Post-transcriptional regulation of thioredoxin by the stress inducible heterogenous ribonucleoprotein A18

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ABSTRACT

Thioredoxin (TRX) is a key protein of the cellular redox metabolism, which expression is increased in several tumors especially gastric tumors. Even though ultraviolet (UV) and hypoxia specifically induce TRX, the mechanisms that lead to increased TRX levels are still ill defined. Here, we show that the heterogenous ribonucleoprotein A18 (hnRNPA18) RNA Binding Domain (RBD) and the arginine, glycine (RGG) rich domain can bind TRX 3′-untranslated region (3′-UTR) independently but both domains are required for maximal binding. Immunoprecipitation (IP) of hnRNPA18-mRNAs complexes and co-localization of hnRNPA18 and TRX transcripts on ribosomal fractions confirm the interaction of hnRNPA18 with TRX transcripts in cells. Moreover, down regulation of hnRNPA18 correlates with a significant reduction of TRX protein levels. In addition, hnRNPA18 increases TRX translation and interacts with the eukaryotic Initiation Factor 4G (eIF4G), a component of the general translational machinery. Furthermore, hnRNPA18 phosphorylation by the hypoxia inducible GSK3β increases hnRNPA18 RNA binding activity in vitro and in RKO cells in response to UV radiation. These data support a regulatory role for hnRNPA18 in TRX post-transcriptional expression possibly through a kissing loop model bridging TRX 3′- and 5′-UTRs through eIF4G.

INTRODUCTION

Thioredoxin (TRX) is a ubiquitous multifunctional protein that has regulatory roles in cellular signaling and gene transcription in addition to cytoprotective activities through the quenching of reactive oxygen species (ROS) (1). The cytoprotective effect of TRX against oxidative stress was best illustrated in experiments where the levels of TRX were reduced with an antisense expression vector (2). These cells became more sensitive to H2O2, a variety of anticancer drugs and ultraviolet (UV) radiation. On the other hand, over-expression of TRX could be detrimental to cellular homeostasis. For example, increased levels of TRX are associated with resistance to chemotherapy and cellular proliferation, probably through inhibition of apoptosis (3). TRX expression is increased in several tumors, especially gastric tumors and colon tumors where TRX mRNA expression is 60 times higher than in normal tissue (4). TRX is also increased in response to a variety of stresses such as X-ray irradiation, UV radiation and other types of oxidative stresses (5). However, the mechanism that leads to increased levels of endogenous TRX in cancer cells is still ill defined (3).

Using a modified Systematic Evolution of Ligands by EXponential enrichment (SELEX) assay, we have identified TRX and 25 other stress-inducible transcripts, as potential targets for the heterogenous ribonucleoprotein A18 (hnRNPA18) (6). hnRNPA18 was the first RNA binding protein (RBP) reported to be inducible by UV radiation (7). hnRNPs are abundant RBPs predominantly found in the nucleus and involved in several cellular activities ranging from transcription and mRNA processing in the nucleus to cytoplasmic mRNA translation and turnover (8). The hnRNPs constitute a family of more than 20 different proteins that are highly conserved (9). hnRNPA18 is different than most hnRNPs in that it contains only one RNA Binding Domain (RBD) and has several arginine and glycine residues (RGG boxes) in its auxiliary domain. In contrast to most hnRNPs that are constitutively expressed, hnRNPA18 mRNA and protein levels can be induced by cellular stress such as UV and hypoxia (10).

The nuclear hnRNPA18 translocates to the cytoplasm in response to UV radiation and binds several stress-responsive mRNA transcripts including TRX (6). hnRNPA18 binds specifically to TRX 3′-UTR and increases translation.
Translation initiation can be regulated at both the 5' and the 3' end of an mRNA transcript. Modulations of the interactions between RBPs, mRNA transcripts and components of the translational machinery dictate the fate of most transcripts. Proteins that bind to 3'-UTRs can affect both the stability and the translation of a transcript. These proteins can directly contact the basal translational machinery and influence activation or repression of translation (11).

Regulation of protein translation is now recognized as an important component of the cellular response to genotoxic stress (12). More than four decades ago, modification of protein synthesis patterns was recognized as an early phenomenon triggered by cellular stress (13). A typical response was characterized by an immediate arrest of protein synthesis followed by an increased rate after exposure to UV radiation (13). The immediate down regulation of protein synthesis is thought to be an adaptive response triggered to protect the cells and conserve the resources required to survive (14). The increase rate of protein synthesis after cellular stress seems to target specific ribosomal proteins and other proteins important for survival (15). Induction of specific ribosomal proteins in response to stress may indicate the involvement of the translational machinery in sensing and responding to cellular stress (15). The association of several ribosomal proteins with the oxidative stress response (1) is additional evidence that translation regulation is a significant component of the cellular stress response. Several types of stress, including heat shock stress and several chemicals, can induce the synthesis of cellular stress response. Several types of stress, including heat shock stress and several chemicals, can induce the synthesis of
translational pathways that can confer protective roles are likely to be important component of the stress response in human cells.

In this report we show that hnRNP A18 RBD as well as the RGG domain confer hnRNP A18 RNA binding activity. More importantly, hnRNP A18 and TRX mRNAs form a complex in RKO cells and co-localize on ribosomal fractions. Our data also indicate that levels of hnRNP A18 correlate with TRX protein levels. Furthermore, phosphorylation of hnRNP A18 by the hypoxia inducible kinase GSK3β (17) increases hnRNP A18 RNA binding activity in vitro and in RKO cells in response to UV radiation. Functional assays indicate that hnRNP A18 can increase TRX translation and interacts with eukaryotic Initiation Factor 4G (eIF4G), a component of the general translational machinery. Collectively, these data indicate that hnRNP A18 could mediate post-transcriptional regulation of TRX possibly through a kissing loop model bridging TRX 3'- and 5'-UTRs by binding eIF4G.

