

Research paper

Subcellular localization of the five members of the human steroid 5 α -reductase family

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Received 1 December 2016; accepted 15 March 2017

Available online 21 March 2017

Abstract

In humans the steroid 5 α -reductase (SRD5A) family comprises five integral membrane enzymes that carry out reduction of a double bond in lipidic substrates: Δ^4 -3-keto steroids, polyprenol and trans-enoyl CoA. The best-characterized reaction is the conversion of testosterone into the more potent dihydrotestosterone carried out by SRD5A1-2. Some controversy exists on their possible nuclear or endoplasmic reticulum localization.

We report the cloning and transient expression in HeLa cells of the five members of the human steroid 5 α -reductase family as both N- and C-terminus green fluorescent protein tagged protein constructs. Following the intrinsic fluorescence of the tag, we have determined that the subcellular localization of these enzymes is in the endoplasmic reticulum, upon expression in HeLa cells. The presence of the tag at either end of the polypeptide chain can affect protein expression and, in the case of trans enoyl-CoA reductase, it induces the formation of protein aggregates. © 2017 Published by Elsevier B.V. on behalf of Société Française de Biochimie et Biologie Moléculaire (SFBBM). This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

Keywords: Steroid 5 α -reductase; Polyprenol reductase; Trans-enoyl-CoA reductase; Subcellular localization

1. Introduction

In humans the steroid 5 α -reductase family (SRD5A) comprises five membrane embedded enzymes that carry out the reduction of a double bond of lipidic substrates. They can be traced back to a common ancestor that split in three subfamilies (SRD5A1-2, SRD5A3 and TECR-TECRL). Members

of the three subfamilies are found in all primary eukaryotic species from plant, amoeba and yeast up to vertebrates, indicating that the split arose in early eukaryota [1]. The duplication of an ancestral gene led to substrate diversification and acquisition of a central role in vertebrates in the context of the complex physiological roles of steroids.

SRD5A1 and SRD5A2 carry out the NADPH-dependent reduction of the Δ^4 group (double bond) of C-19 and C-21 steroids into 5 α -stereoisomers [2]. This reaction yields dihydrotestosterone, a more potent derivative of testosterone [3]. The first identified role of dihydrotestosterone is to trigger the development of external genitalia in the male fetus, as

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dramatically brought to evidence by the identification of familial incomplete male pseudo-hermaphroditism, a congenital condition due to mutations of the SRD5A2 gene [4]. Due to their activity on testosterone, both SRD5A1 and SRD5A2 can be viewed as pivotal factors in several physio-pathological conditions, from benign prostatic hyperplasia and prostatic cancer to acne and male pattern baldness [5,6]. Two commercial drugs, finasteride and dutasteride, are used in therapy for benign prostatic hyperplasia and, in the case of finasteride, also for male pattern baldness.

The action of SRD5A1 and SRD5A2 on progesterone, testosterone and other related steroids is also a committing step for the production of neurosteroids. These are among the strongest modulators of GABA(A) receptors with anticonvulsant, antidepressant and anxiolytic effects [7].

The expression of SRD5A1 and SRD5A2 was studied in several organs and tissues, with results depending on the methodology (antibody, mRNA analysis, *in situ*, after protein extraction, etc.) and on tissue preparation and origin (for a review see Ref. [8]). Expression of SRD5A1 is consistently reported in the brain, liver and sebaceous glands, whereas SRD5A2 is mainly found in the urogenital tract, genital skin, liver and transiently in the brain.

A third member, SRD5A3, has testosterone reductase activity, and seems to play a role in the onset of hormone refractory prostate cancer [9]. Surprisingly, inactivating mutations of the SRD5A3 gene cause a rare congenital neurodevelopmental disorder. This is possibly related to the fact that SRD5A3 is also endowed with polyprenol reductase activity, thus carrying out one of the earliest steps of protein N-linked glycosylation [10]. SRD5A3 was ubiquitously detected in human healthy tissues and is overexpressed in prostate and breast malignancies [11].

