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The human DEVH-box protein Ski2w from the HLA is localized in nucleoli and ribosomes

Xiaodong Qu[1,2,3,4], Zhenyu Yang[1,2,3,4], Shanxiang Zhang[1,3,4,5], Liming Shen[1], Andrew W. Dangel[1], John H. Hughes[1,3,5], Kent L. Redman[7], Lai-Chu Wu[2,4,5,6] and C. Yung Yu[1,2,3,4,*]

[1]Children's Hospital Research Foundation, [2]Molecular, Cellular and Developmental Biology Program, [3]Department of Pediatrics, [4]Comprehensive Cancer Center, [5]Department of Medical Microbiology and Immunology and [6]Department of Medical Biochemistry and Department of Internal Medicine, The Ohio State University, 700 Children's Drive, Columbus, OH 43205-2696, USA and [7]Indiana University School of Medicine, Fort Wayne Center for Medical Education, Fort Wayne, IN 46805-1499, USA

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ABSTRACT

The human helicase gene *SKI2W* is located between *RD* and *RP1* in the class III region of the major histocompatibility complex. Transcripts of *SKI2W* are detectable in RNA samples isolated from multiple tissues. The protein product Ski2w shares striking amino acid sequence similarities to the yeast antiviral protein Ski2p that controls the translation of mRNAs, probably based on the mRNA structural integrity. Whether this translational regulation mechanism for cellular and viral RNAs exists in mammals is under investigation. Antisera against human Ski2w were generated using fusion proteins produced in bacteria or insect cells. Western blot analysis showed that the endogenous Ski2w protein is ~140 kDa in size and is enriched in polysomal fractions of cytoplasmic extracts from HeLa cells. Ribosomal profile studies revealed that Ski2w distributed throughout the entire sucrose gradient in the presence of Mg^{2+}, but co-sedimented with the 18S rRNA-containing 40S subunit and the small ribosomal subunit protein S27a in the presence of EDTA. The co-sedimentation of Ski2w with the 40S subunit is not affected by RNase A treatment of the cell extract, or the addition of KCl to 0.5 M, suggesting that Ski2w is associated with the 40S ribosomal subunit. Indirect immunofluorescence experiments showed that human Ski2w is localized in the nucleoli and in the cytoplasm. In essence, human Ski2w is present at the sites of ribosome biogenesis and protein synthesis.

INTRODUCTION

SKI2W is a putative RNA helicase gene located in the class III region of the major histocompatibility complex (MHC, also known as the HLA in humans) (1). Characterization of the human *SKI2W* gene and its protein product, Ski2w, is of interest because the HLA is associated with >70 diseases (2,3). Human Ski2w is structurally similar to the *Saccharomyces cerevisiae* Ski2p (1,4–7). Yeast Ski2p has been demonstrated to be an antiviral protein involved in the translational regulation of poly(A)$^-$ RNA molecules (4,5). Many RNA viruses in yeast and in humans do not contain a poly(A) tail. Yeast *ski2* mutants with RNA viruses give rise to a superkiller phenotype with increased copy number of RNA viruses or RNA replicons, and an inability to proliferate at a temperature <8°C. Shortening of poly(A) tails and decapping of mRNA are important processes for the degradation of cellular RNAs (8), which is a critical step to achieve the regulation of gene expression. Protein synthesis in eukaryotes occurs on ribosomes in the cytoplasm (9,10). The synthesis of the ribosomal RNA and the assembly of the 40S and 60S subunits of the ribosome take place in the nucleoli. Determining the subcellular localizations of human Ski2w would shed light on its function. Human Ski2w and yeast Ski2p are evolutionary related to another newly discovered group of proteins: KIAA0052 in humans (11) and Mtr4p in yeast (12,13). The yeast Mtr4p is suggested to be involved in the nucleocytoplasmic transport of mRNA.

The open reading frame of human *SKI2W* encodes for a polypeptide with 1246 amino acids. It has a predicted molecular weight of 137 kDa. This putative human Ski2w protein has a typical helicase domain with seven conserved boxes consisting of structural motifs for ATP binding and hydrolysis, and RNA binding and unwinding activities. Transcripts of *SKI2W* are detectable in a great variety of cell lines, suggesting a general cellular function or housekeeping activity of the human Ski2w protein. We hypothesize that the human Ski2w plays a role in the translational regulation, as its yeast homolog does. Here we report the generation of Ski2w specific antisera and immunochemical studies on the subcellular localizations of the endogenous Ski2w proteins. The detection of Ski2w proteins in the nucleoli,

*To whom correspondence should be addressed at: Department of Pediatrics, The Ohio State University, 700 Children's Drive, Columbus, OH 43205-2696, USA. Tel: +1 614 722 2821; Fax: +1 614 722 2774; Email: cyu@chi.osu.edu

polysomes and probably the 40S subunit of the ribosomes suggests that Ski2w proteins may be involved in the translational machinery of the cell.

MATERIALS AND METHODS

Cell cultures

Human cervical carcinoma cell line HeLa was cultured in RPMI 1640 medium (Life Technology, Grand Island, NY) supplemented with 10% heat inactivated fetal bovine serum (Atlanta Biologicals, Hyclone, Logan, UT), 2 mM L-glutamine and 50 U/ml of penicillin–streptomycin. Chinese Hamster Ovary (CHO-K) cells were maintained under the same conditions except that F-12 (HAM) medium was used.

Northern blot analysis of *SKI2W* transcripts in human tissues

Human poly(A)$^+$ RNAs from multiple tissues on the MTN blots (Clontech, Palo Alto, CA) were hybridized with a 1.7 kb 3′ cDNA probe for human *SKI2W* (1).

Generation of human *SKI2W* full length cDNA and production of Ski2w fusion proteins

The full-length, 3.9 kb human *SKI2W* cDNA was constructed. First, a 250 bp 5′ RACE product was ligated to a 339 bp *Bgl*II fragment corresponding to the 5′ region of a 3.6 kb *SKI2W* cDNA clone (1). Second, a 476 bp *Hin*dIII–*Sfi*I fragment containing 12 bp of the 5′ UTR was generated by PCR and restriction digests. This *Hin*dIII–*Sfi*I fragment replaced the 5′ region of the 3.6 kb cDNA at the unique *Sfi*I site. Third, the first 40 bp of the 5′ UTR was removed to eliminate an in-frame stop codon in order to improve the yield of Ski2w proteins.

Fusion protein MBP/W1.7 was expressed in *Escherichia coli* (strain TB1) by ligating a 1.6 kb *SKI2W* cDNA fragment to pMAL-cR1 vector (New England Biolabs, Beverly, MA). MBP/W1.7 is 90 kDa in size and contains human Ski2w amino acid residues 749–1246. Fusion protein GST/Ski2w3.6 was expressed in *Sf*9 insect cells as described previously (1). GST/Ski2w3.6 is 160 kDa in size and contains human Ski2w amino acid residues 50–1246.

Fusion protein T3.9W was produced in *E.coli* by ligating the full length *SKI2W* cDNA to a thioredoxin expression vector, pET-32 (Novagen, Madison, WI). T2.6W was produced by subcloning a 2.6 kb *Bam*HI restriction fragment of *SKI2W* cDNA into pET-32. T2.6W is 110 kDa in size and contains the entire helicase domain of Ski2w. Fusion protein T1.8W is modified from T2.6W by deleting from the pET-32/SKI2W2.6 construct an internal *Sty*I restriction fragment that codes for Ski2w residues 647–931. The helicase Box 6 and part of the Box 5 are absent in the 80 kDa fusion protein T1.8W.

