

Cellular localization studies on human estrogen sulfotransferase

SULT1E1 in human embryonic kidney 293 cells

Ruchita Kapoor¹, Deepthi Nimmagadda¹ and Jonathan J. Sheng

Department of Pharmaceutical Sciences, College of Pharmacy, North Dakota State

University, Fargo, ND58105 (R.K., D.N. and J.J.S.)

RUNNING TITLE

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CORRESPONDING AUTHOR

Jonathan J. Sheng

Department of Pharmaceutical Sciences, College of Pharmacy

North Dakota State University, Fargo, ND58105

Tel.: (701) 231-6140

Fax: (701) 231-8333

E-mail: jonathan.sheng@ndsu.edu

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ABBREVIATIONS

ATCC, American Type Culture Collection; DDT, 1-chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)-ethyl]-benzene; BSA, bovine serum albumin; ER- α , estrogen receptor alpha; ER- β , estrogen receptor beta; ERR- γ , estrogen-related receptor gamma; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GFP, green fluorescent protein; HEK293, human embryonic kidney 293 cell line; PAP, adenosine 3', 5'-diphosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; PBS, phosphate-buffered saline; RT-PCR, Reverse transcription polymerase chain reaction; SULT1E1, human estrogen sulfotransferase.

ABSTRACT

Human cytosolic sulfotransferase SULT1E1 catalyzes the sulfation of endogenous estrogens as well as xenobiotic estrogen-like chemicals. This reaction increases the water solubility of the molecule which may affect its cellular distribution and biological activity. This could alter estrogen signaling to the estrogen receptor in human estrogen receptor positive cells. The current work characterized the cellular distribution of SULT1E1 in human embryonic kidney 293 (HEK293) cell line using green fluorescent protein (GFP)-tagging and immunochemistry methods. The GFP-tagged recombinant SULT1E1 protein was expressed and localized in the cytoplasm of HEK293 cells. By using a commercial anti-SULT1E1 peptide antibody, a 35.7 kDa protein was detected in HEK293 cells via Western blot. The molecular mass of the protein detected suggested that it may be related to native SULT1E1 protein. However, reverse transcription-polymerase chain reaction (RT-PCR) with gene specific primers could not confirm the presence of the SULT1E1 transcript in the total RNA sample of HEK293 cells. The discrepancy between protein and transcript data could be due to the instability of SULT1E1 mRNA or the specificity of the anti-SULT1E1 antibody utilized. In the present work, RT-PCR analysis with gene specific primers also identified a transcript fragment of human estrogen-related receptor ERR- γ . Future studies on the functional relationship between estrogen-related receptors and sulfotransferases are expected to provide additional insights into the physiological and toxicological roles of human estrogen sulfotransferases.

INTRODUCTION

Sulfoconjugation is an important biotransformation reaction for hydroxysteroid hormones including estrogenic compounds (Jakoby et al., 1980; Duffel, 1997; Falany, 1997; Weinshilboum et al., 1997; Glatt et al., 2001). Human sulfotransferase SULT1E1 is a major human hepatic estrogen sulfotransferase isoform that catalyzes the sulfation of both endogenous estrogens and xenobiotic estrogen-like chemicals including drugs (i.e., ethinyl estradiol and diethylstilbestrol) and environmental pollutants (i.e., bisphenol A and DDT)(Nishiyama et al., 2002; Schrag et al., 2004). The reaction catalyzed by SULT1E1 involves the transfer of a sulfuryl group from the donor substrate 3'-phosphoadenosine 5'-phosphosulfate (PAPS) to an acceptor substrate such as β -estradiol. The protein expression of SULT1E1 has been detected in human hepatic and non-hepatic tissues including adult and fetal kidney tissues (Miki et al., 2002). Although the catalytic specificity of SULT1E1 has been studied extensively, little is known about its regulatory role in physiological estrogen actions.

