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Sub-cellular localization of suppressor of potassium transport defect 3 (SKD3) into nucleus and nuclear matrix

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Abstract: Suppressor of Potassium Transport Defect 3 (SKD3) is a mammalian homologue of *Escherichia coli* ClpB and *S. cerevisiae* Hsp104. While the exact cellular functions of SKD3 are not known, the functions of its microbial and yeast homologues have been well documented. The study presents new evidence on the localization of the protein under stress conditions like heat shock and H_2O_2 treatment. Immunofluorescence study of heat stressed cells showed that the protein was redistributed to large foci in the nucleus while cytoplasmic staining remained unaltered. Upon H_2O_2 treatment, the SKD3 protein level is increased both in the cytoplasm and nucleus. The outcome of this study reveals the fact that SKD3 may be a component of nucleus as well as nuclear scaffold which has a direct regulatory role during stress.

Keywords: SKD3, ClpB, HSP104, Nuclear matrix, Oxidative Stress, Heat shock.

Introduction

Cellular homeostasis is one of the important features of living organism. Whenever this steady state is disrupted by physiological changes such as heat shock and ionizing radiation etc., the repair/removal of damaged proteins becomes necessary for cell survival. A number of stress-induced proteins called chaperones whose molecular weight ranges from 10 KDa -110 KDa, catalyzes this function through binding the target protein and folding the protein properly or degrading the misfolded proteins by proteolytic cleavage. Genotypic mutations lead to formation of mutated polypeptides which may have altered protein folding causing a variety of genetic diseases (Thomas *et al.,* 1995). Degradation of damaged or misfolded polypeptides takes place in the lysosome, however recent reports traced such functions to proteosomal degradation pathways (Hilt & Wolf, 1996) mediated by a group of proteases, which include Caseinolytic protease (Clp) family (Gottesman, 1996). Clp family proteins are members of the large AAA+ super family and have diverse cellular functions including degradation of misfolded/aggregated proteins by its ATP dependent proteolytic function (Katayama *et al.,* 1987, 1988; Hwang *et al*., 1987, 1988), DNA repair, replication, membrane trafficking and organelle biogenesis etc. ClpB is a member of Clp family protein first identified in *Escherichia coli* (Gottesman *et al*., 1990), known for its remarkable capacity to rescue the aggregated proteins by its chaperone activity (Barnett *et al.,* 2005; Doyle *et al.,* 2007). Its homologue Hsp104 was first identified in *S. cerevisiae* (Parsell *et al*., 1991) and is well known for its chaperone activity *in vivo* and *in vitro* (Parsell *et al.,* 1994). Hsp104 is involved in the maintenance of mRNA splicing mechanism upon heat shock (Vogel *et al.,* 1995).

ClpB/Hsp104 homologues have been subsequently found in diverse organisms including mammals. Suppressor of potassium transport defect 3 (SKD3) has been isolated recently from mouse macrophage like cell line J 774.1 cDNA library (Perier *et al.,* 1995). SKD3 shares 50 and 57% amino acid homology with *Escherichia coli* ClpB and *S. cerevisiae* Hsp104 respectively. It has four ankyrin repeats that are essential for protein-protein interactions and two nucleotide binding sites (ATP binding site) at the C-terminal end suggesting that SKD3 may be involved ATP dependent proteolytic activity of misfolded and aggregated proteins.

Although SKD3 is expressed in all the mammalian tissues, it has high expression in testis particularly in the sertoli cells (Eiichi *et al*., 2001). But its localization in mammalian tissues and cultured cells was unknown. However, earlier reports in *Drosophila* tissues and some of the mammalian cells have shown Hsp proteins present in both cytoplasm and nucleus under normal conditions. Upon heat shock these proteins were highly expressed and some of these proteins are transported from cytoplasm to nucleus and nucleolus (Velazquez *et al.,*1980; Welch & Feramisco,

1984; Subjeck *et al.,* 1983). Heat shock proteins are also involved in maintenance of RNA splicing proteins (Yost & Lindquist, 1986) and are associated with hnRNP (Kloetzel & Bautz, 1983) and nuclearmatrix (Levinger & Varshavsky, 1981; Sinibaldi & Morris, 1981).