Antibodies

Monoclonal antibody (mAb) against nucleophosmin, B23 (14,15), was kindly provided by Dr P. K. Chan (Houston, TX); αL7a, polyclonal antibodies against a 60S ribosomal subunit protein L7a (16), was kindly provided by Dr Andrew Ziemiecki (Berne, Switzerland); and mAb 170A1 (17) was a gift from Dr Kyuyoung Song (Seoul, Korea). Polyclonal antibodies against the small ribosomal subunit protein S27a was described by Redman and Rochsteiner (37).

Generation and purification of antisera against human Ski2w

MBP/W1.7 and GST/Ski2w3.6 were used for the generation of Ski2w antisera, αW-1.7 and αW-3.6, respectively. Since Ski2w fusion proteins are insoluble or sparingly soluble in physiological buffers, inclusion bodies of fusion proteins MBP/W1.7 and GST/Ski2w3.6 were harvested by centrifugation, solubilized in 2–4 M urea and subjected to SDS–PAGE. Gel slices containing the fusion proteins excised from polyacrylamide gels were lyophilized, ground to powder form, emulsified in physiological buffered saline (PBS), and used for immunization of rabbits and mice using Hunter's TiterMax adjuvant (CyRx), according to a standard protocol (18). Immunoglobulins (IgG) from the antisera were purified by an 'E-Z-sep' kit (Pharmacia, Piscataway, NJ) using the manufacturer's protocol. For affinity purifications of Ski2w-specific antibodies from antisera, purified proteins T2.6W and T1.8W were electroblotted onto nitrocellulose membranes and incubated with antisera αW-1.7 and αW-3.6, respectively, for 3 h at room temperature. Afterwards, the membranes were washed three times with PBS (pH 7.4). Antibodies were then eluted by incubating the membranes with 0.1 M glycine (pH 2.5) for 10 min at room temperature. The eluted antibodies were immediately neutralized with 10 M NaOH and used for immunoblot and immunofluorescence studies. The application of thioredoxin–Ski2w fusion proteins for the purification of antisera against MBP/W1.7 and GST/Ski2w3.6 would ensure the harvests of antibodies specific for Ski2w.

Transient transfection and the establishment of stable transfectants of human Ski2w in mammalian cells

The 3.9 kb human *SKI2W* full-length cDNA was subcloned into a mammalian expression vector, pEBV-His (Invitrogen, Carlsbad, CA). Ski2w was expressed as a fusion protein containing a tag sequence, i.e. an additional 49 amino acids containing the enterokinase recognition sequence and an epitope for the monoclonal antibody Anti-Xpress (Invitrogen). For the transient transfection experiments, HeLa cells were cultured on coverslips until ~50% confluent, transfected with pEBV-HIS/SKI2W using lipofectin reagent (Life Technologies) according to the manufacturer's protocol. Forty-eight hours after the transfection, cells were subjected to indirect immunofluorescence experiments using the Anti-Xpress mAb as the primary antibody. The secondary antibody used was a goat anti-mouse IgG antibody conjugated with Texas red.

Stable transfectants of human Ski2w were established by transfecting CHO-K cells with pEBV-HIS/SKI2W, using the lipofectin reagent. Transfectants were selected by supplementing the culture media with hygromycin B to 400 μg/ml, and subcloned by limiting dilutions and clonal expansions. The stable transfectant used in this report is designated as tCHO-K.

Preparation of total cell lysates and subcellular lysates from HeLa, CHO-K and tCHO-K

For preparation of total cell lysates, cells were harvested by centrifugation at 800 *g*. The cells were lysed on ice with buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl and 0.1% SDS.

The lysed cells were sonicated and spun at 14 000 r.p.m. in a microcentrifuge for 30 min at 4°C. The supernatants were stored at –80°C. Polysomes and the S-130 supernatant were prepared as described previously (19). Briefly, 2×10^8 cells (HeLa cells or tCHO-K cells) were lysed on ice with a Dounce Homogenizer in Solution A (1 mM potassium acetate, 2 mM magnesium acetate, 2 mM DTT and 10 mM Tris–acetate, pH 7.6). The nuclei were removed by centrifugation of homogenates for 10 min at 9000 r.p.m. using an SW41 rotor. The supernatants were loaded to a centrifuge tube with a Solution B cushion (Solution A supplemented with 30% sucrose), and centrifuged at 36 000 r.p.m. for 2.5 h at 4°C. The polysomal pellet (P130) was resuspended in Solution A and the supernatant was saved as a post-polysomal supernatant (S-130).

Ribosomal profiles of cytoplasmic lysates with intact and dissociated ribosomal subunits by sucrose gradient centrifugation and fractionation

A total of 2×10^7 HeLa cells or tCHO-K cells were incubated with cycloheximide (100 µg/ml; Sigma) for 15 min prior to the lysis with buffer containing 20 mM Tris (pH 7.4), 150 mM KCl, 30 mM $MgCl_2$, 1 mM DTT and 0.5% NP-40. Nuclei were removed and the cytoplasmic extracts were obtained after centrifugation at 10 000 r.p.m. for 15 min. Forty-three (OD_{260}) units of cytoplasmic extracts containing proteins and RNAs were loaded onto a linear (15–45%) sucrose gradient and centrifuged for 2 h at 36 000 r.p.m. with a Beckman SW-41 rotor. After centrifugation, the sucrose gradient was fractionated and its absorbance at 254 nm was determined continuously by an Isco UA-5 UV monitor. Proteins in the fractionates were precipitated with isopropyl alcohol. The protein pellets were resuspended in equal volumes of protein sample buffer for immunoblot analyses. RNA was purified from each fraction after phenol extraction and ethanol precipitation, and resolved by agarose gel electrophoresis.

Profiles with dissociated ribosomal subunits were performed by similar procedures except that 30 mM $MgCl_2$ in the lysis buffer for preparation of cytoplasmic lysates was substituted with 30 mM EDTA. Ribosomal subunit studies were performed using purified polysomal fractions, which were subjected to dissociations in the presence of 30 mM EDTA, and separated by centrifugation for 5 h at 38 000 r.p.m. with an SW41 rotor through a 5–35% sucrose gradient.

Profiles with cytoplasmic extracts pretreated with RNase A were performed by adding RNase A to the cytoplasmic extracts to a final concentration of 100 µg/ml. The mixture was incubated on ice for 15 min before loading to the sucrose gradient.

Profiles with cytoplasmic extracts pretreated with KCl were performed similarly by adjusting the final concentration of KCl to 0.5 M.