**MATERIALS AND METHODS**

**Plasmids**

The pcDNA3.1-A18, pcDNA3.1-A18-GFP (green fluorescent protein) and pcDNA3.1-CAT (Chloramphenicol acetyltransferase) expression vectors were constructed as described previously (6). The 3'-UTR of TRX was amplified by PCR from the TRX expression vector pGEM-TRX and cloned into the BamHI/XhoI sites of pcDNA3.1-CAT to generate the plasmid pcDNA3.1-CAT/TRX 3'-UTR. To generate the antisense hnRNP A18 vector, the open reading frame (ORF) of hnRNP A18 was cloned in antisense orientation into the HindIII/XhoI sites of pcDNA3.1. The GST (glutathione S-transferase)-hnRNP A18 expression vectors were constructed with the cDNA of full-length hnRNP A18, RBD with linker (residues 1–92) and RGG domain (residue 84–172) cloned into the BamHI/XhoI site of pGEX-4T-2 vector. These vectors were named pGEX-A18, pGEX-RBD and pGEX-RGG, respectively.

**Cell culture and treatment**

The rationale for using human colon carcinoma RKO cells is that TRX is over-expressed in colon tumors (4) and RKO cells have a wild-type p53 genotype (18) allowing them to respond normally to genotoxic stress. RKO cells were provided by A. J. Fornace, Jr (National Cancer Institute, National Institutes of Health, Bethesda, MD). The cells were grown in RPMI 1640 containing 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA). For transient transfection, cells were cultured to 50% confluency, and the indicated plasmids were transfected with Gene Juice (Roche Molecular Biochemicals). For CAT assay, a total amount of 11 μg of plasmids DNA was used and supplement with pcDNA3.1 when necessary. Each dish received 1 μg of CAT or CAT-UTR expression vector and 0 or 10 μg of hnRNP A18. Forty-eight hours after transfection, cells were harvested and lysed for further analyses. Analyses were performed in triplicate. For stable transfection, hnRNP A18-GFP was transfected into 50% confluent RKO cells. Selection was performed with 600 μg/ml hygromycin B for 2 weeks. The RKO antisense cell line was established as described earlier (6). The UVC source was a Philips 30 W germicidal lamp emitting at 253.7 nm and the intensities of the UV lamp were determined with a UVX Radiometer (UVP Inc., Upland, CA).

**Protein purification**

Expression of GST fusion hnRNP A18 full-length, hnRNP A18 RBD and hnRNP A18 RGG were achieved in *Escherichia coli* BL21(DE3) as recommended by the manufacturer (Novagen) except that the bacteria were grown at 30°C. Glutathione Sepharose 4B was purchased from Amersham Biosciences and the fusion proteins were purified according to the protocol provided by the manufacturer.

**RNA binding activity in vitro**

RNA band shift and Northwestern assay. 3'-UTR of TRX cDNAs plus poly(A) tail (319–485) was amplified by PCR with a T7 promoter (5'-AGTGAATTGTAATACGACTCACAGTATAGGGC-3') engineered at their 5' ends and in vitro transcribed with MAXI-scripts (Ambion Inc.). 32P-radiolabeled TRX 3'-UTR transcripts were gel purified. Binding reactions for gel mobility shift assays were performed with a range of GST–hnRNP A18 fusion protein concentrations and 0.15 nM 32P-labeled RNA in a final volume of 25 μl containing 10 mM HEPEs (pH 7.9), 150 mM KCl, 5 mM MgCl2, 0.2 mM DTT, 10 μl of RNasin (Invitrogen), 8% glycerol and 50 μg/ml yeast tRNA. Reactions were incubated for 20 min at room temperature and immediately fractionated through 1% agarose gel. For Northwestern assay, the RNA–protein binding condition...
was the same as described above except that the recombinant GST or GST–hnRNP A18 fusion proteins were transferred to a nitrocellulose membrane first and the proteins were re- 
erature over-night in a buffer containing 10 mM HEPES (pH 7.9), 40 mM KCl, 3 mM MgCl$_2$, 5 mg/ml BSA, 5% glycerol, 0.2% non-ionic detergent P-40 (NP-40), 0.1 mM EDTA, 1 mM DTT before incubating the membrane with 1 × 10$^6$ CPM/ml of 32P-labeled RNA probes.

**Small interference RNA (siRNA)**

The 21 nt double-stranded RNA was synthesized using Ambion RNAi construction kit. The target sequence (5'-GAGACAGTTACGACAGTTA-3') corresponds to 19 nt from the hnRNP A18 ORF and a two-dT overhang. The scrambled RNA is composed of four random sequences of 19 nt with two a-UU overhang on each side (nonspecific control duplexes XIII, pool of four; Dharmaco, Inc., Boulder, CO). The siRNAs (20 nM) were transfected in RKO cells with TransMessenger transfection reagent (Qiagen) according to the manufacturer’s recommendations. The cells were exposed to UV radiation 24 h after transfection.