Trans-2-Enoyl-CoA reductase (TER or TECR) comes as a more recent addition to the SRD5A family. This gene, when mutated, causes a rare autosomic syndrome, nonsyndromic mental retardation, and the protein carries out the fourth step of very long-chain fatty acid synthesis and sphingosine degradation [12]. TECR expression seems ubiquitous in man and mouse organs by Northern blot analysis [13], with high expression in the nervous system [14] consistently with its involvement in the synthesis of membrane lipids and with congenital neurological impairment arising from mutations.

A fifth gene of unknown function, called TECR-like (TECRL), was identified in vertebrates through sequence homology with TECR [1].

During over 30 years of research on SRD5A enzymes, their purification in an active form has proven elusive. Nevertheless, biochemical studies on the microsomal fraction from liver and prostate led to the characterization of the main enzymatic parameters of SRD5A1, SRD5A2 [15] and, to a lesser extent, SRD5A3 proteins [8,16].

Sequence analysis and biochemical studies of the SRD5A family members did not highlight, to our knowledge, the presence of post-translational modifications, such as specific

proteolytic signals, glycosylation, phosphorylation, acylation or isoprenylation. Similarly, no canonical signal for a specific subcellular localization was reported.

The subcellular localization of the SRD5A enzymes is significant to their functions since it might affect binding of reaction products to nuclear steroid receptors in the same cell or diffusion to other cells. We have undertaken a systematic analysis to address their localization. We have cloned all members of the human SRD5A family in vectors for transient expression in mammalian cells, fusing at either C- or N-terminus the enhanced green fluorescent protein (eGFP). We present a comprehensive assessment of their localization as a guideline for functional analysis in cells and for production and purification for structural and biochemical studies.

2. Material and methods

2.1. Cloning and plasmids

Plasmids containing the cDNA sequence for human SRD5A1, SRD5A2, SRD5A3 and TECR were purchased from the IMAGE Consortium (www.imageconsortium.org), the sequences of the human TECR and TECRL genes were synthesized.

The five genes were cloned into the pTriEx modified vectors pNYCOMPS_RB_30_GFP and pNYCOMPS_RB_33_GFP, using the ligase independent cloning method described in Ref. [17]. These vectors are designed for mammalian, insect and bacterial protein expression and they add at the N-terminus of the protein coded by the cloned gene a cassette containing sequentially: ten histidines tag, FLAG tag, eGFP and TEV protease cleavage site at the amino terminus of the gene of interest for pNYCOMPS_RB_33_GFP. For the pNYCOMPS_RB_30_GFP the C-term is followed by: TEV protease cleavage site, eGFP, 10 histidine tag and FLAG-tag (see Fig. 1). These plasmids allow detection and expression of the cloned protein upon transient transfection of mammalian or insect cells, or expression in bacteria.

2.2. Cell culture and transfection

HEK293 and HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2% L-glutamine, and 2% penicillin/streptomycin in humidified atmosphere at 37 °C in 5% CO₂. Confluent cell layers were trypsinized, and replated on poly-L-lysine coated coverslips in DMEM medium, which was replaced by serum-free media the next day.

Transfections were performed using Lipofectamine 2000, all controls were transfected with pEGFP-N1 vector. Cells were harvested 48 h after transfection and analyzed by Western blot or immunofluorescence. The transfection rate was calculated after staining the cells on the coverslip with DAPI and counting eGFP-positive cells/DAPI-stained nuclei.