SDS–PAGE and immunoblot analysis

Proteins were separated by 4–12% Bis–Tris, precast, polyacrylamide gradient gels (Novex, San Diego, CA) and transferred onto nitrocellulose membranes (BioRad, Hercules, CA) by electroblotting in transfer buffer (48 mM Tris, 39 mM glycine, 10% methanol, 0.04% SDS) at 25 V for 2 h in a minigel transfer apparatus (Novex). The membranes were blocked from non-specific interactions by incubation with a solution containing 4% non-fat milk and 0.2% Tween 20 in PBS (pH 7.4) overnight at 4°C. The membranes were incubated with different antibodies separately (αW-1.7, αW-3.6, mAb 170A1, mAb Anti-Xpress, αL7a, αS27a and mAb1C3) for 2 h at room temperature. For color development (Fig. 3), antigen–antibody complexes were detected using an appropriate secondary antibody conjugated with alkaline phosphatase in conjunction with color developing reagents (BioRad). For chemiluminescence (Figs 4–7), antigen–antibody complexes were detected by horseradish peroxidase (HRP) labeled secondary antibodies and ECL-Plus reagents, according to the manufacturer's instructions (Amersham, Piscataway, NJ).

Indirect single and double immunofluorescence microscopy

HeLa cells were cultured on coverslips until ~70% confluent, supplemented with fresh medium and incubated for an additional 3 h. Afterwards, cells were rinsed with cold PBS, fixed with 2% paraformaldehyde (in PBS) for 20 min at room temperature and permeated with cold acetone at –20°C for 3 min. For single immunofluorescence staining experiments, cells were incubated with primary antibodies αW-1.7, αW-3.6 or monoclonal (mAb) B-23, separately, at 4°C, overnight. Cells were rinsed with PBS, incubated with secondary antibodies (i.e. goat anti-rabbit IgG coupled with FITC for αW-1.7 and for αW-3.6, or goat anti-mouse IgG conjugated with Texas Red for B-23), for 1 h at 37°C, followed by three washes with PBS. For double immunofluorescence staining experiments, cells were incubated with either αW-1.7 or αW-3.6, and with mAb B-23. After washing with PBS to remove free antibodies, cells were incubated with the appropriate secondary antibodies as described above. Fluorescence was observed with a UV microscope with appropriate fluorochrome-specific filters.

RESULTS

Human SKI2W transcripts are detectable in RNA samples isolated from multiple tissues

It has been shown that human *SKI2W* transcripts of ~4.0 kb in size were detectable in RNA samples isolated from cancer cell lines of colon (HT29), neuroblastoma (IMR32) and lymphoid origins (1). Here it is further demonstrated that *SKI2W* transcripts were expressed in RNA samples isolated from every tissue tested, which included spleen, thymus, prostate, testis, ovary, small intestine, colon, peripheral blood leukocytes, heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (Fig. 1). This observation indicates that *SKI2W* is expressed ubiquitously in most if not all human tissues. In addition to the 4.0 kb transcript, slightly larger transcripts ~4.2 kb in size were present in the thymus, ovary, small intestine, colon, brain, placenta, liver, kidney and pancreas.

Production of fusion proteins for human Ski2w

Five fusion proteins for human Ski2w were produced in bacteria or in *Sf9* insect cells using three different types of expression vectors (1; S.X.Zhang and C.Y.Yu, manuscript in preparation). Details of each fusion protein are shown in Figure 2I.

GST/Ski2w3.6, a 160 kDa glutathione *S*-transferase/Ski2w fusion protein, was expressed in *Sf9* cells. It contains the almost full-length Ski2w protein (i.e. residues 50–1246). MBP/W1.7, a 90 kDa maltose binding protein fusion protein, was expressed in *E.coli*. It contains the C-terminal region of Ski2w (residues

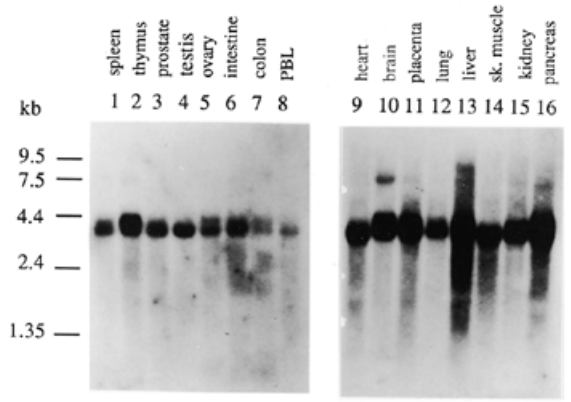
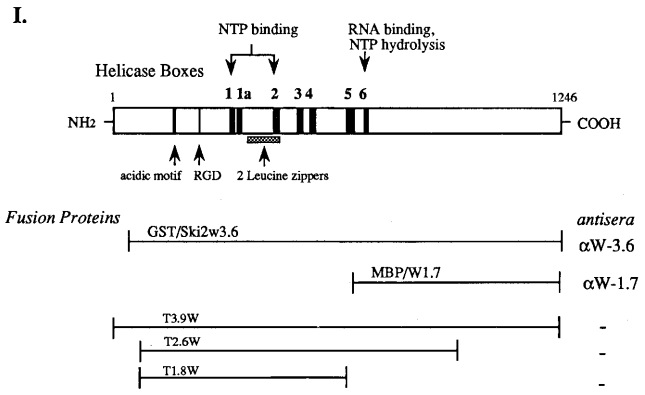


Figure 1. Northern blot analysis of *SKI2W* transcripts in human tissues. Human poly(A)$^+$ RNAs from multiple tissues on the MTN blots (Clontech) were hybridized with a 1.7 kb 3′ cDNA probe for *SKI2W.*



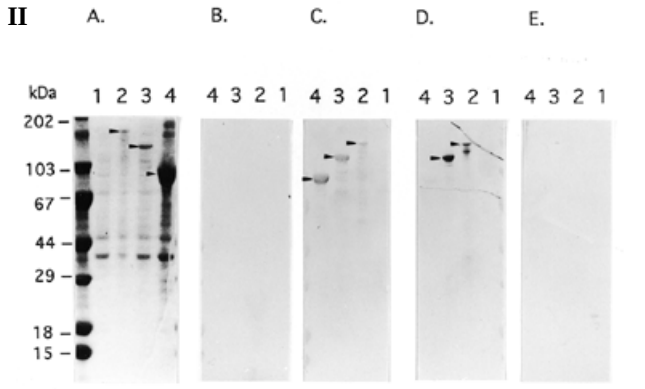


Figure 2. Fusion proteins and antisera of human Ski2w. (**I**) Structural motifs, fusion proteins and antisera of Ski2w. (**II**) Immunoblot analysis of Ski2w fusion proteins: (A) Coomassie blue staining of bacterial lysates after SDS–PAGE; (B–E**)**, immunoblot analysis of the bacterial lysates using (B) preimmune sera for αW-3.6, (C) αW-3.6, (D) αW1.7 and (E) preimmune sera for αW-1.7. For all panels: lanes 1, uninduced total bacterial proteins; 2, induced bacterial proteins containing a full length *SKI2W* cDNA construct, T3.9W; 3, induced bacterial proteins containing a partial *SKI2W* cDNA construct, T2.6 W; and 4, induced bacterial proteins containing partial *SKI2W* cDNA construct, T1.8W.