In mammals, the biological effects of estrogens are primarily attributed to the regulation of target genes through genomic mechanisms. Estrogenic molecules passively diffuse across the cell membrane and bind to the nuclear estrogen receptor. The ligand-receptor complex associates with the chromatin network and initiates target gene transcription in the cell nucleus. Besides the classic human nuclear estrogen receptors ER- α and ER- β , additional estrogen-related nuclear receptors (ERRs) such as ERR- γ encoded by different gene loci have also been reported (Giguere et al., 1988; Kuiper et al., 1996; Gustafsson, 1999; Hong et al., 1999; Giguere, 2002). These estrogen-related nuclear receptors have been located in the cytoplasm as well as the nucleus. Logically, estrogenic actions via cytosolic nuclear receptors could be mediated by human cytosolic estrogen sulfotransferases.

In the present studies, the expression and the cellular localization of human SULT1E1 in human embryonic kidney 293 (HEK293) was investigated by green fluorescent protein (GFP)-tagging, immunocytochemistry, Western blot and RT-PCR methods. The transcriptional status of human estrogen related receptor ERR- γ in HEK293 cells was also evaluated by RT-PCR and gene specific primers in the present work.

MATERIALS AND METHODS

Cell culture-HEK293 cell line was purchased from Invitrogen (Carlsbad, CA) and subcultured in Eagle's minimal essential medium supplemented with 10% fetal bovine serum (FBS) and 1% antimycotic at 37°C in a humidified atmosphere of 5% CO₂. All cell culture experiments were performed in strict aseptic conditions in a class II biological hood.

Localization of a GFP-SULT1E1 chimeric protein in HEK293 cells-To determine the subcellular location of SULT1E1 in human HEK293 cells we expressed a GFP-tagged SULT1E1 and evaluated its subcellular distribution with confocal microscopy techniques. cDNA encoding human SULT1E1 (Genebank accession number: U08098)(Aksoy et al., 1994) was isolated from human hepatic tissue by RT-PCR methods and cloned into a pcDNA3.1/NT-GFP vector (Invitrogen, Carlsbad, CA). The resulting pcDNA3.1/NT-GFP/SULT1E1 vector enabled us to approach the subcellular location of SULT1E1 in living HEK293 cells via confocal laser scanning microscopy. The constructed pcDNA3.1/NT-GFP/SULT1E1 vector was transfected into HEK293 cells on a glass cover slide using Lipofectamine™ 2000 Reagent (Invitrogen, Carlsbad, CA). After 48 hours, the cells were stained with 100 μ M Hoechst33342 for 30 min, rinsed with PBS twice for 5 min each, fixed with 4% paraformaldehyde for 30 min, and examined under an Olympus FV300 confocal laser scanning microscope with appropriate excitation/emission filter pairs.

Western blot analysis-The cultured cells were harvested by centrifugation, followed by protein extraction using the Cytobuster protein extraction reagent (Novagen, Madison, WI) according to the manufacturer's recommendations. Total cell lysate from the cultured cells was resolved by SDS-PAGE and then immunoblotted to detect the sulfotransferase SULT1E1 protein using an anti-SULT1E1 antibody (Calbiochem, La Jolla, CA). Protein concentration was determined using the RC/DC Protein Assay Kit (Bio-Rad, Hercules, CA) according to manufacturer's instructions. Prestained SDS-PAGE standards (Bio-Rad, Hercules, CA) were used to estimate the protein molecular mass. The same blot was similarly probed to detect glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm equivalent protein loading.