**Western blots**

The western blot analyses were performed, as described previously (6) on different amount of protein extracts (indicated in the figure legends). The cells were lysed in radioimmune precipitation assay (RIPA) buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin and 5 mM DTT]. The proteins were run on a 15% polyacrylamide gel, transferred onto nitrocellulose, and reacted with either TRX mouse monoclonal antibody (Oncogene Research Products) at 1:5000 dilution, or actin mouse monoclonal antibody (Bethyl) at 1:1000, GSK3β clonal antibody (6) at 1:200 dilution, eIF4G rabbit monoclonal antibody (BD) at 1:5000 dilution, hnRNP A18 rabbit polyclonal antibody (Upstate) at 1:1000, or GST or GST–hnRNP A18 fusion proteins were transferred to nitrocellulose membrane first and the proteins were reacted with either TRX mouse monoclonal antibody (Oncogene Research Products) at 1:5000 dilution, or actin mouse monoclonal antibody (Bethyl) at 1:1000, GSK3β clonal antibody (6) at 1:200 dilution, eIF4G rabbit monoclonal antibody (BD) at 1:5000 dilution, hnRNP A18 rabbit polyclonal antibody (Upstate) at 1:1000, or actin mouse monoclonal antibody (Upstate) at 1:1000 dilution, or actin mouse monoclonal antibody (Oncogene Research Products) at 1:5000 dilution to control for even protein loading. The blots were then reacted with their respective secondary antibodies conjugated to horseradish peroxidase (HRP) and reacted with a chemiluminescence substrate (ECL, Amersham Biosciences) as recommended by the manufacturer.

**Linear sucrose gradient fractionation**

RKO cells (40 × 10$^6$) exposed or not to UV radiation were treated for 15 min with cycloheximide (100 μg/ml) and then harvested. The cytoplasmic extracts were prepared by resuspension of the cell pellets in polysome lysis buffer [10 mM HEPES (pH 7.0), 100 mM KCl, 5 mM MgCl$_2$, 0.5% NP-40] containing 1 mM DTT, 10 U/ml RNase inhibitor ( Gibco-BRL) and protease inhibitor cocktail (Roche). The cells were homogenized with a Dounce’s homogenizer and centrifuged at 14000 g. The supernatants (800 μl) were loaded on to sucrose gradients (10–50% w/v) in polysome lysis buffer without NP-40. After centrifugation (Solrav AH-629, 25 000 r.p.m., 3 h, 4°C), the samples were fractionated from the bottom into ten 1.5 ml aliquots. For western blot, 50 μl from each fraction was loaded on to SDS–PAGE gel. For RNA analysis, half of each fraction (750 μl) was diluted with an equal volume of water and RNA was isolated by phenol–chloroform followed by isopropanol precipitation. For total RNA analysis, ~40% of the total RNA isolated from each gradient fraction was fractionated on 1.2% agarose gel. For RT–PCR, 5% of isolated total RNA (1 μl) from each fraction was used.

**In vitro phosphorylation**

FLAG-ATM (Ataxia Telangiectasia mutant) and ATR expression vectors were obtained from Dr Mike Kastan (St. Jude Children’s Research Hospital, Memphis, TN) and Aziz Sancar (University of North Carolina, Chapel Hill, NC), respectively. The FLAG-SMG1$_{ATX}$ was obtained from Dr Alan Fields (University of Texas, Galveston, TX). The vectors were stably transfected in RKO cells and the respective kinases were immunoprecipitated with a FLAG antibody after UV radiation. The recombinant CK2, GSK3α and GSK3β kinases were purchased from Upstate Biotechnology. The kinase reaction was performed with 1 μCi of [32P]ATP in 15 μl of 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 10 mM MgCl$_2$, 0.5 mM DTT (kinase reaction buffer) for 30 min at 37°C. Samples were run on an SDS–12% polyacrylamide gel. The gel was dried and exposed to X-ray sensitive film.

**RNA binding activity in vivo**

**Northwestern and GSK3β siRNA treatment.** RKO cells (30% confluency) stably transfected with GFP–hnRNP A18 were transiently transfected with either 20 nM of GSK3β siRNA (SMRTpool GSK3β, Upstate) or scrambled siRNA (Ambion). Forty-eight hours after transfection, the cells were exposed or not to UV (14 J m$^{-2}$) radiation and harvested 4 h later. Cell lysis were performed as described above and GFP–hnRNP A18 pulled down is described below. One mg of total protein from each sample was used to pull down GFP-hnRNP A18. The pulled down GFP–hnRNP A18 was loaded on a 12% SDS–PAGE gel and transferred to a nitrocellulose membrane. Northwestern was performed as described above.

**Co-immunoprecipitation of hnRNP A18 mRNA complexes and hnRNP A18 protein complexes**

RKO cells and RKO cells stably transfected with GFP–hnRNP A18 were exposed to 20 Jm$^{-2}$ UV radiation and harvested 4 h later. Immunoprecipitation (IP) of endogenous hnRNP A18 mRNA complexes were performed essentially as described in (19) except that the cell lysate was separated into two samples; sample (a) was used to immunoprecipitate mRNAs bound to hnRNP A18 and sample (b) was used to immunoprecipitate proteins associated with hnRNP A18. Briefly, the cells were lysed with polysome lysis buffer [10 mM HEPES (pH 7.0), 100 mM KCl, 5 mM MgCl$_2$, 0.5% NP-40] and total lystate (1.5 mg) were incubated in the presence of EDTA (20 mM) with protein A-Sepharose CL-4B beads (Sigma) that had been pre-coated with 30 μg of either anti-GFP (Santa Cruz Biotech.) or anti-IgG1 (Calbiochem). The beads were washed six times with NT2 buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl$_2$ and 0.05% NP-40]. Sample (a): for RNA analysis, the beads were incubated with 100 μl NT2 buffer containing 20 U RNase free DNase I for 15 min at 37°C. Proteins bound to the beads were then digested by adding 0.1% SDS and 0.5 mg/ml proteinase K to the reactions and the incubations were continued for 15 min at 55°C. RNA was
extracted by ethanol precipitation and used to perform RT–PCR. The PCR products were visualized after electrophoresis in 1% agarose gels. Sample (b): for hnRNP A18 co-immunoprecipitation with eIF4G, an RNase cocktail (Ambion) was incubated with the cell lysates at 37°C for 30 min prior to the IP. Following the IP, the proteins were eluted with SDS loading buffer and loaded on an 8% SDS–PAGE gel for western analysis.

