DSCR2, a Down syndrome critical region protein, is localized to the endoplasmic reticulum of mammalian cells

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We used immunocytochemical and fluorescence assays to investigate the subcellular location of the protein encoded by Down syndrome critical region gene 2 (DSCR2) in transfected cells. It was previously suggested that DSCR2 is located in the plasma membrane as an integral membrane protein. Interestingly, we observed this protein in the endoplasmic reticulum (ER) of cells. We also studied whether the truncated forms of DSCR2 showed different subcellular distributions. Our observations indicate that DSCR2 probably is not inserted into the membrane of the endoplasmic reticulum since the fragments lacking the predicted transmembrane (TM) helices remained associated with the ER. Our analyses suggest that, although DSCR2 is associated with the endoplasmic reticulum, it is not an integral membrane protein and it is maintained on the cytoplasmic side of the ER by indirect interaction with the ER membrane or with another protein.

Key words: Down syndrome, DSCR2, endoplasmic reticulum, immunolocalization.

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Down Syndrome (DS) is the major cause of mental retardation with an incidence of approximately 1/700 live births (Hassold et al 1984). The main features of DS include facial and dermatological features, congenital heart defects and immunological and endocrinal abnormalities. DS patients have a 20-fold increased risk of developing leukemia (Fong et al 1987; Robinson 1992; Yang et al 2002) as well as an increased risk of testicular cancer (Hasle et al 2000).

Most DS patients carry a complete trisomy of chromosome 21 (Lejeune et al 1959), but DS patients carrying a partial trisomy of chromosome 21 have also been observed. Analyses of partial trisomy of chromosome 21 have helped in determining the minimal regions potentially associated with DS features. The so called Down Syndrome Critical Region 2 (DCR-2), located between markers D21S55 and MX1 on sub-bands 21q22.2 and 21q22.3, is the determinant of several facial and dermatological features as well as mental retardation, congenital heart disease and duodenal stenosis (Rahmani et al 1986; Korenberg et al 1992; Delabar et al 1993; Korenberg et al 1994).

The DSCR2 gene is located in this region and codes for a 288 aa leucine-rich protein comprised of 34% hydrophobic amino acid residues. Little is known about the DSCR2 protein characteristics but many assumptions have been made according to in silico predictions. According to PSORT II (*http://psort.nibb.ac.jp/form2.html*), the DSCR2 protein lacks signal sequences to specific organelles and is located in the cytoplasm (69,6%) or in the mitochondria (17,4%) of cells (Vidal-Taboada et al 1998). Based on its amino acid sequence it was also suggested that DSCR2 was a plasma membrane integral protein (Vidal-Taboada et al 1998). Two topological models have been proposed to illustrate this suggestion. In the first case, the protein would have a single transmembrane domain (TM1) with the N-terminus located intracellularly. In the second

model, the protein would have both predicted TM domains with the N- and C-terminus located intracellularly (Vidal-Taboada et al 1998; Vidal-Taboada et al 2000).

Expression analyses have demonstrated that the DSCR2 gene is highly expressed in all proliferating tissues and cells, such as fetal tissues, adult testis and cancer cell lines (Vidal-Taboada et al 2000). Additionally, it was observed that DSCR2 expression is upregulated during the cell cycle proliferative phases (Vidal-Taboada et al 2000). Recently, analysis of gene expression using microarray technology demonstrated that the expression of DSCR2 is also upregulated during DS brain development (Mao et al 2003). Thus, we can suppose that the function of DSCR2 is related to cell cycle mechanisms and proliferation processes.

The function of DSCR2 remains unclear and although some of its features are well known, others are based on predictions that need to be confirmed. The association of DSCR2 gene expression with proliferation may correlate with the higher incidence of leukemia and testis tumors in DS patients. In this study, we investigate the subcellular location of DSCR2 in different cell lines. Analysis of the DSCR2 full length and truncated forms lacking putative domains were performed by immunocytochemistry and GFP based assays.