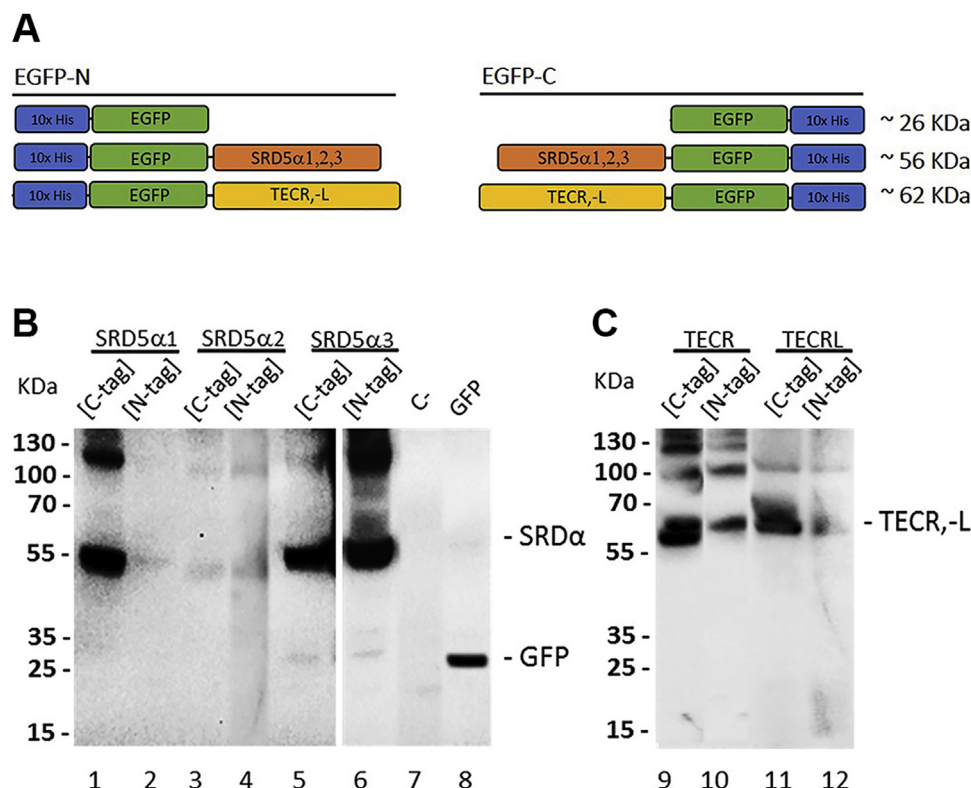


Fig. 1. **Schematic cartoon and expression of human SRD5As, TECR and TECRL eGFP-fusion proteins.** (A) Diagram of SRD5A constructs showing position of eGFP both at N- or C-term; the FLAG tag is omitted. (B) Extract from HeLa cells transfected with human SRD5A1, SRD5A2, SRD5A3 expression plasmids for C-term and N-term eGFP fusions, untransfected and eGFP-transfected cells. Different exposure times are shown (50 s for lanes 1–5, 25 s for lanes 6–8). (C) Extract from HeLa cells transfected with human TECR, TECRL (C-term and N-term eGFP fused). Proteins were visualized using anti-eGFP antibody after 10 s exposure (lanes 9–12).

2.3. Western blot

Total protein lysate was extracted with Normal Salt Buffer (20 mM Hepes, pH 7.0, 200 mM NaCl, 2 mM 2-Mercaptoethanol) supplemented with 1% β -D-Maltopyranoside and protease inhibitor cocktail (Roche). After incubation for 90 min at 4 °C, the lysates were clarified by centrifugation at 12,000 $\times g$ at 4 °C for 20 min. Protein concentration was determined by the Bradford method. Equal amounts of proteins (40 μ g) were loaded on 4–12% SDS-PAGE. Samples were electrotransferred onto nitrocellulose membranes. The membranes were blocked with 5% milk in TBS/0.1% Tween 20 (TBST) on an orbital shaker for 1 h and incubated for 1 h with rabbit monoclonal anti-GFP (1:1000, Roche). Following five TBST washes, the membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit antibody in TBST. Membranes were developed by enhanced chemiluminescence, and detected proteins were identified by their molecular sizes.