Immunofluorescence staining and confocal laser scanning microscopy-Monolayer cultured cells grown on a glass cover slip were stained with 100 μ M Hoechst33342 for 30 min. The stained cells were then washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were rinsed in PBS for 5 min and permeabilized in 1% Triton X-100 for 10 min. Cells were probed with an anti-SULT1E1 antibody (Calbiochem, La Jolla, CA) at 1:50 dilution with 3% BSA in PBS at 37°C for 45 min and then washed with 1% BSA and 0.02% Tween 20 in PBS for 5 min. Cells were then incubated with a goat anti-rabbit IgG fluorescein conjugate (Calbiochem, La Jolla, CA) at 1:100 dilution in 3% BSA in PBS for 45 min at room temperature. After washing in 0.02% Tween 20 in PBS for 10 min and rinsing briefly in PBS, the cells were then mounted with mounting media (90% glycerol; 3% n-propyl gallate in PBS) on a glass slide. Three controls were included in each experiment, in which we omitted the primary or secondary antibody or Hoechst33342. Samples were analyzed using an Olympus FV300 confocal laser scanning microscope with appropriate excitation/emission filter pairs.

Reverse transcription-polymerase chain reaction (RT-PCR)-Total RNA was isolated from HEK293 cells using the RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. An oligo-dT 18-mer (Sigma-Genosys, Inc., Woodlands, TX) was used for the reverse transcription step. First strand cDNAs were synthesized using AMV reverse transcriptase (Promega, Madison, WI) at 42 °C for 30 min and then subjected to PCR amplification using primers derived from human ERR- γ (Genbank accession number: NM_206594) and SULT1E1 (Genbank accession number: U08098) mRNA sequences. Two pairs of PCR primers were used for detection of the transcripts of human ERR- γ (forward primer, 5'-ATGGCCATCAGAACGGACTTGACT-3'; reverse primer, 5'-CGGTTTCAGCCACCAACAAATGTGA-3') and human SULT1E1 (forward primer, 5'-TTCCAGGCAAGACCAGATGATCT-3'; reverse primer, 5'-TTAGATCTCAGTTCGAAACTTCAGTG-3') respectively. PCR reactions were carried out with a GeneAmp PCR system 2400 (Perkin Elmer, Branchburg, NJ) using the Easy-A™ high-fidelity PCR master mix (Stratagene, La Jolla, CA). The PCR cycling parameters were as follow: 35 cycles; denaturation, 94°C for 10 sec; annealing, 50°C for 20 sec; and extension, 72°C for 1min. The PCR products were analyzed by electrophoresis with a 1% agarose gel stained with ethidium bromide.

RESULTS AND DISCUSSION

(1) *Assess the SULT1E1 expression level in human HEK293 cells using an anti-SULT1E1 peptide antibody.*

The protein expression of SULT1E1 has been detected in human hepatic and non-hepatic tissues including reproductive tissues (Song et al., 1995; Song et al., 1997). As the human fetus is believed to be exposed to a hyper estrogenic environment during pregnancy, estrogen sulfotransferase could be present in human fetal cells and may play an important role in estrogen homeostasis and signaling during fetal development. Recent studies have demonstrated that ablation of the mouse *sult1e1* gene

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causes placental thrombosis and spontaneous fetal loss (Tong et al., 2005). HEK293 cell line is a transformed cell line derived from human fetal kidney tissue. As a transformed cell line, it may have lost the original gene expression profile of the primary cells (Shaw et al., 2002). However, because of its rapid growth rate and high expression vector transfection efficiency, HEK293 cell line has been used widely to study various intracellular physiological processes. In the present work, the cellular distribution of SULT1E1 was examined using HEK293 cell line as a model cell system. The protein expression level of SULT1E1 in HEK293 cells was assessed with Western blot analysis using an anti-SULT1E1 antibody provided by Calbiochem, La Jolla, CA. The immunogen used for this antibody was a synthetic peptide corresponding amino acid 1-13 at the N-terminal of SULT1E1. With this antibody, western blot analysis detected a band of 35.7 kDa (Figure 1) and its molecular mass indicated that the native SULT1E1 is present in the cytosol of this human embryonic kidney derived cell line. To further confirm the presence of SULT1E1 in HEK293 cells, RT-PCR analysis with SULT1E1 specific primers was performed. Unexpectedly, the transcriptional level of SULT1E1 in human embryonic kidney derived cell line was beyond the limit of detection by RT-PCR methods using SULT1E1 derived primers (Lane B; Figure 3). Furthermore, β -estradiol sulfation activity of the HEK293 cell lysate was also evaluated using a published methylene blue method (Nose and Lipmann, 1958), and the results indicated that there was no discernable increase of β -estradiol sulfation activity for HEK293 cell line compared to other SULT1E1-negative cell lines such as CA-HPV-10 cell line (Data not shown). Because of the negative results from RT-PCR and catalytic activity studies, the potential instability of SULT1E1 mRNA and/or the potential cross reactivity to other sulfotransferase isoforms or other cytosolic proteins of the anti-SULT1E1 peptide antibody used in the Western blot also can not be excluded. Thus, the quantity of the native SULT1E1 protein in HEK293 cells remains inconclusive.