CAT assays
The CAT activity was measured essentially as described previously (6). Cellular extracts containing either 50 or 25 μg of protein were incubated at 37°C for 1–3 h and separated on TLC plates. The conversion of chloramphenicol was quantified on a PhosphorImager (Molecular Dynamics STORM) with the ImageQuant software.

RNase protection assay
RKO cells were transiently transfected with hnRNP A18 and either a CAT or a CAT-TRX 3’-UTR construct as described previously (6). Forty hours later the cells were treated with actinomycin D (10 μg/ml) for 8 h before harvesting. RNase protection was performed using the ribonuclease protection kit RPAIII (Ambion) and the procedures were performed as described in (6) with the exception that a new set of primers was used to prepare the antisense CAT RNA probe: 5’-TACGCTGGTGCTAGCCGCTG-3’ and 3’-GACGC-TACTCACCCTATCGGATATATCACCTAGAAAT-5’ (T7 polymerase binding site is underlined).

RESULTS
Specific interaction between hnRNP A18 and TRX 3’-UTR mRNA
We have previously shown that hnRNP A18 specifically binds to several stress-activated transcripts including TRX (6). Binding of hnRNP A18 to TRX was specific to TRX 3’-UTR since no binding was detected with the ORF or the 5’-UTRs of TRX. In order to further characterize the interaction between hnRNP A18 and TRX mRNA in solution, mobility shift assays were performed with GST fusion hnRNP A18 and 32P-labeled TRX 3’-UTR RNA. Because of the size of the RNA probe (220 nt) we used 1% agarose gel. Data in Figure 1B show that hnRNP A18 formed a single complex with 32P-labeled TRX 3’-UTR RNA in a dose dependent manner (0.12 to 4 μg, lanes 3–8). No binding was observed with GST alone (lane 2). We also performed the RNA band shift with deletion mutants of the TRX 3’-UTR. Our data (lanes 9–13) indicate that the first 120 nt of the 3’-UTR are sufficient to confer binding. No binding was observed with the last 70 nt of the 3’-UTR (lanes 14–18). These data confirm the specific interaction between hnRNP A18 and TRX mRNA previously observed by Northwestern (6).

Most hnRNP proteins exert their RNA binding function through their RBD. However, it has been reported that RGG boxes may increase the general RNA binding affinity (20). To determine whether the hnRNP A18 domain containing the RGG boxes was involved in RNA binding, we engineered deletion mutants and analyzed them by Northwestern with TRX mRNA. The data shown in Figure 1C indicate that both the RBD (lanes 5–7) and the RGG domain (lanes 2–4) can bind RNA independently of each other but maximal binding is only observed when both domains are present (lanes 8–10).

hnRNP A18 and TRX transcripts form a complex and co-localize on ribosomal fractions
To verify if TRX is a target for hnRNP A18 in cells and to support a role for hnRNP A18 on TRX regulation, we performed RNA IP of cellular lysates extracted from RKO cells exposed to UV radiation. We initially used RKO cells stably transfected with GFP–hnRNP A18 to facilitate the detection of the immune complex. The cells were exposed to UV radiation to translocate hnRNP A18 to the cytosol (6) and the proteins were extracted 4 h later. Polysomes were then extracted under conditions that preserve the association of RBPs with target mRNA essentially as described before (19). To facilitate the detection of the bound mRNAs, the polysomes were disrupted by the addition of EDTA during the IP step. hnRNP A18-bound mRNAs were then eluted and used for RT–PCR to amplify the products. The data shown in Figure 2A indicate that endogenous TRX can be amplified from the material immunoprecipitated with anti-GFP antibody to pull down GFP–hnRNP A18 (lane 2) but not when non-specific antibodies (IgG) (lane 1) were used. To verify the specificity of the interaction, we also amplified two oxidative stress-responsive transcripts (21,22) not targeted by hnRNP A18. Our data indicate that neither vascular endothelial growth factor (VEGF) nor GADD45 were preferentially amplified from the mRNAs pulled down by hnRNP A18. To determine whether the same amount of input material was used in both samples we also amplified trace amounts of the non-target GAPDH mRNA. Our data indicate that GAPDH could be amplified to the same extent in both samples but rather inefficiently since three times as much material as TRX had to be loaded to detect its presence. To verify whether endogenous hnRNP A18 could also associate with TRX transcripts in the cells, we repeated the experiment described above but used purified hnRNP A18 antibodies to immunoprecipitate the complex. The data shown in Figure 2B indicate that endogenous hnRNP A18 also specifically associates with TRX transcripts (lane 2). These data confirm the in vitro binding of TRX transcript to hnRNP A18 (Figure 1) and indicate that TRX mRNA is a bona fide target for hnRNP A18 in cells.