2.4. Immunofluorescence and microscopy

Cells grown on coverslips were washed in PBS and fixed at room temperature by: (a) 3.7% PFA/30 mM sucrose in PBS, 10 min, (b) 5 min of membrane permeabilization in 0.1%

TritonX-100 in PBS, and (c) 10 min in 0, 1 M Glycine. Blocking and incubations were performed in PBS/0.05% Tween 20/3% BSA. Incubation with primary antibodies was performed for 16 h at 4 °C. Secondary antibodies were incubated for 45 min at room temperature. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.1 μ g/ml) and mounted using Vectashield (Vector Laboratories).

Primary antibodies were: mouse anti-Erp57 (1:200, ab13506, Abcam), rabbit anti-Giantin (1:500, ab24586; Abcam), rabbit anti-pericentrin (2 μ g/ml, ab4448; Abcam) and mouse anti- α -tubulin (1:2000, B-5-1-2, Sigma–Aldrich). Secondary antibodies were conjugated to Cy3, Texas red, FITC or Alexa647 (Vector Laboratories). Samples were analyzed using a Nikon Eclipse 90i microscope with a Qicam Fast 1394 CCD camera (QImaging). Image acquisition, deconvolution and Extended Depth of Focus on Z-serial optical sections were performed using Nis-Elements AR4.2 (Nikon); images were further processed with Adobe Photoshop CS 8.0.

The ProteoStat[®] aggresome detection kit was used to detect protein aggregates in HeLa cells transfected for SRD5A protein expression. Samples were analyzed using an Olympus FLUOVIEW FV10i-DOC Confocal Laser Scanning Microscope, equipped with a 60 \times phase contrast water-immersion objective (N.A.1.2) and an analog detector (PMT), using a

Texas Red filter set for the ProteoStat[®] dye, and an Midoriishi-Cyan filter set for the eGFP. Image acquisition and analysis were performed using an Image Processing and Analysis in Java software (ImageJ); images were further processed with Adobe Photoshop CS 8.0.

3. Results

3.1. Protein expression

Protein expression was assessed by immunoblot on detergent-solubilized whole lysates of HeLa cells transfected with the five members of the human SRD5A family. The antibody recognized eGFP fused either at the N- or at the C-term. We observed expression for all constructs, albeit with significant quantitative variations, as high as 100-fold (Fig. 1). SRD5A2, tagged at C- or N-term, and SRD5A1 when tagged at the N-term, show faint bands by immunoblot analysis. In parallel, we observed on duplicate slides that their transfection efficiency was only between 5% and 2%, thus explaining the weak signal detected in Western blots. The not transfected control shows no reaction with the anti-GFP antibody as expected. In extract from cells transfected with the vector for eGFP expression, the antibody recognizes a single polypeptide with the correct molecular weight (~27 kDa). Intensely stained bands, of apparent molecular weight corresponding to eGFP-fused SRD5A proteins (~56 kDa), are evident for cells transfected with C-term tagged eGFP-SRD5A1 (Fig. 1; lane 1), and with SRD5A3 eGFP-tagged at either end (Fig. 1; lanes 5 & 6). On the other hand, TECR and TECRL have a higher expected molecular weight (~62 kDa) and consistently their transient expression yielded slower-migrating bands on the immunoblot (Fig. 1; lanes 9–12).

In all samples, the anti-GFP antibody also recognized higher molecular weight species, which we attribute to an oligomeric state of SRD5A membrane proteins, which were not dissociated in SDS PAGE due to their very high hydrophobic character. Indeed, no high molecular weight signals were detected in control samples expressing eGFP not fused to SRD5A proteins.

3.2. Subcellular localization of eGFP tagged SRD5A proteins

The localization of each eGFP-tagged protein was examined relative to relevant markers of the membrane compartments, i.e. the ER resident protein Erp57 and the Golgi compartment resident protein Giantin [18–20]. Due to the fact that preliminary assays depicted diversified patterns for these markers, especially Giantin, in individual cells, we first evaluated the distribution of Erp57 and Giantin in co-staining with cell cycle markers, i.e. the centrosomal protein pericentrin (to identify interphase stages: one spot, G1 phase; two mature spots, late S and G2 phases) and the microtubule constituent α -tubulin (to identify mitosis). This allowed us to exclude that Erp57 and Golgi distribution patterns undergo significant remodeling during the cell cycle

and avoid inaccurate evaluation of SRD5As co-localization (Fig. S1).