(2) *Subcellular localization of SULT1E1 in HEK293 cells.*

It is generally accepted that cytosolic sulfotransferases are present in the cytosol. However, previous studies have also suggested that certain sulfotransferase isoforms can be present in other subcellular compartments, such as the nucleus (Whitnall et al., 1993; He et al., 2004; Wang et al., 2004). There are significant differences in the subcellular location and intracellular distribution among different sulfotransferase isoforms, which could be related to the functional status of the enzymes in human cells.

A GFP-tagged SULT1E1 protein was localized in the cytoplasm of HEK293 cells (Panel A, Figure 2). The cytosolic location of the larger chimeric GFP-SULT1E1 protein in HEK293 cells was also confirmed by Western blot analysis on the HEK293 cell extract (Lane A; Figure 1). In addition, the immunoreactivity of HEK293 cells to the anti-SULT1E1 antibody used in the Western blot analysis was only visualized within the cytoplasm (Panel B, Figure 2) when it was examined using the immunocytochemistry and confocal laser scanning microscopy methods described in the *Methods and Materials* section.

The subcellular distribution of the SULT1E enzyme could be associated with the enzyme's functional status and the location of endogenous estrogen substrates in mammalian cells. Results from the current studies on the subcellular localization of SULT1E1 is consistent with results from several previous studies on different cytosolic sulfotransferase subfamilies (Whitnall et al., 1993; Schwartz et al., 1998; He et al., 2004; Wang et al., 2004). In the current studies, the SULT1E1 protein and the nucleus of HEK293 cells were not co-localized (Figures 2). This is in contrast to a previous report that the SULT1E homologous enzyme was not only present in the cytosol but also in the nucleus of guinea pig adrenal cells (Whitnall et al., 1993). One potential reason for this difference is that the chimeric GFP-tagged SULT1E1 protein has a much larger molecular mass.

(3) *The estrogen-related nuclear receptor gamma (ERR- γ) transcript was detected in HEK293 cells by RT-PCR analysis.*

In reproductive organs, estrogen receptors are mainly localized in the nucleus and their location is ligand dependent (Htun et al., 1999). Studies on the co-expression of SULT1E1 and estrogen nuclear receptors may provide new insights into the physiological roles of SULT1E1. Previous studies have showed that ER- α and ER- β were not detectable in HEK293 cells (Thomas et al., 2005). Results from our recent studies also indicated that the classic ER- α and ER- β proteins were below the limits of detection by Western blot methods (data not shown). However, primers derived from a human gene encoding ERR- γ were able to amplify a RT-PCR product of the predicted size (521 bp) from total RNA extracted from HEK293 cells (Lane A, Figure 3). It is not surprising that human ERR- γ transcripts are detected in HEK293 cells, as previous studies have shown that they are present in various human fetal tissues including brain, kidney, lung and liver (Eudy et al., 1998). Previous studies have also indicated that diethylstilbestrol is an active ligand of human estrogen-related receptor ERR- γ (Eudy et al., 1998). The metabolism of this synthetic estrogenic chemical is also reportedly mediated by SULT1E1 (Nishiyama et al., 2002; Schrag et al., 2004). Thus, future studies on the functional relationship between the estrogen-related receptor and sulfotransferase using HEK293 cell line as a model system would provide additional insights into the intracellular roles of human estrogen sulfotransferase in drug and xenobiotic metabolism.