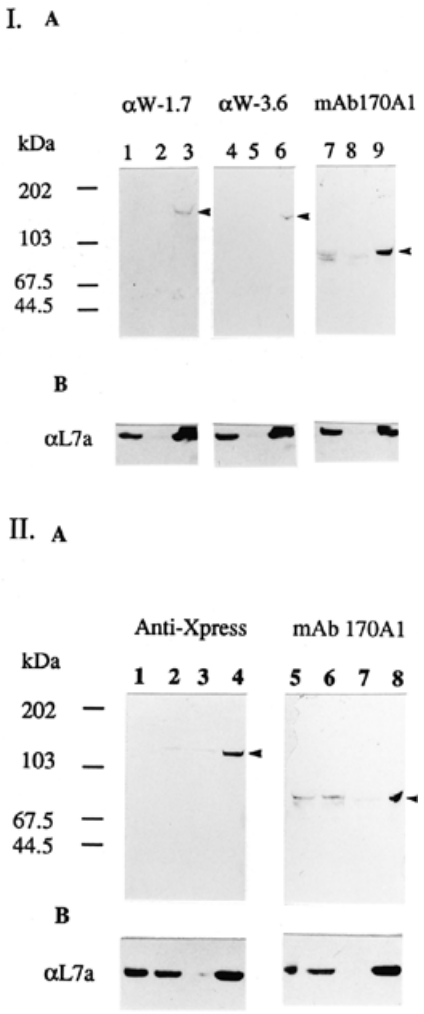


Figure 3. Immunoblot analysis of HeLa cell extracts and CHO-K cell lysates. (**I**) Lanes 1, 4 and 7 are total HeLa cell extracts. Lanes 2, 5 and 8 are S130 fractions. Lanes 3, 6 and 9 are P130 fractions. (**II**) Lanes 1 and 5 are untransfected CHO-K total cell lysates. Lanes 2 and 6 are total cell lysates from transfected CHO-K cells with PEBV His/ SKI2W 3.9. Lanes 3 and 7 are transfected, S-130 fractions. Lanes 4 and 8 are transfected P130 fractions. Samples were resolved by SDS–PAGE, transferred onto nitrocellulose membranes and probed with primary antibodies as indicated. Alkaline phosphatase conjugated secondary antibodies were used. The antibody–antigen complexes were visualized by color development.

749–1246). Polyclonal antibodies against GST/Ski2w3.6 and MBP/W1.7 were generated from rabbits and designated as αW-3.6 and αW-1.7, respectively.

The other three Ski2w fusion proteins generated for this study contained thioredoxin (Trx) as the fusion partners. T3.9W contains the full length Ski2w protein sequence. T2.6W contains amino acid residues 86–957 of Ski2w that includes the entire helicase domain. T1.8W is a derivative of T2.6W that contains a deletion from amino acid residues 647–931 of Ski2w. These three Trx fusion proteins were used to evaluate the specificities of αW-1.7 and αW-3.6. Figure 2II (panel A) shows a Coomassie blue stained SDS–PAGE gel with total bacterial proteins from uninduced bacteria with T3.9W (lane 1), induced bacteria containing T3.9W (lane 2), T2.6W (lane 3) and T1.8W (lane 4). It is apparent

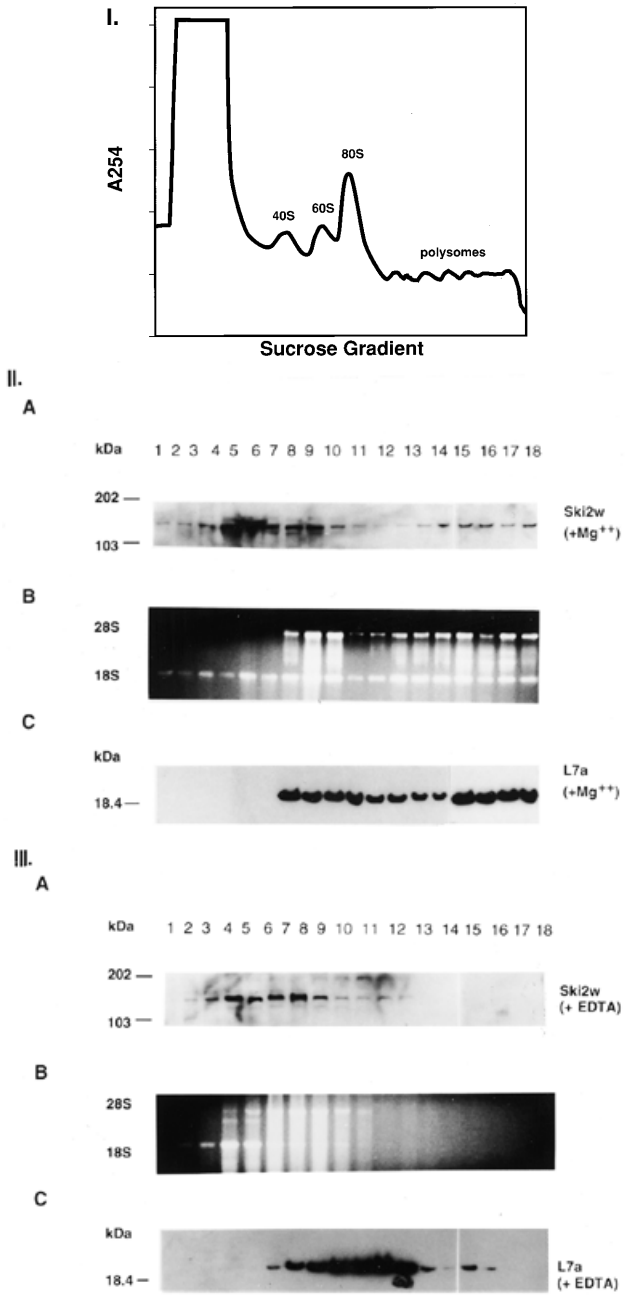


Figure 4. Immunoblot analysis of HeLa cytoplasmic extracts after sucrose gradient centrifugation. HeLa cell extracts were subjected to sucrose density gradient centrifugation in the presence of Mg^{2+} or EDTA. (**I**) The ribosomal profile in the presence of Mg^{2+}. (**II** and **III**) Immunoblot analysis using (A) αW-1.7 and (C) αL7a, in the presence of Mg^{2+} and in the presence of EDTA, respectively. (II, B) and (III, B) show the distribution of total cytoplasmic RNAs without and with EDTA treatment in correlation with the ribosomal profiles, respectively. (II, C) and (III, C) show the distribution of L7a in the presence of Mg^{2+} and in the presence of EDTA, respectively. For Figures 4–7, antibody–antigen complexes were detected by a chemiluminescence method.

that considerable amounts of Trx/Ski2w fusion proteins were produced (marked by arrows). Figure 2II (panels B–E) shows the results of immunoblot experiments of these bacterial fusion proteins using αW-3.6 (panel C), αW-1.7 (panel D), and the corresponding preimmune serum for GST/Ski2w3.6 (panel B) and for MBP/W1.7 (panel E). None of the preimmune sera reacted with the bacterial proteins on these blots. The antiserum αW-3.6 reacted with all three Trx/Ski2w fusion proteins (panel C, lanes 2–4) but not with the background bacterial proteins (panel C, lane 1). On the other hand, the antiserum αW-1.7 (panel D) only recognized T3.9W (lane 2) and T2.6W (lane 3), but not T1.8W (lane 4) or background bacterial proteins (lane 1). This is because the MBP/W1.7 fusion protein used to generate αW-1.7 contains sequence corresponding to the C-terminal region of Ski2w and only overlaps with T1.8W by 26 residues. In conclusion, αW-3.6 and αW-1.7 contain specific antibodies against Ski2w.