To determine whether hnRNP A18 interaction with TRX transcripts could influence translation of TRX, we first performed sucrose gradient analysis (Figure 3) to evaluate whether hnRNP A18 and TRX transcripts co-localize on ribosomal fractions. The RKO cells were exposed or not to UV radiation and the cells extracts were processed as described above in the absence of EDTA before (19). To facilitate the detection of the bound mRNAs, the polysomes were disrupted by the addition of EDTA during the IP step. hnRNP A18-bound mRNAs were then eluted and used for RT–PCR to amplify the products. The data shown in Figure 2A indicate that endogenous TRX can be amplified from the material immunoprecipitated with anti-GFP antibody to pull down GFP–hnRNP A18 (lane 2) but not when non-specific antibodies (IgG) (lane 1) were used. To verify the specificity of the interaction, we also amplified two oxidative stress-responsive transcripts (21,22) not targeted by hnRNP A18. Our data indicate that neither vascular endothelial growth factor (VEGF) nor GADD45 were preferentially amplified from the mRNAs pulled down by hnRNP A18. To determine whether the same amount of input material was used in both samples we also amplified trace amounts of the non-target GAPDH mRNA. Our data indicate that GAPDH could be amplified to the same extent in both samples but rather inefficiently since three times as much material as TRX had to be loaded to detect its presence. To verify whether endogenous hnRNP A18 could also associate with TRX transcripts in the cells, we repeated the experiment described above but used purified hnRNP A18 antibodies to immunoprecipitate the complex. The data shown in Figure 2B indicate that endogenous hnRNP A18 also specifically associates with TRX transcripts (lane 2). These data confirm the in vitro binding of TRX transcript to hnRNP A18 (Figure 1) and indicate that TRX mRNA is a bona fide target for hnRNP A18 in cells.

To determine whether hnRNP A18 interaction with TRX transcripts could influence translation of TRX, we first performed sucrose gradient analysis (Figure 3) to evaluate whether hnRNP A18 and TRX transcripts co-localize on ribosomal fractions. The RKO cells were exposed or not to UV radiation and the cells extracts were processed as described above in the absence of EDTA to preserve the polysomes integrity. The extracts were layered on 10–50% sucrose gradients and centrifuged. Our data (Figure 3) indicate that in the absence of UV radiation (Control), hnRNP A18 is mainly localized in fractions 3 and 4. According to the conventional distribution of the polysomal fractions and the localization of the ribosomal S6 protein and the ribosomal 18 and 28S RNA, these fractions correspond to the mono-ribosomal and low molecular weight polysomal fractions. hnRNP A18 also localized to some extent to fraction 8 which corresponds to a high molecular weight polysomal fraction. In contrast, the
non-ribosomal protein GAPDH is exclusively found in fraction 1, which corresponds to the unbound, non-ribosomal fraction. RT–PCR of TRX transcript indicates that TRX mRNA is detected in fraction 2 but is more abundant in fractions 3–8. Interestingly, the presence of hnRNP A18 in fraction 3 correlates with an increase in TRX mRNA levels. Levels of GAPDH transcript and ribosomal RNAs are relatively constant throughout the gradient. The co-localization of hnRNP A18 and TRX transcript in the mono-ribosomal and low molecular weight polysomal fractions suggest a role for hnRNP A18 in ribosomal assembly and/or translation initiation. Exposing the cells to UV radiation did not change the distribution of either hnRNP A18 or TRX transcript. However, UV radiation increased the levels of both, hnRNP A18 protein and TRX transcript. Again, hnRNP A18 and TRX transcripts co-localized in fractions 3–4. The presence of TRX transcript in high molecular weight polysomal fractions, which have no or low levels of hnRNP A18 (fractions 5–8), would suggest that hnRNP A18 is not involved in translation elongation.

Figure 1. hnRNP A18 binds to TRX 3′-UTR in solution. (A) Schematic representation of the different probes used. FL; full-length, nt; nucleotides. (B) RNA band shift was performed as described in the text. Increasing amounts (0.12 to 4 μg) of GST–hnRNP A18 was incubated with the indicated labeled TRX probe and run on a 1% agarose gel. (C) hnRNP A18 RBD and RGG domain are required for maximal RNA binding. Northwestern analysis was performed with increasing amounts (0.5 to 2 μg) of either full-length (FL) GST–hnRNP A18 (lanes 8–10), GST–hnRNP A18 RGG domain (RGG; lanes 2–4), GST–hnRNP A18 RBD (lanes 5–7) or GST alone (lane 1) and labeled TRX 3′-UTR as described in the text.
hnRNP A18 protein levels correlate with TRX protein levels

hnRNP A18 increases TRX translation in cells

To determine whether hnRNP A18 binding and co-localization with TRX transcript could affect TRX protein levels, we then compared the levels of hnRNP A18 and TRX in response to UV radiation. Our data (Figure 4A) indicate that hnRNP A18 protein levels increase in a dose dependent manner in response to UV radiation. The basal levels of TRX are also increased in response to UV radiation and are the highest at 14 J m$^{-2}$ where hnRNP A18 protein levels are also the highest. To determine whether hnRNP A18 contributed to TRX increased protein levels in response to UV radiation, we measured the levels of hnRNP A18 and TRX protein in RKO cells stably transfected with an hnRNP A18 antisense vector. Our data (Figure 4B) indicate that the levels of hnRNP A18 and TRX are markedly reduced in the antisense cell line. Because stable transfectants may sometimes lead to cellular aberrations, we also transiently transfected RKO cells with a 21 nt small interfering RNA (siRNA) matching only hnRNP A18 sequence in the GenBank database. The data shown in Figure 4C indicate that down regulation of hnRNP A18 (lane 2) reduced the accumulation of both hnRNPA18 and TRX in response to UV radiation (lane 3). This effect is specific since a scrambled RNA sequence of the same length had no effect on TRX (Figure 4C, lane 1). Interestingly, hnRNP A18 had no significant effect on TRX basal levels in absence of radiation (data not shown). This is in good agreement with our data showing that hnRNPA18 is mainly in the nucleus in absence of stress (6).