Materials and Methods

Plasmid constructs

The cDNA encoding DSCR2 was amplified from a fetal kidney cDNA panel (Clontech) using primers F2/R2 and cloned into the pGEM-T vector (Promega). PCR fragments were digested with Bgl II/Xba I (Amersham Biosciences) and inserted in-frame into p3XFLAG-myc-CMV25 (Sigma) to generate FLAG-DSCR2-myc fragments. To make GFP-tagged protein, the gene was amplified from pGEM-T using F2/R3 and F3/R3 and digested using Sac I/Bam HI and Xho I/Bam HI (Amersham Biosciences). These fragments were subcloned in-frame into pEGFP-C1 and pEGFP-N1 (Clontech), respectively to generate GFP-DSCR2 and DSCR2-GFP. The truncated form lacking TM1 ($DSCR2_{91\text{-}288}$) was cut off pGEM-T using Eco RI and Xba I and subcloned in frame into p3XFLAG-myc-CMV25. $DSCR2_{1\text{-}217}$ and $DSCR2_{91\text{-}217}$ truncated forms (lacking TM2 and lacking both TM) were amplified using primers F2/R4 and F4/R4, respectively, digested with Bgl II and Xba I and subcloned in-frame into p3XFLAG-myc-CMV25. All constructions were confirmed by DNA sequencing using Dyenamic ET Dye (Amersham Biosciences) in an automated DNA sequence analyzer ABI PRISM 377 (Applied Biosystems). Primer sequences were as follows: F2 (5′-CCGCTCGAGATCTCATGGCGGCCACGTTCTTC-3′); R2 (5′-GCTCTAGATGTATAAATGTTACTCTG-3′); F3 (5′-CGGGAGCTCATGGCGGCCACGTTCTTC-3′); R3 (5′-CGCGAATCCCGTGTATAAATGTTACTCTG-3′); F4 (5′-GAAGATCTAGGAGTCTGGG-3′); R4 (5′-GCTCTAGAAGGAAGGTCG-3′).

Cell culture and transient transfection

COS-7 (SV-40 African Green Monkey Kidney), CHO-1 (Chinese Hamster Ovary) and HEK-293 (Human Embryonic Kidney) cells were grown in a 5% CO_2 humidified atmosphere at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen), 100U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen). Cells were plated 12-24 hours prior to transfection on 35 mm culture dishes at 10^4-10^5 cells/dish containing DMEM 10% FBS. Transient transfection was performed with 2 μg of purified plasmid DNA using LipofectAMINE 2000 reagent (Invitrogen).

Expression analysis by confocal microscopy

Forty-eight hours following transfection, cells were fixed in 3,7% (w/v) paraformaldehyde (Merck) for 30 min. For immunocytochemistry, cells transfected with epitope-tagged constructs were washed in phosphate buffer saline pH 7,4 (PBS) and then permeabilized in 0.5% (v/v) Triton X-100 (Merck) in PBS for 5 min. Fixed, permeabilized cells were blocked with 1% BSA (bovine serum albumin) (Invitrogen) in PBS + 5 mg/mL donkey IgG (Jackson ImmunoResearch) for 30 min and then incubated for 1 h with murine monoclonal antibody anti-c-myc (1:50) (Sigma) against the C-terminal epitope conjugated to Cy3 and murine monoclonal antibody anti-FLAG M2 conjugated to FITC (1:100) (Sigma) against the N-terminal epitope. For ER co-localization studies, Cy3 labeled cells were incubated with goat polyclonal anti GRP-78 (glucose-regulated protein) as the primary antibody (15 mg/mL) (Santa Cruz) and donkey anti goat IgG (Fab'2) FITC conjugated antibody (1:50) (Jackson ImmunoResearch) for 1 h each.

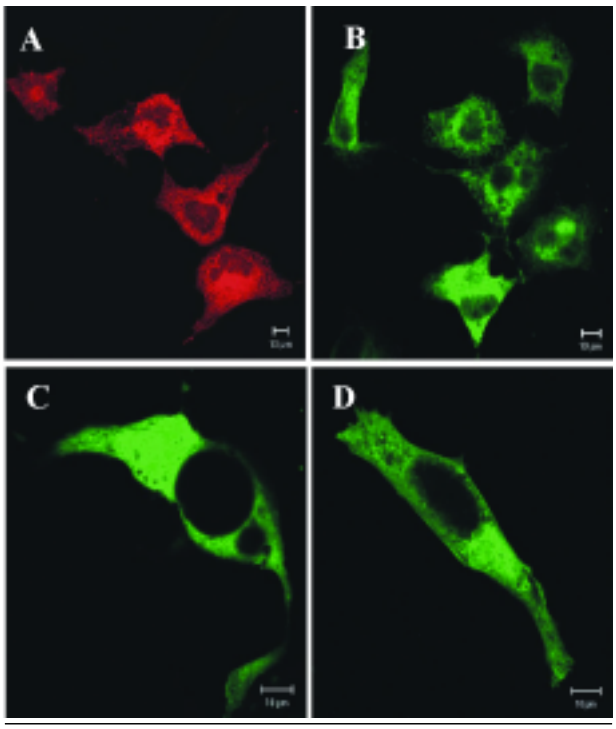

Figure 1. Confocal microscopy for subcellular localization of DSCR2. COS-7 cells transfected with the myc-DSCR2-FLAG construct were detected in the cytoplasm using antibodies against the epitopes c-myc (A) and FLAG M2 (B). GFP-DSCR2 (C) and DSCR2-GFP (D) were also visualized in the cytoplasm of cells.