Fig. 2A shows that N-term and C-term eGFP-tagged SRD5A1, SRD5A2 and SRD5A3 proteins expressed upon transfection reside in the ER, as indicated by co-localization with Erp57 and the overall distribution of the green fluorescence relative to Erp57. No nuclear localization was observed. On the other hand, eGFP alone localizes in the nucleus (Fig. S1). These observations are consistent with the ER localization of SRD5A proteins being genuine and not attributable to the presence of the eGFP tag.

Also for TECR and TECRL members, both the N- and C-term eGFP-tagged proteins, with one exception, showed a pattern and co-localization behavior corresponding to ER membrane insertion (Fig. 2B). A remarkable exception was observed in cells expressing the C-term eGFP-tagged TECR, where only sparse bright dots were detected, moreover the localization of the ER marker Erp57 was less diffuse than in non-transfected cells (Fig. S1). In some cases, there was some limited overlap between Erp57 and C-term eGFP-tagged TECR proteins (individual z-stack scanning are given in Fig. S2). On the other hand, the lack of co-localization with Giantin for TECR and TECRL, in both the C- and N-term eGFP-tagged proteins (Fig. 3A), ruled out the possibility that they might localize to the Golgi.

Staining with ProteoStat[®] that highlights the presence of aggregates and protein aggregates [21], showed that the green fluorescent dots observed for C-term eGFP-tagged TECR form foci that are contained within larger aggregates, providing evidence consistent with the fact that the presence of eGFP at the C-term of TECR promotes protein misfolding, at least in part, resulting in aggregation (Fig. 3B).

4. Discussion

The subcellular localization of members of the SRD5A family in humans and also in mouse is controversial, especially as far as SRD5A1 and SRD5A2 are concerned. Russell and Wilson reported indications of a differential organ-specific subcellular localization, nuclear in the prostate and cytoplasmic in the liver, regardless of the enzyme variant (SRD5A1 or SRD5A2) [15,22], or that the localization for both is in the endoplasmic reticulum [23]. Early findings based mainly on cellular component fractionation [24,25], but later also on immunohistochemical studies [26,27], indicate that the SRD5A1 enzyme localization is mainly within nuclear or perinuclear membranes whereas the SRD5A2 protein is found in the cytoplasm, most likely in the ER.

Protein expression in mammalian (human HeLa and HEK293 and simian COS-M6), as well as yeast and insect cells, again yielded contradictory results. Some authors have reported that human SRD5A1 is associated with nuclear fractions upon expression in insect Sf9 cells [28,29] but later papers [30] have instead reported retrieval of enzyme activity from the microsomal fraction. Expression of SRD5A2 and SRD5A1 in HEK293 and in LNCa-9 PCa cells yielded activity in the nuclear and microsomal fractions [31,32]. Upon