hnRNP A18 interacts with the translation initiation factor eIF4G

Recent evidence indicate that 3'-UTR binding proteins can regulate the rate of translation initiation by interacting with 5' cap binding proteins (24). Here, we used co-immunoprecipitation assays to evaluate the capacity of hnRNP A18 to interact with components of the basal translational machinery. More specifically we evaluated the capacity of hnRNP A18 to interact with the eukaryotic initiation factors eIF4G,

hnRNP A18 interacts with TRX mRNA in the cells. (A) Polysomes were extracted from RKO cells exposed to 20 J m$^{-2}$ UV radiation. IP with either anti IgG or anti-GFP to pull down GFP-A18 hnRNP (A18) was performed in the presence of 20 mM EDTA to disrupt the polysomes but preserve the association of RBP with target mRNAs. IP was followed by RT–PCR to detect endogenous TRX. As a negative control, VEGF, GADD45 and GAPDH were amplified from the same fractions. To facilitate detection of GAPDH, three times as much material as the TRX products was loaded. (B) Same as (A) except that anti hnRNP A18 was used to pull down mRNAs bound to endogenous hnRNP A18.
one component of the cap binding complex eIF-4. The cells were exposed to UV radiation and lysed under conditions that preserve the integrity of polysomes (Figure 5). The lysates were then incubated with RNAse in the presence of EDTA to dissociate the ribosome. The presence of eIF4G in the cell extracts (InPut) was verified on a small fraction (Figure 6, lane 3). GFP–hnRNP A18 was then immunoprecipitated with GFP antibodies (Santa Cruz) and a negative control was performed with IgG antibodies. The data (Figure 6) indicate that indeed eIF4G form a complex with hnRNP A18 on cells exposed to UV radiation (lane 2). The interaction is specific since eIF4G was not detected in the fraction immunoprecipitated with the negative control IgG (lane 1). Interaction of hnRNP A18 with eIF4G could thus also contribute to the increased translation observed in Figure 5.

Phosphorylation of hnRNP A18

Post-translational modification such as phosphorylation has been reported to affect the translocation and RNA binding activity of a number of RBPs. For example, phosphorylation of hnRNP A1 is associated with its translocation to the cytosol (25) and phosphorylation of the RBP nucleolin increased its RNA binding activity (26) while dephosphorylation of the hnRNP C is required for its binding to RNA (27). Thus, it is important to determine whether hnRNP A18 could be phosphorylated and, if so, whether it affects its RNA binding activity. We first used several purified protein kinases and recombinant hnRNP A18 to investigate which protein kinase may target hnRNP A18 in vitro. The analysis of hnRNP A18 primary sequence reveals several consensus phosphorylation sites for stress-activated kinases. The hnRNP A18 primary sequence indicates two possible Casein Kinase 2 (CK2) sites, several glycogen synthase kinase 3 (GSK3) and potential ATM and Rad3-related kinase ATR. CK2 targets several stress-activated proteins such as p53 (28), the oxidative stress-inducible protein A170 (29) and the murine STI1 protein (30). GSK3 was originally described as a constitutively active regulator of glycogen synthase but has recently been described as being activated during development and under prolonged hypoxia (17). ATR phosphorlysates several substrates in response to UV radiation (31) including the RBP
nucleophosmin (NPM) (32). Hypoxia can activate ATR as well (33). Recently another member of the ATM/ATR protein kinase has been isolated. This kinase was originally named ATX and subsequently renamed SMG1. Like ATM and ATR, SMG1 ATX is activated by UV and X-ray radiation and share substrates specificity with these kinases (34).

Therefore, we investigated whether hnRNP A18 could be phosphorylated by CK2, ATM, ATR, SMG1 ATX and the two GSK3 isoforms, $\alpha$ and $\beta$. The in vitro kinase assay was performed as described before (32). The data in Figure 7A indicate that hnRNP A18 is a good in vitro substrate for GSK3 $\alpha$ and $\beta$ (lanes 1 and 8), CK2 (lane 10) and to some extent for SMG1 $^{\text{ATX}}$ (lane 9) but not for ATM or ATR.

To determine if phosphorylation of hnRNP A18 could affect its RNA binding activity, we phosphorylated hnRNP A18 in vitro with different kinases and cold phosphate and performed Northwestern assay with TRX 3'-UTR. We used only 0.25 and 0.5 $\mu$g of recombinant protein in order to prevent saturation and facilitate the detection of the effect of phosphorylation. The data shown in Figure 7B indicate that even though hnRNP A18 is a good in vitro substrate for CK2 and SMG1 $^{\text{ATX}}$ (Figure 7A), these kinases have no effect on hnRNP A18 RNA binding activity. However, phosphorylation by GSK3$\beta$ (Figure 7B lanes 3 and 4) increases considerably hnRNP A18 binding to 3'-UTR. Interestingly, five consensus GSK3 phosphorylation sites (S144, S148, S152, S155 and S159) are located in hnRNP A18 RGG domain. This domain is required for maximal RNA binding activity (Figure 1C).