Human endogenous Ski2w is 140 kDa in size and is present in polysome-enriched cell lysates

Immunoblot analysis was employed to investigate the expression of the endogenous Ski2w proteins, using polyclonal antibodies αW-3.6 and αW-1.7. In most cases the antibodies were affinity purified, using immobilized Trx/Ski2w fusion proteins, T1.8W and T2.6W respectively, to minimize non-specific interactions. The predicted molecular weight for human Ski2w is 137 kDa before post-translational modifications. Human HeLa cell lysates were centrifuged at 130 000 *g*. The original total cell lysate, the supernatant (S130) and the pellets after centrifugation were subjected to SDS–PAGE and immunoblot analysis (19–22). Both αW-1.7 and αW-3.6 detected a polypeptide of ~140 kDa in size in polysome-enriched fractions of HeLa cell extracts (Fig. 3I, panel A, lanes 3 and 6, respectively). By this less sensitive method of using color development for immunoblot analyses, αW-1.7 and αW-3.6 did not readily detect the 140 kDa polypeptide in total cell lysates (lanes 1 and 4) and in the S130 fractions (lanes 2 and 5). Figure 3I (panel B) displays results of an immunoblot of HeLa cell extracts using polyclonal antibodies against L7a, a protein of the 60S subunit of the ribosome. This ribosomal protein was present in the total cell extracts (lanes 1, 4 and 7) and in the P130 fractions (lanes 3, 6 and 9). Previously, Song and co-workers used the monoclonal antibody mAb 170A1 to isolate from an expression library a cDNA clone with sequence similar to *SKI2W*. It was shown that mAb 170A1 reacted with two closely migrated polypeptides ~90 kDa in size (17). Although it was confirmed that mAb 170A1 detected the doublet 90 kDa polypeptides in HeLa cells (Fig. 3I, panel A, lanes 1–3), this mAb failed to detect the ~140 kDa polypeptide in the same cell lysates, and any of the Ski2w bacterial fusion proteins used in this study (data not shown).

To further substantiate the above observation, the full-length human *SKI2W* cDNA was subcloned into a mammalian expression vector pEBV-His. The unique feature of this vector is that between its start codon and its multiple cloning sites, there is a sequence tag that can be recognized by a commercially available mAb, anti-Xpress. Thus, the expression of human Ski2w protein using this expression vector can be monitored. A stable transfectant of the pEBV-His/SKI2W3.9 in CHO-K cell line was established. Total cell lysate of the stable transfectant for pEBV-His/SKI2W3.9 (tCHO-K) and untransfected CHO-K cells were subjected to immunoblot analysis. A polypeptide with the expected size of 140 kDa was detected in tCHO-K cells (Fig. 3II, lane 2), but not in the untransfected CHO-K cells (lane 1). The cell lysates from the stable transfectant and untransfected CHO-K were centrifuged at 130 000 *g*. The proteins in the supernatant (S130) and in the pellet (P130) were analyzed by immunoblotting. The

anti-Xpress mAb detected an ~140 kDa polypeptide in the polysome-enriched P130 fraction of the tCHO-K stable transfectants (Fig. 3II, panel A, lane 4). This polypeptide was slightly detectable in the total cell lysates in the tCHO-K cells (lane 3). It was not detectable in the untransfected CHO-K total cell lysates (lane 1) by anti-Xpress mAb. On the other hand, mAb 170A1 could only detect the 90 kDa polypeptides (Fig. 3I, panel A, lanes 7 and 9, and II, lanes 5, 6 and 8). It also failed to recognize the tagged-Ski2w fusion proteins in the polysome-enriched fractions of the tCHO-K cell lysates (Fig. 3II, lane 8). As a positive control to show the presence of proteins in each sample, αL7a (16) was used (Fig. 3I, panel B and Fig. 3II, panel B). The L7a ribosomal protein was detectable both in the total cell lysates of the untransfected CHO-K cells (II, panel B, lanes 1 and 5) and the tCHO-K cells (lanes 2 and 6). It was enriched in the P130 fractions (lanes 4 and 8). As expected, it was not detectable in the S130 fractions (lanes 3 and 7). Based on these observations, it is concluded that (i) human Ski2w is ~140 kDa in size and is likely to be associated with the polysomes, and (ii) mAb 170A1did not react with human Ski2w in immunoblot experiments.

Human Ski2w is present in polysomes and probably associates with the 40S subunit of the ribosome

To investigate the possible association of Ski2w with ribosomes or polysomes, ribosomal profile studies were performed. Cytoplasmic extracts from HeLa cells were prepared and subjected to 15–45% sucrose gradient centrifugation. The cultured cells were pretreated with cycloheximide to prevent the dissociation of polysomes. The sucrose gradient buffer was supplemented with either 30 mM Mg^{2+} or 30 mM EDTA. After centrifugation, the sucrose gradient was fractionated and its absorbance at 254 nm was continuously measured by an Isco UA-5 UV monitor (Fig. 4I). Proteins from each fraction were precipitated and assayed for the presence of Ski2w proteins by immunoblot analysis (Fig. 4II, panel A). Aliquots from each fraction of the sucrose gradient were used to isolate total RNA. The RNA samples were electrophoresed on 1% agarose gels and stained with ethidium bromide (II, panel B). The 18S and 28S ribosomal RNAs are used as markers to indicate the locations of the 40S and the 60S ribosomal subunits in the sucrose gradient, respectively. The more sensitive chemiluminescence method of signal detection was employed for immunoblot analyses of protein samples. In the presence of 30 mM Mg^{2+}, Ski2w present throughout most of the sucrose gradient (Fig. 4II, panel A), but it was more concentrated in lower density sucrose (fractions 4–8). It was also present in the polysome-containing fractions 14–18. The distribution pattern of Ski2w superimposed with that of 18S rRNA, which is a structural component of the 40S ribosome subunit (Fig. 4II, panels A and B). On the other hand, the distribution pattern of an integral protein of the large ribosomal subunit, L7a, superimposed with that of the 28S rRNA, which is a structural component of the 60S ribosomal subunit. This confirmed the accuracy of the ribosomal profiles (Fig. 4II, panels B and C).

The association of the 40S and 60S subunits to form the 80S ribosomes requires Mg^{2+}. The presence of the chelating agent EDTA promotes the dissociation of the polysomes to the 80S monosomes and to the 40S and 60S subunits (23). Ski2w proteins in the sucrose gradient were redistributed (Fig. 4III, panel A) in the presence of EDTA. Ski2w proteins were totally diminished from the high sucrose density fractions and shifted to the lower

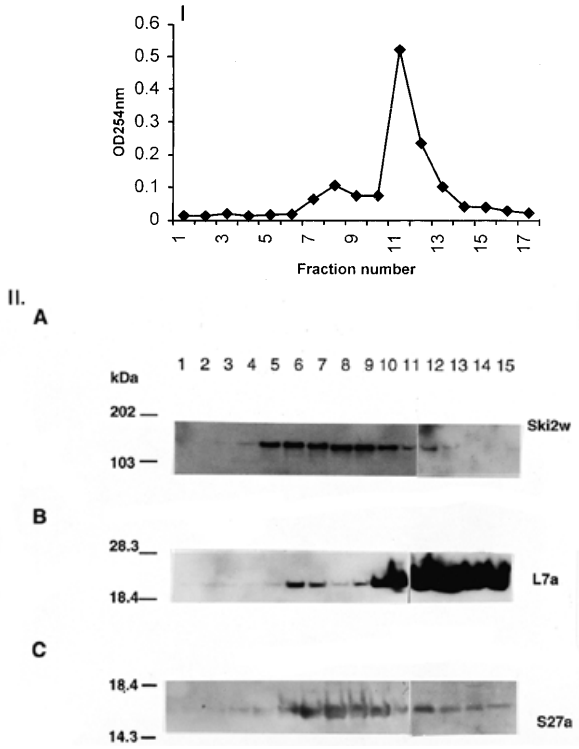


Figure 5. Human Ski2w is present in 40S ribosomal subunits. Polysomes were isolated from HeLa cell extracts supplemented with 30mM EDTA and subjected to sucrose gradient (5–35%) centrifugation. (**I**) OD_{254} of the fractionated sucrose gradient. (**II**) Results of immunoblot analyses using αW-3.6 (A), αL7a (B) and αS27a (C).