pEYFP-Mito (Clontech) was used as a mitochondria marker in co-transfection experiments. Non-transfected cells were used as negative control in immunofluorescence assays. All steps were carried out at room temperature. Cells were analyzed in a ZEISS LSM 510 Laser-Scanning Confocal Microscope using LSM Image Browser R3.0 software (Zeiss).

Results and Discussion

The determination of the subcellular compartment in which a protein is located provides substantial help in designing experimental strategies. The in silico prediction of a plasma membrane location for DSCR2 has provided some ideas about the role of DSCR2, although they have not been confirmed by experimental analysis. In an attempt to investigate the subcellular location of DSCR2, we used an expression system containing short nucleotide sequences encoding c-myc and FLAG M2 epitopes appended to the 3′ and 5′ ends of the DSCR2 gene. In a different construction, the DSCR2 gene was fused to green fluorescent protein generating GFP-DSCR2 and DSCR2-GFP constructs. Both N- and C-terminals of the protein were tagged because (a) little was known about the properties of the amino acid sequence of DSCR2; (b) two putative transmembrane helices had been predicted and (c) no signal peptide had been identified. Therefore, no certainty could be attributed to the functionality of each domain and it was not possible to know which region would be important for the location of DSCR2.

DSCR2 fused constructs were used to transiently transfect COS-7, CHO-1 and HEK-293 cells. Immunocytochemical assays using antibodies against the epitope-tagged DSCR2 (anti-myc-Cy3 and anti-FLAG M2-FITC) were used to detect protein expression in cells transfected with the FLAG-DSCR2-myc construct. We expected that the protein would be localized in the peripheral region of the cells, since this is a characteristic of plasma membrane proteins and agrees with previous suggestions (Vidal-Taboada *et al.* 2000). However, the expressed FLAG-DSCR2-myc proteins were observed in the cytoplasm of transfected COS-7 cells with a more intense fluorescence in the perinuclear region, a pattern common to proteins associated with the ER (Figure 1A). No differences were observed in the pattern of immunostaining obtained with antibodies against c-myc and FLAG M2 epitopes (Figure 1B) and GFP-tagged constructs also had a similar subcellular distribution (Figure 1C, D). Similar results were observed in CHO-1 and HEK-293 cells (*data not shown*). COS-7 cells were preferentially used in further experiments since they are larger than CHO-1 and HEK-293 cells and have a distinct ER network, which facilitates the identification of ER proteins.

To rule out the possibility of a mitochondrial location, cells were co-transfected with pEYFP-MITO and FLAG-DSCR2-myc and no similarity in the subcellular distribution was observed (Figure 2). The ER distribution of DSCR2 was confirmed when transfected cells were co-stained with anti-c-myc and anti-GRP-78, a protein known to reside in the endoplasmic reticulum. Merging of the two images showed a complete superimposition of the immunostaining (Figure 2), demonstrating that both proteins co-localize in the ER.