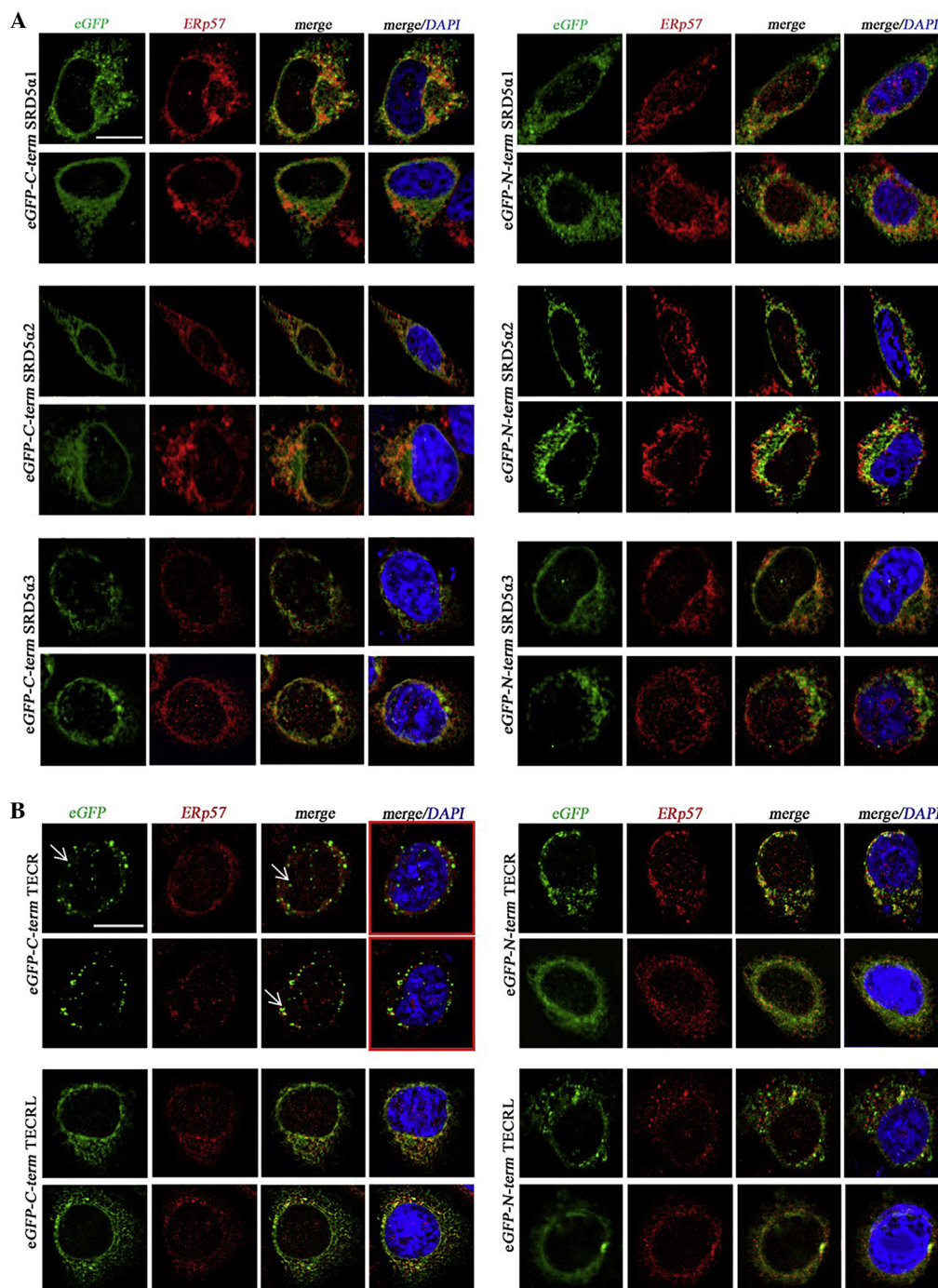


Fig. 2. Fluorescence of human SRD5As (A) and TECR, TECRL (B) proteins in relation to endogenous ERp57. SRD5A1, SRD5A2 and SRD5A3 proteins eGFP-tagged at C- or N-term (A), N-term eGFP-tagged TECR and N- and C-term eGFP-tagged TECRL proteins (B) reside in the ER, as indicated by co-localization with Erp57 and overall distribution of green fluorescence relative to Erp57. eGFP C-term TECR (B, left column) shows diffuse bright dots indicated by white arrows, with only limited overlap with Erp57. Deconvolutions plus Extended Depth of Focus (EDF) were performed on Z-stack series (13 frames-0, 6 mm each). The scanning of Z-stacks for red-framed images in B, with eGFP C-term TECR is given in [supplementary Fig. 2](#). Scale bar = 10 μ m, Magnification = 100 \times .

heterologous expression and purification in yeast, SRD5A1 appears to be associated to nuclei, whereas SRD5A2 is in the microsomal fraction [33]. Immunostaining for SRD5A2 showed presence of the enzyme in the cytoplasm throughout most regions of the brain [34]. Finally, it was reported that

glioma cells express SRD5A1 in the perinuclear space or in the cytoplasm, depending on culture conditions [35].