sucrose fractions. It appears that the Ski2w protein co-sedimented with the 40S ribosomal subunit as indicated by the distribution of the 18S rRNA (III, panel B). On the other hand, the L7a proteins were optimally detectable in the 60S containing fractions, as shown by the distribution of the 28S rRNA (Fig. 4II and III, panels B and C).

To better separate the 40S and 60S subunits, purified polysomes were treated with 30 mM EDTA and subjected to centrifugation in a lower concentration sucrose gradient, 5–35%. After centrifugation, a total of 15 fractions were collected, and 15 µl from each fraction was used to measure the optical density at 254 nm to correlate the protein concentration with each fraction (Fig. 5I). Immunoblot analysis showed that Ski2w, under this experimental condition, co-sedimented with the 40S ribosomal subunit. The Ski2w protein began to be detectable in fraction 4, and became more concentrated in fractions 5–10 (Fig. 5II, panel A). These fractions corresponded to the 40S peak of the ribosomal profile (Fig. 5I). On the other hand, small quantities of Ski2w could be detected in the 60S ribosome containing fractions (fractions 11–15), as shown by the ribosomal profile and the distribution of L7a in the western blot (Fig. 5II, panel B).

To further confirm that Ski2w co-sedimented with the 40S subunit in the ribosome profile, we employed the polyclonal antibodies raised against S27a for western blot analysis. S27a is an integral protein of the small ribosomal subunit. Figure 5II, panel C shows that S27a was more concentrated in fractions 5–10 that corresponded to the 40S ribosomal subunit. It began to decrease in concentration after fraction 10, which marked the

appearance of the 60S ribosomal subunit. Both Ski2w and S27a could be detected in the 60S subunit containing fractions, which reflected that the separation of the 40S and the 60S subunits in the sucrose gradient was incomplete. Overall, these results suggested that Ski2w is associated with ribosomes and polysomes, and more specifically with 40S ribosomal subunits. Alternatively, Ski2w is present in a protein complex which has a sedimentation pattern similar to polysomes, ribosomes and 40S subunits of ribosomes.

The association of Ski2w with ribosomes are not mRNA dependent. To investigate whether Ski2w associates with polysomes through binding to mRNA in a manner similar to the FRMP (24), the cellular extract was subjected to RNase A treatment on ice for 15 min prior to the sucrose gradient centrifugation. RNase A digests the mRNA linking ribosomes together in a polysome. A ribosomal profile is shown in Figure 6I. In comparison with the control, the peaks of the polysomes were completely eliminated upon RNase A treatment. On the other hand, the amplitude of 80S peak was increased (Fig. 6I, panels A and B), which consists of the original monomers and the 80S ribosomes derived from the breakdown of the polysomes by RNase A. To better correlate the peak with each fraction, a total of 18 fractions were collected and 15 µl of each fraction was used to measure their OD_{254} values. The profile was plotted against the fraction numbers (Figure 6I, panel C). From the plotted ribosomal profile, it shows that the OD_{254} values of fractions 10, 11 and 12 increased significantly after RNase A treatment when compared with the control. In addition, the OD_{254} values of fractions 16, 17 and 18 decreased greatly. Figure 6II, panel A showed that Ski2w protein, in the absence of RNase A, distributed throughout the entire sucrose gradient. This was consistent with the previous observations. However, upon RNase A treatment, Ski2w protein existing in the polysome fractions were totally diminished and a significant increase of Ski2w protein was observed in the middle of the gradient (Fig. 6II, panel B, fractions 6–12). The Ski2w protein was most abundant in fraction 10, which corresponded to the peak of the 80S monosomes (I, panel B). To assess the efficiency of RNase A digestion, the distribution in the sucrose gradient of the FMRP protein, which has been demonstrated to be an mRNA dependent, polysome-associated protein (mRNP) (24–26) was determined. As expected, without RNase A treatment, FMRP protein was present throughout the entire sucrose gradient (Fig. 7III, panel A). However, FMRP was totally relocated to the lower density fractions 3 to 5 (Fig. 6II, panel C), which is similar to a previous observation (25). In essence, Ski2w protein associates with ribosomes and polysomes and this association is unlikely to be mRNA dependent.

Ski2w associates with ribosomes tightly. To further determine the strength of association of Ski2w with ribosomes, HeLa cell extracts were supplemented with KCl to 0.5 M and subjected to sucrose gradient centrifugation. Under such a high salt concentration, most protein–protein interactions would be disrupted. As shown in Figure 7, no obvious alteration in the polysome distribution was observed (I), nor was Ski2w's distribution changed by the 0.5 M KCl treatment when compared with the control (II, panels A and B). However, FMRP, as reported previously (26), dissociated from the ribosomes/polysomes, and migrated to the very first few fractions (III B) upon 0.5 M KCI treatment. This result suggests that the Ski2w protein is associated with ribosomes and polysomes with relative high affinity.