In the present investigation, focus has been made on the sub cellular localization of SKD3 in normal and stressed conditions in mammalian cells since its homologue proteins ClpB and Hsp104 have been shown to play important roles in bacteria and yeasts in response to various stresses. Accordingly, the subcellular localization of SKD3 was studied in Hela cells by immunostaining and western blot analysis. The present investigation led to the conclusion that under normal conditions this protein is present both in the cytoplasm and nucleus in a diffused pattern. Upon heat shock at 45^0C the nuclear SKD3 gets reorganized into larger foci. Upon H_2O_2 treatment the protein level increased several fold both in the cytoplasm and nucleus. Also the study revealed the property of SKD3 including resistance to detergent treatment, high salt extraction and nuclease treatment and remain insoluble protein as a part of the nuclear matrix.

Material and Methods

Recombinant expression of SKD3 and raising antibody against SKD3

Mouse SKD3 cDNA (spanning amino acid residues 219-677) was cloned in a pET 21a (Novagen) vector and transformed into BL21(DE3) plyS strain of *Escherichia coli.* Protein expression was induced by the addition of 1 mM IPTG (Rosenberg *et al.,* 1987) in midlog phase culture. A recombinant SKD3 protein molecular weight of ~50 KDa was purified from SDS-PAGE gels by passive elution method (Hager & Burgess, 1980) and the protein sequence was confirmed by N-terminal sequencing. Approximately 300 μg of protein was emulsified with complete Freund's adjuvant (Sigma) and immunized the rabbit at multiple sites subcutaneously. Three booster doses of 150 μg of purified protein were given with incomplete Freund's adjuvant (Sigma) at three week intervals. After the third booster, the animal was bled, serum was separated and stored at -20^0 C.

Mammalian Cell culture

Hela cells (Human cervical carcinoma cell line) were maintained as monolayer cultures in DMEM supplemented with 10% Fetal Calf Serum (Gibco BRL) containing antibiotics Penicillin, Streptomycin, and Kanamycin (Sigma). The cells were maintained at 37^0 C in a humidified 5% CO_2 incubator and were subcultured at a 70-80% confluence.

Heat shock and Oxidative stress treatment

Hela cells were seeded on glass cover slip and allowed to grow up to 60% confluence and either given heat shock at 45^0 C for 15 minutes followed by one hour recovery at 37^0 C or H_2O_2 treatment (600 μM) for 1 hour at 37^0 C.

Immunostaining

The cells were washed with PBS (0.01 M sodium phosphate pH 7.5, 0.15 M NaCl) and fixed in 3.7% formaldehyde for 10 minutes, followed by permeabilization in 0.5% Triton X-100 in PBS for 6 minutes, and blocked in 5% Horse serum in PBS for 1 hour. Subsequently cells were incubated with SKD3 antibody (1:25 dilution, protein A purified IgG) for one hour, followed by one hour incubation with FITC or Cy3 conjugated secondary antibodies (goat anti rabbit IgG Alexa flour 488 conjugate 1:250 dilution, molecular probes or biotinylated avidin Cy3 conjugate 1:700 dilution, Jackson laboratory). The cells were washed thoroughly with 1X PBS at the end of each incubation. For dual staining with mitotracker, the cells were incubated in incomplete medium containing mitotracker for 30 minutes and then fixed and immunostained with SKD3 antibody in dark. The cells were permeabilized in 0.1% of saponin for 10 minutes at room temperature and then Immunostained with SKD3 antibody. Samples were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA) containing 1 mg/ml DAPI.

Samples were either scanned in Zeiss LSM 510 META Confocal laser-scanning microscopy (CLSM) and the Images were analyzed using LSM 510 META software version 3.2 (Carl Zeiss, Germany) or Olympus BX60 fluorescence microscope with a cooled camera device and the images were analyzed using ImagePro software. Images were assembled in Adobe Photoshop 6.0.

Nuclear matrix preparation

Nuclear matrix was prepared from Hela cells (De Conto *et al.,* 2000). The cells were grown on a cover slip (for immunostaining) or in a 35 mM dish (for western blot) to a 70% confluence. The cells were then washed in TM buffer (50 mM Tris - HCl pH 7.5 and 3 mM $MgCl_2$) and incubated for 15 minutes on ice in TM buffer containing 0.5% Triton X-100, 0.5

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mM $CuCl_2$ and 0.2 mM PMSF (for permeablization). Subsequently the permeablized cells were rinsed thrice with TM buffer and then incubated at 37^0 C for 20 minutes in TM buffer containing 20-40 U/ml of DNase I and 20 μg/ml of RNase A (Pharmacia) (nuclease treatment). The Cells were then washed with TM buffer and incubated on ice for 5 minutes in TM buffer containing 2 M NaCl (High salt extraction). Residual NaCl was removed by washing with TM buffer and processed for immunostaining or lysed in 2X Laemmli buffer (50 mM Tris. Hcl pH 6.8, 100 mM DTT, 7% SDS, 0.1% bromophenol blue, 10% glycerol) for immunoblotting.