As far as the third isoform of testosterone α -reductase (SRD5A3, also known as polyprenol reductase), available data report recovery from the microsomal fraction [8] and transient

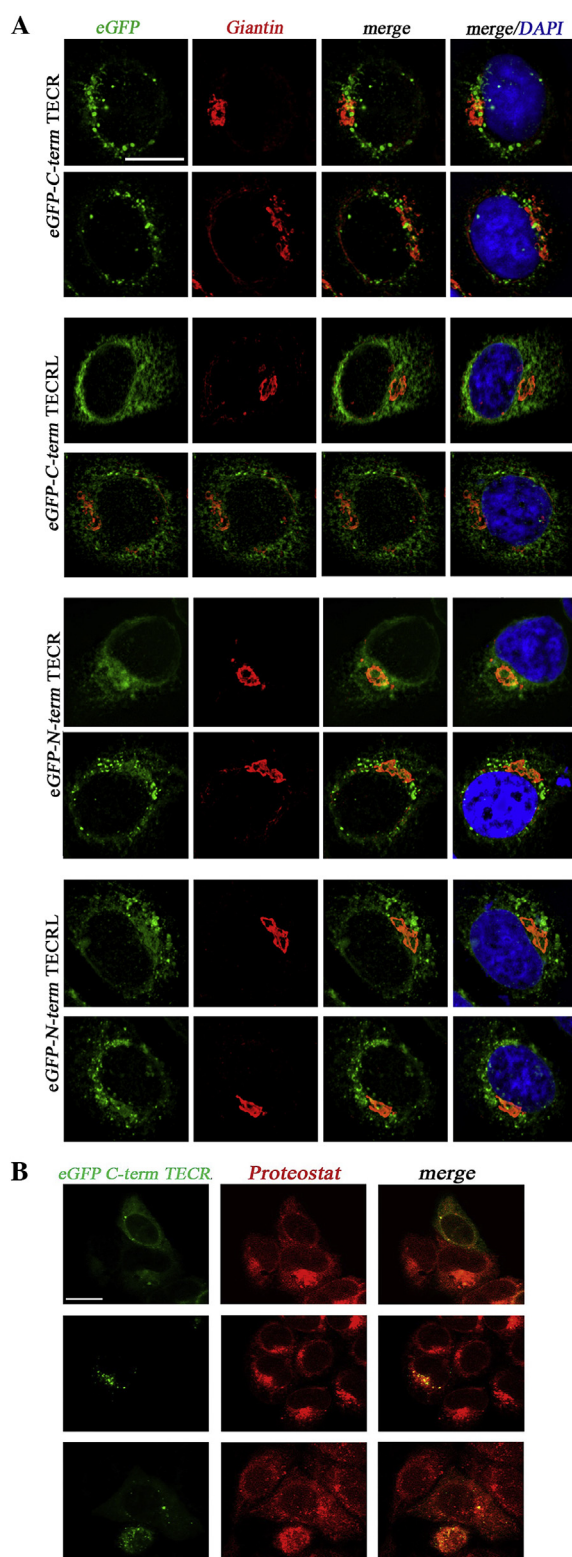


Fig. 3. Fluorescence analysis of human TECR and TECRL relative to Giantin and fluorescence of eGFP C-term TECR and aggregates/aggregates in the cytoplasm. (A) TECR proteins do not localize in the Golgi apparatus, as associated eGFP fluorescence shows a broader distribution around, but not coinciding with, the Giantin signal. Scale bar = 10 μ m, Magnification = 100 \times . (B) Localization of eGFP C-term TECR and aggregates in HeLa cells after treatment with ProteoStat[®] dye for 30 min. Fluorescence of C-term eGFP-tagged TECR partially co-localizes with aggregated proteins. Scale bar = 10 μ m, Magnification = 60 \times .

expression in HeLa cells yielded cytoplasmic localization, and no detectable activity [36]. For the TECR enzyme, immunohistochemistry has clearly suggested a localization in the ER [12]. No data is available for the TECRL that has been described only at the gene level.