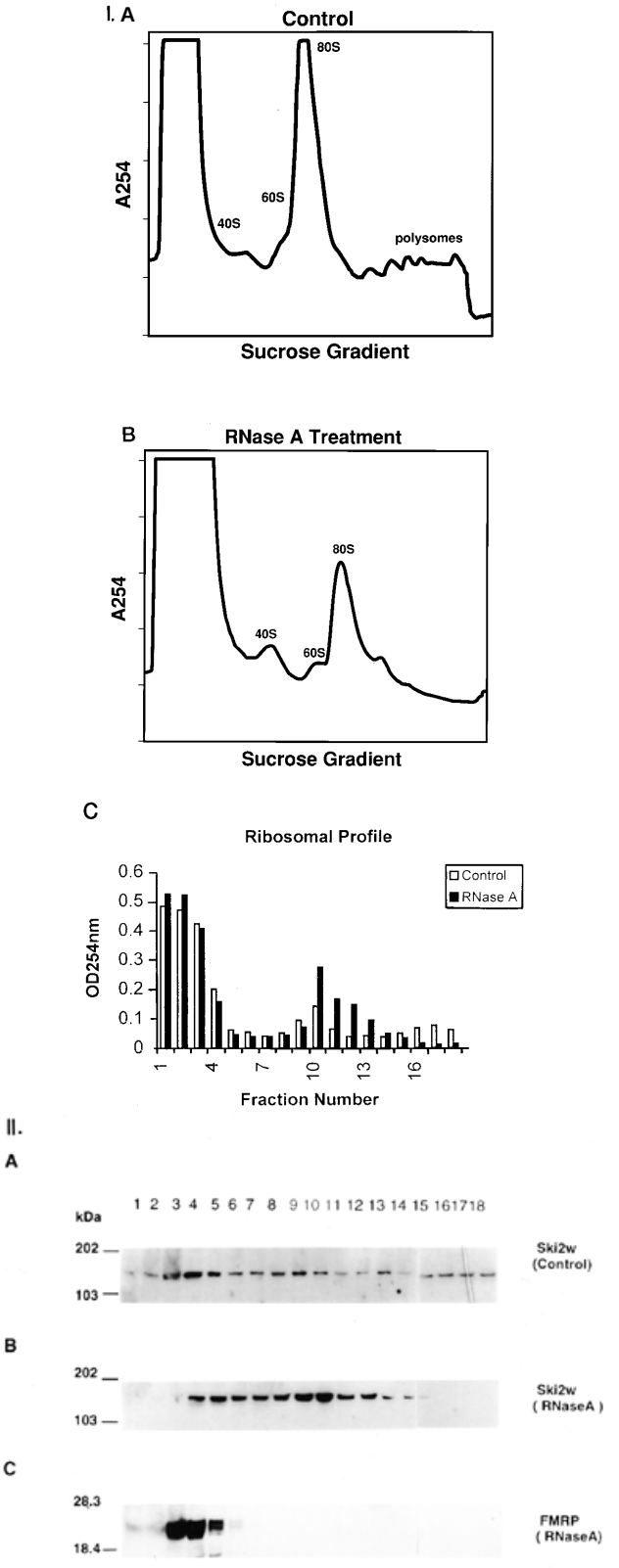


Figure 6. Immunoblot analysis of Ski2w in fractionated ribosomal profiles with and without RNase A treatment. (**I**) A and B, ribosomal profiles in the absence of RNase A and with RNase A treatment, respectively; C, ribosomal profile based on OD_{254} of fractionated samples. (**II**) Distribution of Ski2w related to the ribosomal profile with (B) and without (A) RNase A treatment. (II, C) Distribution of FMRP protein in the same profile upon RNase A treatment.

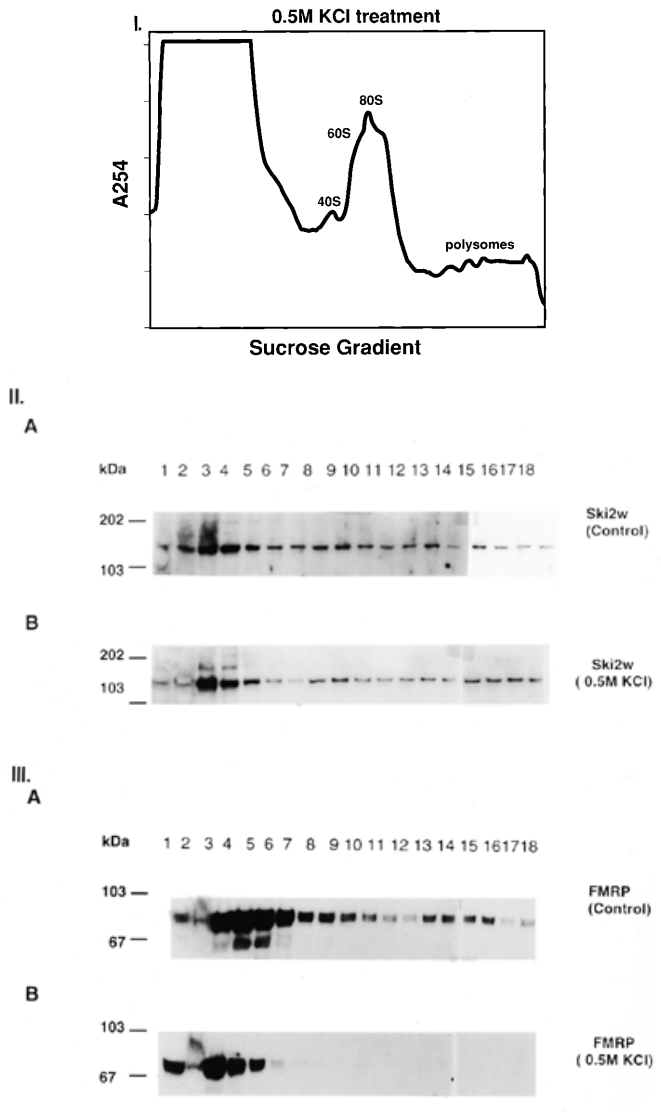


Figure 7. Distribution of Ski2w in fractionated ribosomal profiles with and without 0.5 M KCl treatment. (**I**) Ribosomal profile of HeLa cell extract upon 0.5 M KCl treatment. (**II**) Immunoblot analysis of fractionated protein extracts using αW-3.6. (**III**) Immunoblot analysis using mAb against FMRP, 1C3; controls, without 0.5 M KCl treatment.

Indirect immunofluorescence (IF) microscopy shows that Ski2w is localized in nucleoli and cytoplasm

To determine the subcellular localization of Ski2w, indirect IF experiment was performed using HeLa cells and αW1.7. A mouse monoclonal antibody against the nucleolar protein nucleophosmin, αB23, was used as a control to show the localization of the nucleolus. The secondary antibodies were goat anti-rabbit IgG conjugated with FITC, and goat anti-mouse IgG conjugated with Texas red, respectively. When mAb B23 was used, a brilliant red staining of the nucleolus was observed (Fig. 8B). When αW1.7 was used, an intense fluorescence was observed in the nucleoli and in the cytoplasm, suggesting human Ski2w is present in these subcellular regions. Co-localization of the Ski2w (green) and nucleophosmin (red) at the nucleoli led to a yellow fluorescence (Fig. 8A).

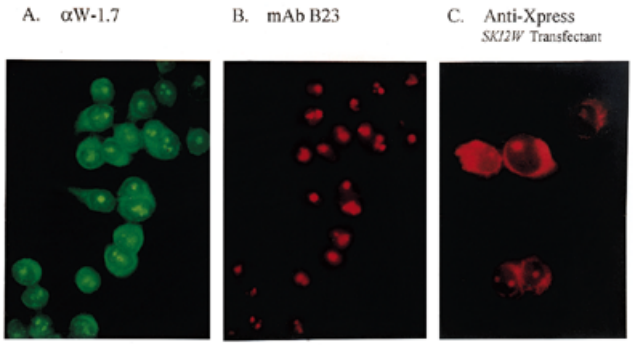


Figure 8. Localization of Ski2w proteins *in vivo*. (**A** and **B**) Immunofluorescent staining of Ski2w and nucleophosmin in HeLa cells (on the same slide) using rabbit antisera αW-1.7 and mouse mAb B23, respectively. The secondary antibodies were goat/anti-rabbit IgG conjugated with FITC for antisera αW-1.7, and goat/anti-mouse IgG conjugated with Texas red for B23. (**C**) Subcellular localization of the enterokinase epitope tagged-Ski2w proteins in HeLa cells transiently transfected with pEBV-His/SKI2W3.9. The primary antibody used was mAb anti-Xpress. The secondary antibody was goat anti-mouse IgG conjugated with Texas red.