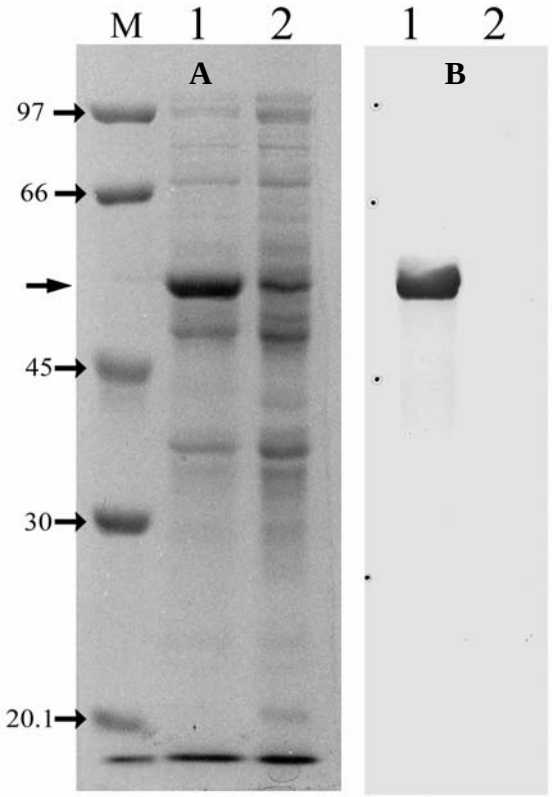
Purification of nuclear and cytoplasmic fraction

Nuclear and cytoplasmic fractions were purified from Hela (Andrews & Faller, 1991). The cells were grown in a 100 mm dish to about 80% confluence (~$5X10^6$ cells) and washed with PBS and extracted with one ml of hypotonic solution containing 10 mM KCl, 10 mM HEPES-KOH, pH 7.4, 1.5 mM $MgCl_2$ (KHM buffer), 0.5% Triton X-100, 0.5 mM DTT, 1 mM PMSF and 25μl of 25X protease inhibitor cocktail (Roche) and incubated at 4^0 C for 10 minutes. Cells were scraped and passed through 21 G needle 5 to 6 times. The sample was centrifuged at 10,000 g for 5 minutes at 4^0 C and the pellet containing nuclei and supernatant containing cytoplasm were saved separately. Equal volume of 2X Laemmli buffer was added and boiled for 5 minutes for SDS-PAGE and western blots.

Protein analysis by SDS-PAGE and Immunoblotting

Protein samples were subjected to SDS-PAGE (Laemmli, 1970), and the proteins were electroblotted onto PVDF or nitrocellulose membrane by semi-dry transfer method. Membrane containing electroblotted proteins were incubated in 5% skimmed milk powder in 1X TBST (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 0.1% Tween-20) for one hour at room temperature for blocking and incubated with the primary antibody SKD3 (1:1000 dilution, protein A purified IgG) diluted in TBST containing 0.5% milk powder for 1-2 hour at room temperature, subsequently washed and incubated with anti rabbit IgG HRP conjugate (1:4000 dilution, Amersham) secondary antibody. The blots were washed in TBST and developed using ECL kit (Roche).

Fig. 1. SKD3 recombinant protein expression



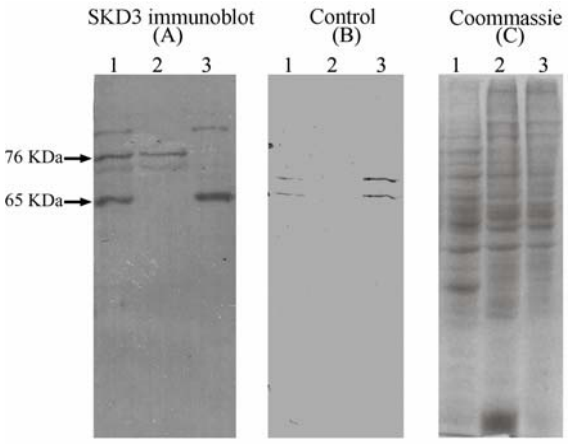
A=coomassie-blue stained gel of SKD3 recombinant protein; B= western blot gel of anti SKD3 antibody.
M=Molecular weight markers viz Phosphorylase b - 97 KDa, Bovine serum albumin-66 KDa, Ovalbumin-43 KDa, Carbonic anhydrous- 30 KDa, Soyabean trypsininihibitor - 20.1 KDa.
lane 1= IPTG induced; lane 2=uninduced; asterisk points to show the recombinant protein.