The UV-activated hnRNP A18 RNA binding activity is GSK3$\beta$ dependent

The hnRNP A18 was originally identified based upon its rapid induction by UV radiation (7). We subsequently showed (6) that the nuclear hnRNP A18 is also translocated to the cytoplasm in response to UV radiation. Accumulation of hnRNP A18 in the cytoplasm does not require de novo protein synthesis since pre-treatment of the cells with cycloheximide (100 $\mu$M for 4 h) does not prevent the UV-induced translocation (data not shown). To determine whether hnRNP A18 RNA binding activity is affected by UV radiation, we used a GFP antibody to immunoprecipitate GFP–hnRNP A18 from cells exposed or not to UV radiation and measured the RNA binding activity of the immunoprecipitated proteins by Northwestern. The data shown in Figure 8A indicate that the binding activity of hnRNP A18 is increased 2-fold upon exposure to UV radiation (lane 3). The level of induction of the RNA binding activity is in the same range as what has been reported for other RBPs (26) when exposed to cellular stress. Western blot analysis of the same extracts (Figure 8B) indicates that the overall levels of GFP–hnRNP A18 protein levels are not affected by UV radiation (lanes 1 and 3). This suggests that post-translational modifications could contribute to enhance hnRNP A18 RNA binding activity. Our in vitro data (Figure 7B) indicate that GSK3$\beta$ can increase hnRNP A18 RNA binding activity. To determine whether the UV-induced activation of hnRNP A18 was GSK3$\beta$ dependent, we down regulated GSK3$\beta$ with specific siRNA and repeated the Northwestern. Our data indicate that the siRNA were indeed specific for GSK3$\beta$ since they decreased the levels of GSK3$\beta$ considerably but did not affect the levels of GSK3$\alpha$ (Figure 8B, lane 2). Moreover, down regulation of GSK3$\beta$ prevented the activation of hnRNP A18 RNA binding activity upon UV exposure (Figure 8A, lane 4). This effect is specific since scrambled siRNA did not prevent the activation of hnRNP A18 RNA binding activity (lane 3) that is normally observed following UV exposure (lane 6). These data suggest that phosphorylation of hnRNP A18 by GSK3$\beta$ following...
UV radiation exposure is necessary to increase hnRNP A18 RNA binding activity. The presence of GSK3β phosphorylation sites in hnRNP A18 RGG domain and the fact that it co-immunoprecipitates with GSK3β, but not GSK3α (data not shown), also support that hnRNP A18 is a bona fide oxidative stress-responsive GSK3β substrate.

DISCUSSION

It is now becoming increasingly evident that oxidative stress is implicated in several human diseases. The molecular understanding of the oxidative stress response is particularly relevant to solid tumor development and neurodegenerative diseases. The small (12 kDa) oxidoreductase TRX is a ubiquitous multifunctional protein that plays important parts in the oxidative stress response. TRX can regulate cellular signaling and gene transcription in addition to protecting the cells through the quenching of ROS (1). The redox activity of TRX increases the DNA binding and transactivating activity of transcription factors. A recent report (35) indicates that TRX increases the expression and activity of the hypoxia inducible factor (HIF1) and its downstream effector gene the VEGF. This underscores the pivotal role of TRX in the oxidative stress response. Because of its role in stimulating cancer cell growth and inhibiting apoptosis, TRX offers a target for the development of drugs to treat and prevent cancer. Although, studies have focused on the TRX gene expression at the transcriptional level, little is known on the

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**Figure 5.** hnRNP A18 increases TRX translation. (A) Schematic representation of the constructs used in the CAT assay. The poly A tract of the bovine growth hormone (bGh) was placed downstream of the CAT gene under the control of a constitutive pCMV promoter. (B) Representative CAT assay performed by transient co-transfections in RKO cells of either the CAT construct or the CAT construct containing the TRX 3’-UTR, with hnRNP A18. (C) Average of two CAT assays performed with 25 μg of cellular extracts. The conversion of the chloramphenicol was quantified with a Phosphorimager and expressed as a relative fold conversion of total counts of [14C]chloramphenicol compared to basal levels (0 μg hnRNP A18) of % conversion. (D) RNase protection assay was performed as described in the text. The controls yeast RNA (lanes 1 and 2) were digested (+) or not (−) with RNase. Assays were performed in the absence (0) or presence (10 μg) of hnRNP A18 expression vector with the indicated CAT construct. Levels of transcripts were quantified with a Phosphorimager and expressed as relative fold induction compared to basal levels (0 μg hnRNP A18, lane 3).

**Figure 6.** hnRNP A18 forms a complex with eIF4G. The protein fractions of the polysomes extracts (2 mg) were treated with RNase and immunoprecipitated in the presence of EDTA (20 mM) with either control antibodies (IgG) or GFP antibodies to immunoprecipitate GFP-hnRNP A18. The protein complexes formed on dissociated ribosomes in the absence of RNA were resolved on SDS–PAGE and the blot was reacted with eIF4G antibodies (western blot). In lane 3, a fraction of the protein extract (50 μg, 2.5%) was used to assess the amount of eIF4G present in the InPut. The position of the eIF4G is indicated.
post-transcriptional or translational regulation of TRX in response to stress.