To further substantiate the localization of Ski2w in cells, HeLa cells were transiently transfected with pEBV-His/SKI2W. The cellular localization of the Ski2w protein tagged with an enterokinase epitope at the N-terminal region was shown by IF experiments, using anti-Xpress mAb as the primary antibody and the goat anti-mouse antibody coupled with Texas red as the secondary antibody. As shown in Figure 8C, the tagged Ski2w fusion proteins were present in the nucleoli and in the cytoplasm but not in the nucleoplasm, which is consistent with results of the previous experiment using αW-1.7. No fluorescent signals were observed when untransfected cells were used (data not shown).

DISCUSSION

The human *SKI2W* gene was discovered by molecular characterization and DNA sequencing of the HLA class III region (1). The presence of an RNA helicase domain suggests that the Ski2w protein would unwind RNA secondary structures under appropriate conditions. The extensive structural similarities of human Ski2w to yeast Ski2p suggest a possible antiviral function. This antiviral function could be achieved through regulating translation of RNA molecules based on their structural integrities such as the presence of the 5′ cap and the 3′ poly(A) tail. There is strong evidence showing that both of the 5′ cap and the 3′ poly(A) tail of a eukaryotic mRNA play an important role in the initiation of translation (9,27). Although there are elegant genetic studies on the antiviral role of yeast Ski2p, the biochemical and physiological properties of this protein are largely unknown. As an initial step to understand the function of human Ski2w, we employed immunological techniques to elucidate the subcellular localizations of Ski2w proteins. Indirect immunofluorescence experiments showed that the endogenous Ski2w proteins were localized in the nucleoli and the cytoplasm. Immunoblot analysis demonstrated that Ski2w proteins are ~140 kDa in size. It is associated with ribosomes and polysomes, or more specifically, with the 40S subunit of ribosomes and polysomes, as revealed by co-sedimentation of Ski2w with the 18S rRNA-containing proteins and S27a of the small ribosomal subunit protein. The association of Ski2w with ribosomes is unlikely to be mRNA dependent because this

association is refractory to RNase A treatment. Moreover, the association of Ski2w with ribosomes was not abolished by 0.5 M KCl treatment, suggesting high affinities of Ski2w to ribosomes (30).

The presence of Ski2w in the nucleoli and cytoplasm/polysomes indicates that Ski2w is present at the sites of ribosome biogenesis and protein synthesis. In the nucleoli, the Ski2w helicase could help refolding of rRNAs and facilitate the interaction of rRNA and ribosomal proteins. Although the yeast homolog Ski2p was originally shown to be an antiviral protein (4), the high degree of conservation of this protein from yeast to *Caenorhabditis elegans* to humans, and its ubiquitous expression in almost all human tissues tested are indicative of a more fundamental cellular function. In yeast, mutation of the 5′→3′ exonuclease gene *XRN1* (*xrn1)* leads to the accumulation of poly(A)-shortened and uncapped RNA in the cell. The synthetic lethality of yeast *ski2* with *xrn1* suggests that Ski2p may be involved in an RNA degradation pathway (28). This is intriguing as in *E.coli*, a DEAD-box RNA helicase protein, RhlB, is present in the multi-enzyme complex of the RNA degradosome. RhlB acts by unwinding RNA structures that impede the processive activity of the 3′→5′ exoribonuclease polynucleotide phosphorylase (PNPase) (29). Moreover, one of the subunits for the yeast mitochondrial 3′→5′ exonuclease, Svu3p, is a putative NTP-dependent RNA helicase (30). Among all helicases showing structural similarities to human Ski2w and yeast Ski2p, and their related protein human KIAA0052 (or Ski2k) (1), and yeast Mtr4p (also named as Dob1p, a homologue of human Ski2k) (12), Suv3p is one of the closest proteins in overall sequence similarities to the Ski2 protein family. Therefore, it is interesting when it was shown (after submission of this manuscript) that yeast Ski2p is required for the 3′→5′ degradation of mRNAs (31). It was also shown that other yeast proteins involved in a pathway for restricting proliferations of yeast RNA viruses, Ski3p, Ski6p and Ski8p are also related to mRNA turnover. In fact Ski6p is an exoribonuclease present in the so-called exosome (31,32) and is structurally similar to the *E.coli* RNase PH. The exosome is a multienzyme 3′→5′ ribonuclease complex consisting of five essential proteins with a sedimentation coefficient ~14S or a molecular weight of 300–400 kDa (32). Apart from playing a role on the mRNA turnover, the exosome complex is also involved in the 3′ end formation of the 5.8S rRNA. Yeast Mtr4p probably interacts with the exosome and is involved in the processing of the 5.8S rRNA in the nucleus (33). Therefore, it has been suggested that Ski2p plays a role on the 3′→5′ degradation of mRNAs in the cytoplasm, while its related protein Mtr4p contributes to the 3′→5′ RNA turnover in the nucleus (33), a phenomenon similar to the 5′→3′ degradation by Rat1p and Xrn1p, respectively (34). A homologous protein for a subunit of the yeast exosome, Rrp4p, has been discovered in humans and shown to complement the temperature sensitive growth phenotype of the yeast mutant, *rrp4-1* (32). Therefore, it is likely that a similar degradation pathway exists in humans and human Ski2w may be involved in a pathway for RNA turnover, similar to that of the yeast Ski2p.

Again it needs to be stressed that studies on the roles of Ski2p and Mtr4p on RNA turnovers were mainly performed using yeast mutants, and the modes of action of the endogenous proteins have not been examined directly. In this paper we have shown that human Ski2w is present in the nucleolus, is associated with the polysomes and probably with 40S ribosomal subunits, although this does not exclude the possibility for the association of Ski2w with another large protein complex of the similar size (~40S). It will be important to investigate how the exosome complex interacts with ribosomes/polysomes to control the RNA stability and turnover. We propose that Ski2w plays a role in linking the polysomes and exosomes to achieve one of the crucial steps on the temporal control of gene expression: to degrade mRNAs and to prohibit the degrading mRNAs from being translated.

In yeast, it was shown that *ski2* is a suppressor of the *mak7* mutant. *mak7* is defective in the L4 protein of the 60S ribosome subunit, which leads to a marked decrease in free 60S ribosomal subunits in the cell and inability for the propagation of dsRNA replicons such as M_1 (5,35,36). However, the *mak7 ski2* double mutants did not restore the distribution of free 60S ribosomal subunit in the ribosomal profile, as compared with those from the *mak7* mutant and from the wild type cells (5). It was suggested that Ski2p or its 'controlling protein' would be present in or interacting with the 60S subunit. The detection of human Ski2w in polysomes, ribosomes and in the 40S subunit of ribosomes is not inconsistent with the observations for the yeast Ski2p. It is possible that yeast Ski2p is present on the 40S subunit and interacts with a protein on the 60S subunit of the ribosome in the active translation machinery, such as the intact ribosomes or polysomes. The de-repression of translational inhibition of poly(A)$^-$ RNAs in yeast *ski2* mutants suggests that wild type Ski2p plays a role on the regulation of translation, probably by controlling the access of the translational machinery to different types of RNAs (5). Therefore, on the 40S ribosomal subunit, the human Ski2w could serve as a gatekeeper to differentiate intact and degrading/degraded mRNA for the initiation of translation. This process may insulate cells from synthesizing truncated proteins (from partially degraded transcripts) that could severely interfere with regular protein function. The mechanism to achieve the differentiation of various RNA molecules for translation remains to be elucidated.

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