Results

Distribution of SKD3 in mammalian cells

Rabbit polyclonal antibody was raised against recombinant SKD3 protein and confirmed its specificity by western blot using recombinant protein. The antibody specifically recognized the recombinant protein of 50 KDa (Fig 1). In order to determine the specific reactivity of the antibody in the mammalian cells, the antibody was checked in western blot of Hela whole cell lysate. It was found that the antibody specifically recognizes the full length protein of 76 KDa, along with an additional specific band of 65 KDa. Further to characterize the additional band, the nuclear

Fig. 2. Localization of SKD3 in the cytoplasm and nucleus of Hela cells



(A) SKD3 Immunoblot: Hela whole cell lysate (Lane1), cytoplasmic fraction (Lane 2), and nuclear fraction (Lane 3). (B) Control western blot of Lamin A to show the purity of cytoplasmic and nuclear fraction. (C) Coomassie- blue stained gel to show equal loading

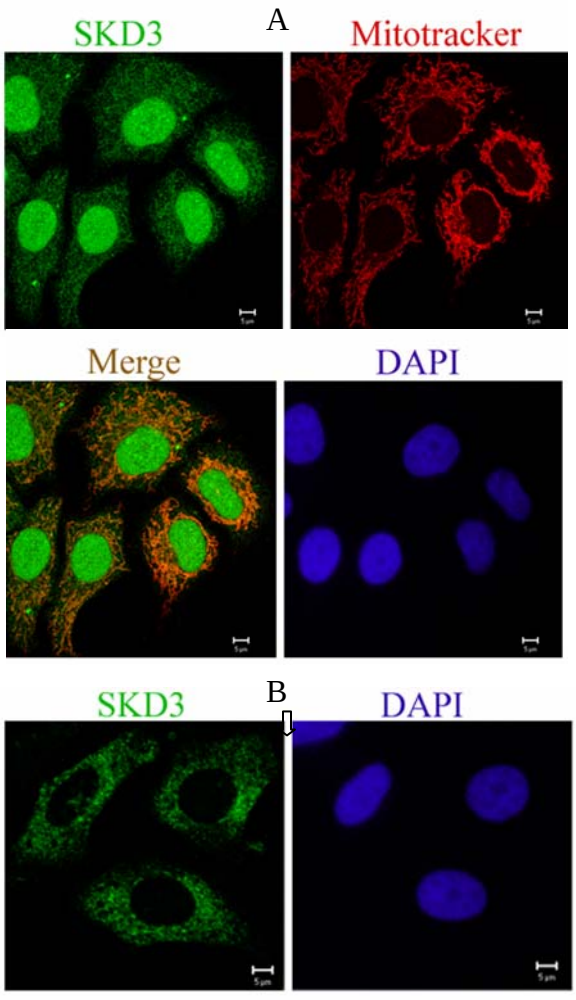
and cytoplasmic fractions from Hela cells were purified and analyzed using western blots. The western blot showed that the 76 KDa protein was found in the cytoplasmic fraction and the 65 KDa protein was exclusively found in the nuclear fraction (Fig 2A). Purity of the fractions was confirmed by a nuclear protein lamin A (Fig 2B). The resultant additional band could either be a truncated product of the full-length protein or an alternatively spliced form.

Immunostaining of normal Hela cells showed diffused staining throughout the cytoplasm and nucleus. Dual immunostaining of SKD3 antibody with mitotracker treated cells revealed the presence of SKD3 as colocalized with mitotracker within the mitochondria (Fig 3A). When the cells were permeabilized using 0.1% saponine, the antibody staining was observed only in the cytoplasm and not in the nucleus, indicating the nuclear staining is specific and not an artifact (Fig 3B).