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FOOTNOTES

¹ Indicating authors with equal contribution

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LEGENDS FOR FIGURES

Figure 1. Western blot analysis on SULT1E1 expression in HEK293 cells-The Western blotting was performed as described under the *Materials and Methods* section. 25 μ g of total cell lysate protein was used for Western blot analysis. Lane A, HEK293 cells transfected with the GFP-SULT1E1 vector. Lane B, control HEK293 cells (without SULT1E1-GFP transfection).

Figure 2. Cellular localization of SULT1E1 in HEK293 cells-*Panel A.* Co-visualization of the GFP-SULT1E1 fusion protein with the nucleus of HEK293 cells. HEK293 cells were transiently transfected with the pcDNA3.1/NT-GFP/SULT1E1 vector encoding a GFP-SULT1E1 fusion protein (excitation/emission wavelengths, 395/507 nm). The confocal microscopy image shows no co-localization of the recombinant GFP-SULT1E1 protein with the nucleus of HEK293 cells. Hoechst33342 (Molecular Probes, Eugene, OR) was used for co-localization with the nucleus. *Panel B.* Immunochemical localization of SULT1E1 in HEK293 cells. Cell immunoreactivity to the anti-SULT1E1 antibody used in the Western analysis (Figure 1) was shown in the cytoplasm of HEK293 cells. Co-visualization studies show no immunoreactivity in the nucleus. A goat anti-rabbit IgG fluorescein conjugate (Calbiochem, LA Jolla, CA) was used for confocal imaging analysis. Hoechst33342 (excitation/emission wavelengths, 350/461 nm; Molecular Probes, Eugene, OR) was used for co-localization of the nucleus.

Figure 3. ERR- γ mRNA expression in HEK293 cells-The total RNA sample, extracted from HEK293 cells, was amplified with the primers derived from genes encoding ERR- γ and SULT1E1 by RT-PCR methods as described in the *Methods and Materials* section. *Lane A:* A pair of ERR- γ derived primers was able to produce an RT-PCR amplicon of the predicted size (521 bp) from the first strand cDNAs of HEK293 cells. *Lane B:* no RT-PCR product was shown when a pair of SULT1E1 derived primers was used to determine the SULT1E1 mRNA expression in HEK293 cells. *Lane C*

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(Positive control). The same SULT1E1 primers used in *Lane B* were able to amplify a PCR product of 788 bp using the constructed pcDNA3.1/NT-GFP/SULT1E1 vector as template. *Lane M*, GeneRuler DNA ladder mix, Fermentas, Burlington, Ontario, Canada.

