Slater MR, Urh M, Wood KV. HaloTag7: A genetically engineered tag that enhances bacterial expression of soluble proteins and improves protein purification. *Protein Expression and Purif*. 2009; 68:110–120.
24. Chumanov RS, Burgess RR. Expression, purification, and refolding of active Nrf2 transcription factor fused to protein transduction TAT tag. *Protein Expression and Purif*. 2010 In Press, Corrected Proof.
25. Wessel D, Flügge UI. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. *Anal Biochem*. 1984; 138:141–143. [PubMed: 6731838]
26. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A*. 1995; 92:7297–7301. [PubMed: 7638184]
27. Lee J, Bedford MT. PABP1 identified as an arginine methyltransferase substrate using high-density protein arrays. *EMBO Rep*. 2002; 3:268–273. [PubMed: 11850402]
28. Cheng X, Collins RE, Zhang X. Structural and sequence motifs of protein (histone) methylation enzymes. *Annu Rev Biophys Biomol Struct*. 2005; 34:267–294. [PubMed: 15869391]
29. Herrmann F, Pably P, Eckerich C, Bedford MT, Fackelmayer FO. Human protein arginine methyltransferases *in vivo* - distinct properties of eight canonical members of the PRMT family. *J Cell Sci*. 2009; 122:667–677. [PubMed: 19208762]
30. Xu J, Li Q. Review of the *in vivo* functions of the p160 steroid receptor coactivator family. *Mol Endocrinol*. 2003; 17:1681–1692. [PubMed: 12805412]

Figure 1. Purification plan and expression and purification of N-terminal HaloTag tagged CARM1 from *E. coli*

(A) Purification plan for HaloTag - CARM1 overexpressed in Rosetta 2(DE3)pLys *E. coli* strain or in transiently transfected HEK293T cells. (B) Coomassie - stained SDS-PAGE gel showing different steps in the HaloTag-CARM1 purification from a Rosetta 2(DE3)pLys pellet. (C) Fluorescent scan of the TMR ligand staining of the gel in (B). ON – onput (cleared cell lysate) to the HaloLink resin; FT – flowthrough, lysate after the overnight incubation with HaloLink resin; elut – TEV protease cleavage of the HaloLink resin to release the bound CARM1; IB's – solubilized CARM1 inclusion bodies. Solid arrow – CARM1 (66 kDa), dotted arrow – HaloTag-CARM1 (101 kDa).

Figure 2. Enzymatic activity of purified CARM1

(A) Western blot of solubilized HaloTag-CARM1 inclusion bodies (IBs), CARM1 purified from *E. coli* (N-terminal HaloTag), and purified CARM1 from HEK293T (N-terminal and C-terminal HaloTag) (B) Methylation of PABP1 fragment (1 μ g) (a known CARM1 substrate) with *E. coli* - and mammalian - purified CARM1. Coomassie stain. (C) Autoradiograph of gel in (B). (D) Relative activity of *E. coli* - and HEK293T - purified CARM1. The activity was normalized to the relative abundance (22 %) of a 60 kDa protein (corresponding to CARM1) purified from the *E. coli*, as seen in Figure 1B. Solid arrow – CARM1 (66 kDa), dotted arrow – PABP1 fragment (38 kDa).

Figure 3. Expression and purification of N- and C-terminal HaloTag – tagged CARM1 from HEK293T cells

(A) Fluorescent microscopy images of MEF20 (CARM1 null) cells transiently transfected with overexpression constructs for N-terminal or C-terminal HaloTag – tagged CARM1 proteins. I, II – N-terminal HaloTag-CARM1; III, IV – C-terminal HaloTag-CARM1; V, VI – Flag-CARM1; DAPI (blue), TMR or A546 staining (red). (B) Coomassie staining of the stages of purification. (C) Fluorescent scan of the TMR ligand staining of the gel in (B). ON – onput (cleared cell lysate) to the HaloLink resin; FT – flowthrough, lysate after the overnight incubation with HaloLink resin; elut – TEV protease cleavage of the HaloLink resin to release the bound CARM1. Solid arrow – CARM1 (66 kDa), dotted arrow – HaloTag-CARM1 (101 kDa).

Figure 4. Optimization of wash conditions for the HaloLink resin for purification of CARM1
 (A) Coomassie – stained SDS-PAGE gel of different elutions (15 μ l each) after resin washes with different concentrations of GdnHCl, urea, and Sarkosyl. (B) Silver stained gel from (A). (C) Methylation of PABP1 fragment with 1 μ l of each elution from (A). Corresponding methyltransferase activity, normalized to binding buffer wash, and compensating for purity and protein concentration. Solid arrow – CARM1 (66 kDa), dotted arrow – TEV protease (27 kDa).

Figure 5. Affinity purification of CARM1 – interacting proteins in the presence of SAM analog, sinefungin

(A) Coomassie – stained SDS-PAGE gel for the HaloTag-CARM1 and HaloTag-GST interacting proteins. 1 M urea – elution, affinity resin washed with 1 ml of 1 M urea in binding buffer for 20 min at RT, each lane contains 200 μ l of eluate precipitated with methanol-chloroform; TEV – elution, affinity resin eluted with TEV protease for 2 hrs at 22 $^{\circ}$ C, 15 μ l of eluate loaded on the gel (10 % of total volume); lane 1, HEK293T nuclear lysate from AdoX – treated cells; lanes 2,6, CARM1 affinity column; lanes 3,7, GST affinity column; lanes 4,8, CARM1 affinity column pretreated with 100 μ M sinefungin; lanes 5,9, GST affinity column pretreated with 100 μ M sinefungin. (B) Silver stained gel from (A). (C)

Autoradiograph showing the ^3H - methylation of CARM1 targets bound to the HaloTag-CARM1 affinity resin and eluted with 1 M urea and TEV protease. Solid arrow – CARM1, dotted arrow – GST.