Association of SKD3 with nuclear matrix

The immunostaining and fractionation experiment strongly suggests the presence of SKD3 both in the nucleus and cytoplasm. Earlier reports have shown some of the nuclear Hsp proteins in *Drosophila* were highly resistant to detergent , high salt and nuclease extraction and purified as an insoluble protein (Levinger & Varshavsky,1981; Sinibaldi & Morris, 1981). In order to check whether the

Fig. 3. Immunolocalization of SKD3 in Hela cells



(A) Confocal microscopic picture of SKD3 localization in the cytoplasm and nucleus, mitotracker staining to show the protein localization in the mitochondria, Merged images of SKD3 and mitotracker (B) Saponine treated cells were stained with SKD3 antibody to show the cytoplasmic staining.

nuclear SKD3 is also retained in the insoluble matrix fraction, the cells were subjected to detergent and nuclease treatment followed by high salt extraction to prepare the nuclear matrix fraction. Immunostaining of the nuclear matrix with SKD3 antibody have shown that the antibody strongly recognized the nuclear matrix (Fig 4A). In western blot analysis the SKD3 antibody recognized a 65 KDa protein as seen in an earlier nuclear fractionation experiment (Fig 4B) suggesting the presence of SKD3 in the insoluble nuclear matrix.

Redistribution of SKD3 upon stress

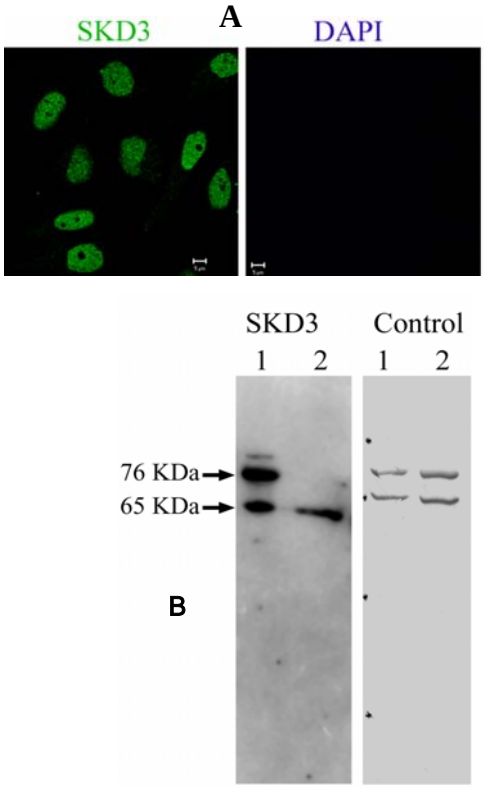
As SKD3 is a known stress response gene, its localization under stress conditions such as heat shock and H_2O_2 treatment has been of interest. Immunofluorescence study of heat stressed cells showed that the protein was redistributed to large foci in the nucleus; no changes were observed in the cytoplasmic staining. Upon H_2O_2 treatment, the SKD3 protein level increased in cytoplasm as well as in nucleus (Fig 5) suggesting its role in heat and oxidative stress.

Discussion

The present investigation demonstrates that SKD3 is localized to the cytoplasm and nucleus under normal condition in Hela cells. Similar staining pattern was observed in other mammalian cell lines like C2C12, NIH3T3 and A375 (data not shown). Colocalization of SKD3 with mitotracker suggests the presence of SKD3 in the mitochondria. The exact role of SKD3 in mitochondria is not known. However, GFP-BCL-xL transfected cells were treated with Cisplatin followed by SKD3 staining showed SKD3 colocalization with GFP-BCL-xL (Data not shown) suggesting that it may be involved in apoptotic pathway induced by DNA damage. The nuclear staining of SKD3 antibody was confirmed by immunostaining, when cells are permeablized with saponine instead of Triton X-100. Since saponine cannot permeablize the inner nuclear membrane the staining was observed only in the cytoplasm in contrast to the cells that were permeablized with Triton X-100 wherein the staining was observed both in the nucleus as well as in the cytoplasm suggesting that the SKD3 antibody staining in the nucleus was specific and not an artifact.