According to Vidal-Taboada *et al.*, DSCR2 has

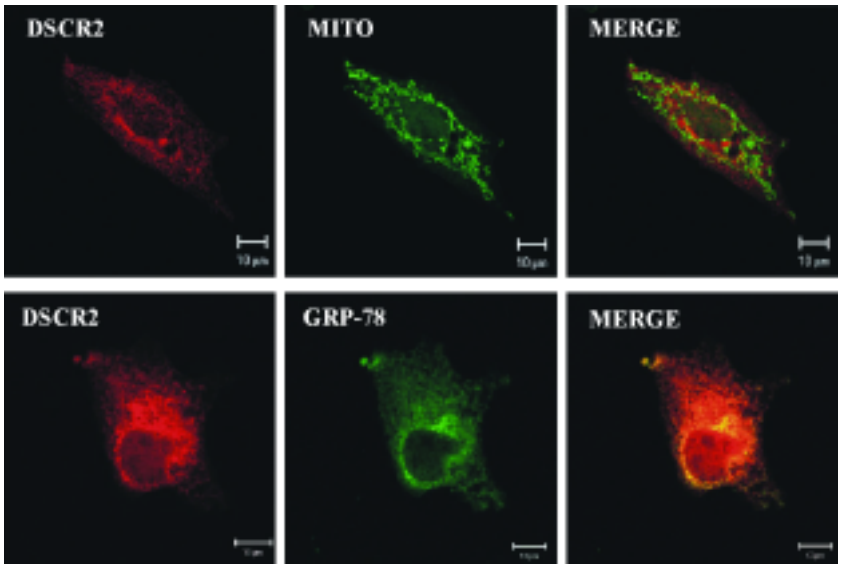

Figure 2. Co-localization analysis of DSCR2 in COS-7 cells. For mitochondria labeling, cells were co-transfected with myc-DSCR2-FLAG and pEYFP-MITO, incubated with anti-c-myc Cy3 conjugate, and the two images were merged. For endoplasmic reticulum labeling, cells transfected with myc-DSCR2-FLAG were incubated with anti-c-myc Cy3 conjugate, anti-GRP-78 primary antibody following an incubation with anti-IgG FITC conjugated secondary antibody, and the two images were overlaid.

two putative transmembrane spanning regions. However, the amino acid sequence of TM1, located between amino acids 72-91, is more likely to be a transmembrane helix, while the C-terminal one, TM2, is less likely to be a TM (Vidal-Taboada *et al.* 1998). In addition to TM domains, most ER lumenal and membrane proteins require a signal sequence to direct the synthesis and transport of the protein to the ER as well as a recognition signal responsible for the retrograde transport from the Golgi apparatus to the ER (Jackson *et al.* 1993; Pelham 1995; Martine *et al.* 1996; Teasdale *et al.* 1996; van Vliet *et al.* 2003). Although the DSCR2 amino acid sequence lacks a typical target sequence, our first experiments demonstrated that DSCR2 is localized in the endoplasmic reticulum. To characterize the region responsible for the ER localization of DSCR2, we carried out mutational analyses of the DSCR2 gene. Thus, truncated forms of the gene in fusion with c-myc and FLAG epitopes were subcloned, generating $DSCR2_{1\text{-}217}$, $DSCR2_{91\text{-}217}$, $DSCR2_{91\text{-}288}$ fragments (Figure 3). The $DSCR2_{1\text{-}217}$ fragment encodes the N-terminal portion of DSCR2, including TM1 while the $DSCR2_{91\text{-}288}$ fragment encodes the whole C-terminal of the protein, including TM2. Additionally, a third fragment comprising only the sequence between the putative TMs, $DSCR2_{91\text{-}217}$, was subcloned. The latter prevents the protein from having any signal sequence typical of ER resident proteins (Teasdale *et al.* 1996; van Vliet *et al.* 2003).

Cells were transfected with the constructions and we observed that all truncated forms displayed distribution patterns similar to that of the DSCR2 protein (Figures 3 A-D). The fragment predicted to be the region between the putative TMs (amino acids 91 to 217) was common to all constructions indicating that this sequence must play a role in the mechanism responsible for the ER distribution pattern of the DSCR2 protein. We suggest that DSCR2 is not an integral membrane protein but is somehow maintained in the ER through an indirect interaction with the ER membrane. Since no signal sequence that addresses the protein to the ER was mapped and the region between the two putative TMs was sufficient to retain the protein in the ER, we propose that DSCR2 is associated with the cytoplasmic side of the ER. The identification of a protein that interacts with DSCR2 in the ER membrane would explain how DSCR2 is associated with the ER.

In the present study, we observed the DSCR2 protein associated with the endoplasmic reticulum. However, the mechanism involved in the transport of this protein to the ER and the association of this protein with the ER differs from the mechanisms described for transmembrane proteins and our analysis suggests that DSCR2 is associated with the ER as a resident protein probably by interaction with other molecules on the cytoplasmic side of the membrane. If the DSCR2 protein is indeed an endoplasmic reticulum protein, its attributed function should be reviewed. The endoplasmic reticulum is a highly dynamic organelle responsible for many vital