Since UV and hypoxia generate ROS and both induce hnRNP A18 expression (10), we believe that hnRNP A18 is an oxidative stress-inducible protein that can participate in the cellular response to oxidative stress. Our immunofluorescence data indicate that UV (6) and hypoxia (CoCl$_2$ 200 µM for 6 h, data not shown) can induce translocation of the nuclear hnRNP A18 to the cytoplasm. One function of the hnRNP A18 in the cytoplasm could be to interact with and regulate the expression of targeted mRNAs transcripts. Using a modified version of the SELEX technique 46 potential hnRNP A18 mRNA targets were identified (6). Among these transcripts, more than 20, including TRX, were identified as stress-responsive mRNA targets (6). In this work, IP of mRNA–protein complexes (Figure 2) verified that TRX is a bona fide target of hnRNP A18 in vivo. In addition, our in vitro binding data indicate that hnRNP A18 bind to TRX 3'-UTR in a dose dependent manner (Figure 1). Interestingly, both the RBD and the RGG domain can bind TRX 3'-UTR independently but maximal binding is only observed when both domains are present (Figure 1C). The hnRNP A18 RGG domain (residues 84–172) contains the RGG boxes (residues 93–119) and several phosphorylation sites. Our in vitro data (Figure 7) indicate that phosphorylation by the hypoxia inducible GSK3β (17) increases hnRNP A18 RNA binding activity considerably. The GSK3β phosphorylation sites are all located within the RGG domain between residues 144 and 159. Because the phosphorylation of hnRNP A18 is likely to increase its net negative charge and should be more repulsive of the negatively charged RNAs targets we believe that phosphorylation of the RGG domain triggers a conformational change that facilitates RNA binding. This however remains to be investigated. Our data underscore the uniqueness of hnRNP A18 in that the RGG domain does not merely increase the general RNA binding activity but is actively involved in the binding. So far, only hnRNP U had been reported to use an RGG box in specific RNA binding (36). In fact, the RGG box constitutes the entire RBD in this protein (36).

The significance of hnRNP A18 binding to TRX 3'-UTR is highlighted by our data on translation. Our modified CAT assay (Figure 5) indicates that over-expression of hnRNP A18 increases general translation and more specifically TRX translation. Addition of the TRX 3'-UTR downstream of the CAT gene reduced the basal translation of this transcript (Figure 5B) suggesting that the sequence destabilizes or represses the translation of the transcript. However, addition of hnRNP A18 increased translation to similar or even higher levels than the ones observed on construct lacking the TRX 3'-UTR (Figure 5B). This suggest that hnRNP A18 could bind to the TRX 3'-UTR, as shown in Figure 1, and stabilize the transcript, as shown in Figure 5D, to increase translation. Another
The possibility is that hnRNP A18 could compete out repressive proteins bound to TRX 3'-UTR and thus de-repress translation. This possibility is currently under investigation. The capacity to destabilize or repress translation seems to be particular to transcripts targeted by hnRNP A18 since adding the 3'-UTR sequence of another hnRNP A18 target, RPA also reduced translation of this gene (6) but the sequence of an unrelated transcript (GADD45) had no effect on this gene basal translation (Chakravarty, D. and Carrier, F., unpublished data). We have recently shown (6) and also observed here (Figure 5) that hnRNP A18 could stimulate translation at higher levels than it stabilizes mRNA transcripts. This led us to suggest that hnRNP A18 could also interact with components of the basal translational machinery to stimulate translation. The data presented here indicate that indeed, hnRNP A18 interacts with the translation initiation factor eIF4G (Figure 6). The effect of hnRNP A18 on translation is thus reminiscent of the effect of certain transcription factor such as p53 on transcription, p53 is a specific transcription factor that can also interact with components of the basal transcription machinery such as TBP and TAFs to regulate transcription (37).

Recently, biomedical evidences have been provided to indicate that many 3'-UTR binding proteins can regulate translation initiation at the 5' end. This phenomenon led to a model of circularization of mRNA (38) in which 3' and 5' ends of RNA form a ‘kissing’ loop through the interaction between 3' RBPs and the cap binding protein eIF4F. Such end-to-end complex is thought to increase translation efficiency and permits 3'-UTR mediated translation control (23). There are three important elements involved in this mRNA circularization model: first, the binding of an RBP to the 3'-UTR; second, interaction with the 5' end through bridge protein(s); and third, the effect of this end-to-end complex on translation. The data presented here indicate that all three elements are present in the case of hnRNP A18; (i) binding to 3'-UTR (Figure 1), (ii) interaction with 5' end through bridge protein, eIF4G (Figure 6) and (iii) effect on translation (Figures 4 and 5). hnRNP A18 might thus fit into the ‘kissing’ loop model to increase translation in general and in particular stress related transcripts such as TRX. The specificity of hnRNP A18 translational control on TRX transcript is further supported by the correlation of the down regulation of hnRNP A18 with the down regulation of TRX protein levels (Figure 4).

Regulation of RBPs activity can be mediated through post-translational modifications (25). The data presented here indicate that hnRNP A18 is an in vitro substrate for several stress related kinases including CK II, SMG1 and GSK3 but not ATR and ATM. Most interestingly, only GSK3 phosphorylation increased hnRNP A18 RNA binding activity in vitro (Figure 7B). GSK3 was originally thought of as a constitutively expressed cytosolic protein involved in glycogen metabolism. The phosphorylation of several transcription factors by...
GSK3β implied a nuclear localization as well (17). Moreover, certain cellular stresses can lead to GSK3β accumulation in the nucleus (39). Actually phosphorylation of the transcription factor NFATc by GSK3β induces its transport out of the nucleus (40). It thus seems likely that GSK3β could affect hnRNPA18 similarly in response to oxidative stress. Because GSK3β, hnRNPA18 and TRX are all activated by hypoxia, our data suggest an interesting regulatory pathway where in response to hypoxia or oxidative stress generated by UV radiation, hnRNPA18 would be phosphorylated by GSK3β (Figure 7), translocate to the cytosol (CoCl2 100 μM, 4 h data not shown), bind to TRX transcript (Figures 2 and 3) and increase its translation (Figures 4 and 5) by interacting with the general translational machinery (Figure 6). Even though the basic principles of this model have been presented here the molecular details and the kinetics of this model remain to be elucidated.

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