So far there is no concrete evidence available for the involvement of SKD3 in stress response in mammalian cells. But its homologues ClpB/Hsp104 has been thoroughly studied by many groups upon various stress conditions. During heat shock, these proteins are highly expressed and assume a chaperone like function to protect the cells from elevated stress conditions (Barnett *et al.,* 2005; Doyle *et al.,* 2007; Parsell *et al.,* 1991, 1994). Also Hsp104 play a major role in the rescue of mRNA splicing protein upon heat shock (Vogel *et al.,* 1995). We have studied the expression of SKD3 after heat shock by immunostaining and observed that SKD3 protein level did not increase either in cytoplasm or nucleus instead the nuclear protein was rearranged to larger foci or speckle like structures in the nucleus. Similar results have been reported (Welch & Feramisco, 1984; Subjeck *et al.*, 1983) in the case of mammalian heat shock proteins 72 and 110 KDa. These proteins normally localize in the cytoplasm, nucleus and nucleolus. Upon heat shock the expression of these proteins increased both in the cytoplsam and nucleus. Importantly the nuclear targeted proteins were relocalized to into larger nuclear foci, suggesting that SKD3 may play similar physiological roles as Hsp72 and 110 KDa

Fig. 4. Association of SKD3 in the nuclear matrix

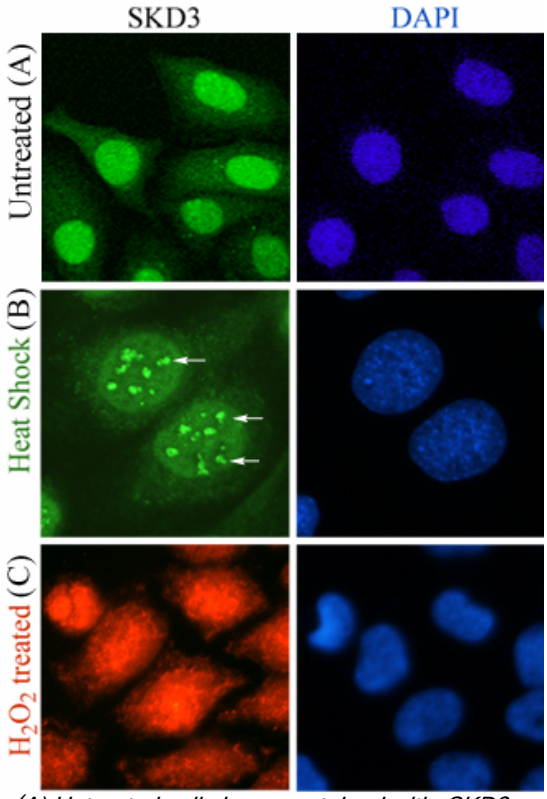


(A) Immunostaining of the nuclear matrix with SKD3 antibody. (B) Western blot of nuclear matrix: Hela whole cell lysate (Lane1), nuclear matrix (Lane 2).

under heat shock. When the cells were subjected to oxidative stress, SKD3 level increased substantially both in the cytoplasm and nucleus indicating its importance in protecting the cells from oxidative stress. The relocalization of the nuclear protein upon heat shock and its elevated expression upon H_2O_2

treatment suggest SKD3's role in the regulatory changes associated with stress response.

Fig. 5. Immunostaining of Hela cells after Heat shock and H_20_2 treatment



(A) Untreated cells Immunostained with SKD3 antibody. (B) Heat treated cells Immunostained with SKD3 antibody asterisk points to show the protein aggregation in the nucleus after heat shock. (C) H_20_2 treated cells stained with SKD3 antibody.

Western blot of cytoplsamic and nuclear fractions clearly indicates that the 76 KDa polypeptide is a full length protein of SKD3 having mitochondrial signal peptide in the N-terminal region localized exclusively in the cytoplasm and the 65 KDa protein is an isoform present in nucleus. The results strongly suggest the presence of SKD3 in the nucleus but nothing is known about its native association. To answer this question nuclear matrix were made and analyzed the protein using western blot and immunostaining. Immunofluresence study confirmed the presence of SKD3 in the nuclear matrix. Western blot result showed the 65 KDa nuclear targeted SKD3 in the nuclear matrix. Previous reports (Levinger & Varshavsky, 1981; Sinibaldi & Morris, 1981) also show some of the Hsp protein of *Drosophila* as highly resistant to nuclease and high salt treatment and extracted as insoluble protein complexes suggesting SKD3 protein as also a part of nuclear matrix.

The physiological functions of SKD3 homologues ClpB/Hsp104 have been well studied by many groups and were reported to play important roles in various cellular functions viz proteolysis, chaperone activity, maintenance of mRNA splicing etc. But little is known about the mammalian counter part SKD3. Our study provides valuable insights with respect to its subcellular localization under normal and stress conditions, although its exact molecular functions during stress are yet to be identified. We strongly believe that SKD3 is an important stress response protein that could serve as a crucial molecular chaperone and may be involved in the rescue of major cellular functions. Therefore, SKD3 could possibly help in the maintenance of cellular integrity under various conditions.

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