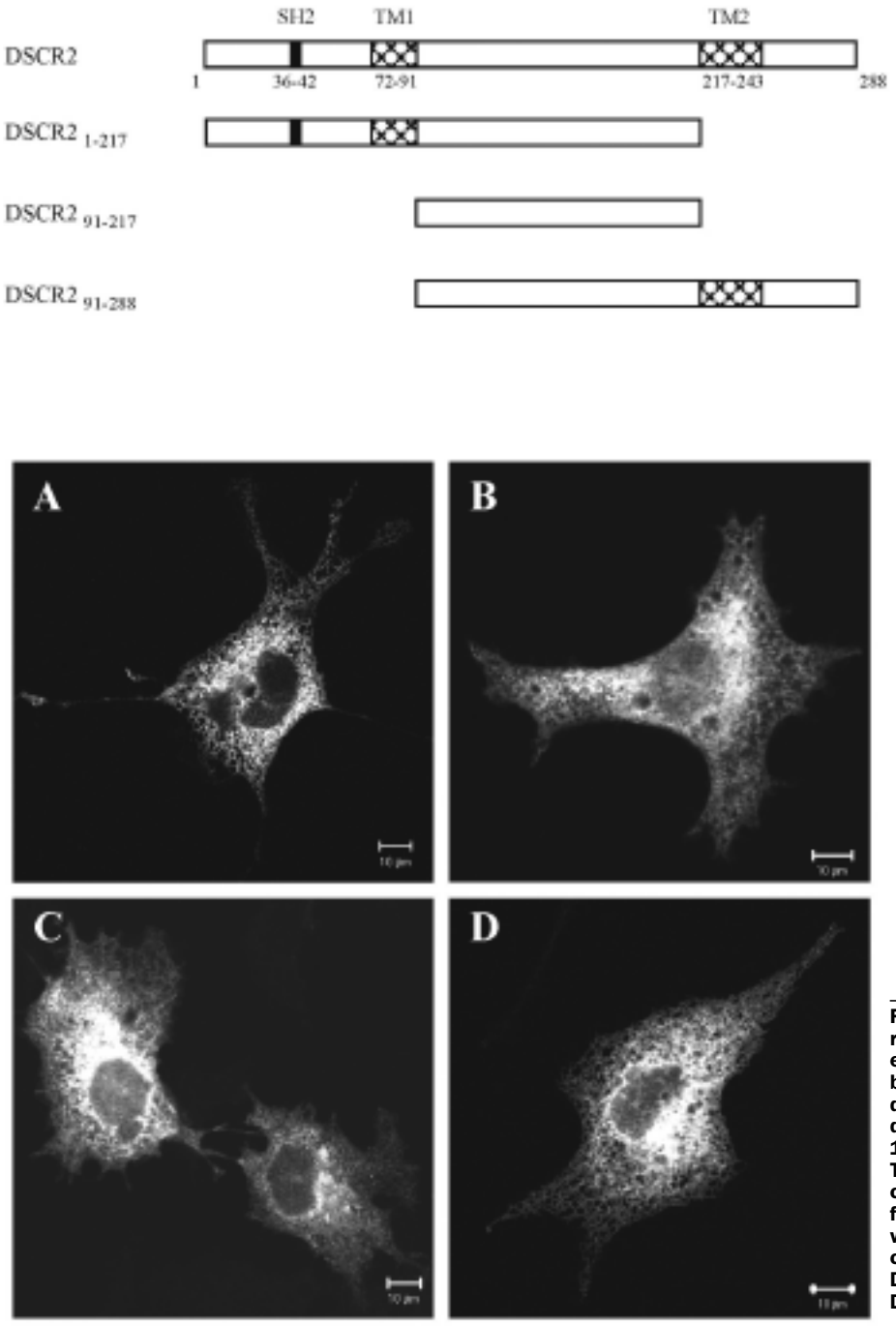

Figure 3. On the top, schematic representation of DSCR2 truncated forms. Predicted transmembrane helices (TM) and SH2 domain have been previously described (Vidal-Taboada *et al.* 1998, Vidal-Taboada *et al.* 2000). The images show immunofluorescence of DSCR2 and its truncated forms. Transfected COS-7 cells were labeled with anti-c-myc Cy3 conjugate. (A) DSCR2, (B) DSCR2$_{1\text{-}217}$, (C) DSCR2$_{91\text{-}217}$, (D) DSCR2$_{91\text{-}288}$.

cellular functions, which include synthesis and packaging of proteins (Berridge 2002). These processes involve transcription and translation factors that regulate protein synthesis and apoptosis (Cheng *et al.* 1996). It also plays a central role in many signaling processes. Earlier expression studies of DSCR2 have provided evidence that its function could be related to signaling mechanisms involved in cell cycle and proliferation processes (Vidal-Taboada *et al.* 2000). The high incidence of leukemia and testis tumors in DS patients, as well as the elevated expression of DSCR2 in HL60 (a leukemia cell line) and testis tissues (Vidal-Taboada *et al.*, 2000), combined with the results reported herein, suggest that this protein may be involved in the regulation of the cell cycle through the ER, possibly playing a role in the proliferative response that leads to leukemia and testis tumors in

DS patients. Further studies are required to investigate this protein's involvement and participation in cell cycle processes.

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