FIGURE 1

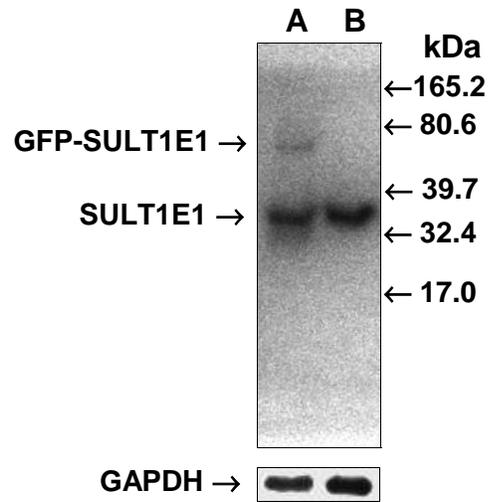
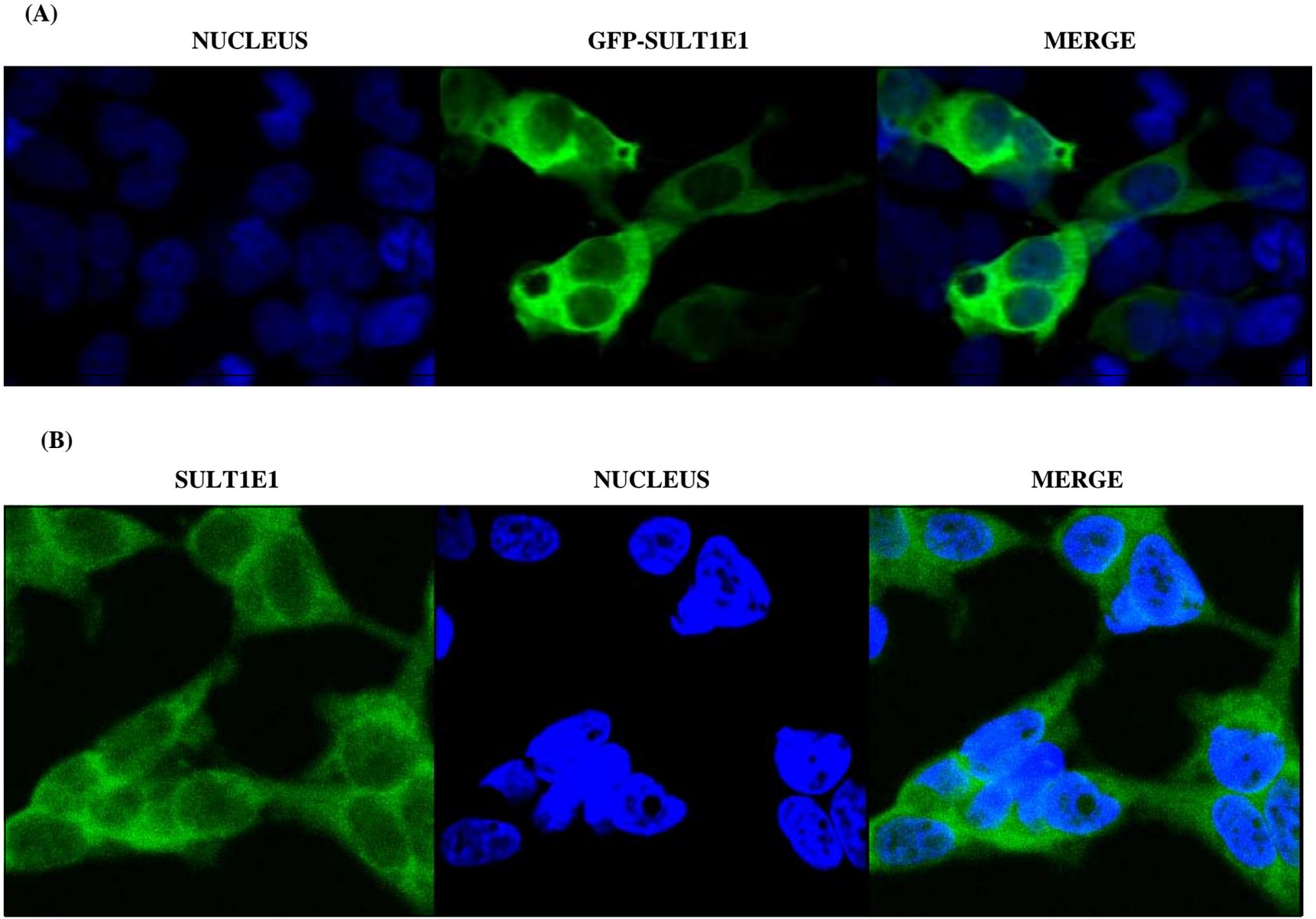


FIGURE 2



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FIGURE 3