SRD5A family members share significant sequence similarity, having likely evolved via duplication from an ancestral gene (sequence alignment is shown in Fig. S3). In localization studies, in order to eliminate any potential ambiguities due to antibody specificity, we directly examined each of the five human SRD5A family members by taking advantage of the fluorescent emission of the eGFP fusion tag in transfected HeLa cells. The caveat of aberrant subcellular localization due to the presence of the eGFP tag calls for careful consideration, and we therefore systematically compared the localization of C-term and N-term tagged paired constructs for each SRD5A member in parallel experiments.

The effect of a GFP tag on membrane protein localization and expression can vary, nevertheless this method is reported as a valid tool in subcellular localization studies [37–39]. The positioning on the C- or N-term of the polypeptide chain can affect protein expression and stability and its effect varies depending on the system under analysis. In an extended study on the effects on GFP tagging on subcellular localization [40] C-term tagging is reported to maintain physiological localization, whereas N-term affected it, contrarily to what we observed with human TECR.

The data we obtained with proteins fused with eGFP at the N-term indicate that all members of the SRD5A family reside in the endoplasmic reticulum, when transient expression is carried out in HeLa cells. This is also evident for protein constructs where the fusion with eGFP was at the C-term, with the exception of TECR. TECR was previously localized in the endoplasmic reticulum by immunofluorescence staining in transiently transfected HeLa cells [10]. This, and the patterns obtained here with the C-term chimera, allowed us to conclude that the presence of eGFP at the C-term affects TECR folding, leading to the accumulation of aggregated protein.

In the SRD5A proteins, N-term eGFP-tagging reduces expression for SRD5A1, TECRL and TECR, and for the latter C-term eGFP-tagging promotes aggregation. On the other hand, for SRD5A3, expression is somewhat higher in the N-term eGFP-tagged form. Thus, even within a family of proteins, the effect of fusion tags on protein expression is not predictable and has to be assessed for each member. Our results indicate that membrane protein tagging with eGFP, which is commonly used to assess protein expression by fluorescence-coupled size-exclusion chromatography (FSEC) [41], can lead to very different expression yields, depending on the tag position, and that higher expression levels, as in the case of C-term tagged TECRL with respect to the N-term tagged form, can underlie unfolding and aberrant subcellular localization.

In summary, the present studies support the conclusion that isozymes 1 and 2 of testosterone 5 α -reductase (SRD5A1 and SRD5A2) are both localized in the ER, upon transient expression in HeLa cells, our data are a valuable contribution to clarify the contradictory reports detailed above, although

any ultimate conclusion on the intracellular localization of SRD5As should be validated in other cell lines and complementary experimental settings.

Moreover, the subcellular localization in the ER for SRD5A3 is consistent with the reported microsomal sub-fractionation. Finally, we show that the previously uncharacterized TECRL gene can be expressed in human cells and that its product is a protein residing in the ER membrane.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

Supported by Regione Lazio (*ProTox*) *Prot. FILAS-RU-2014–1020* and by Sapienza Grant C26H154L5A. Support from H2CU Center to BV and GC is acknowledged. This work was also supported by NIH grants U54 GM095315 and P41 GM116799 (Hendrickson, PI) and R01 GM111980 (Mancia, PI) and by the fellowship awarded by the Italian Academy for Advanced Studies in America at Columbia University (Val-lone). We thank Dr. Valeria De Turreis for access to the Microscopy Facility of CLNS-IIT@Sapienza.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biopen.2017.03.003>.

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