A Point Mutation in the Exon Junction Complex Factor Y14 Disrupts Its Function in mRNA Cap Binding and Translation Enhancement*

Received for publication, November 16, 2015, and in revised form, February 5, 2016. Published, JBC Papers in Press, February 17, 2016, DOI 10.1074/jbc.M115.704544

Tzu-Wei Chuang, Kuo-Ming Lee, Yuan-Chao Lou, Chia-Chen Lu, and Woan-Yuh Tarn

From the Institute of Biomedical Sciences, Academia Sinica, Taipei 11529, Taiwan

Eukaryotic mRNA biogenesis involves a series of interconnected steps mediated by RNA-binding proteins. The exon junction complex core protein Y14 is required for nonsense-mediated mRNA decay (NMD) and promotes translation. Moreover, Y14 binds the cap structure of mRNAs and inhibits the activity of the decapping enzyme Dcp2. In this report, we show that an evolutionarily conserved tryptophan residue (Trp-73) of Y14 is critical for its binding to the mRNA cap structure. A Trp-73 mutant (W73V) bound weakly to mRNAs and failed to protect them from degradation. However, this mutant could still interact with the NMD and mRNA degradation factors and retained partial NMD activity. In addition, we found that the W73V mutant could not interact with translation initiation factors. Overexpression of W73V suppressed reporter mRNA translation in vitro and in vivo and reduced the level of a set of nascent proteins. These results reveal a residue of Y14 that confers cap-binding activity and is essential for Y14-mediated enhancement of translation. Finally, we demonstrated that Y14 may selectively and differentially modulate protein biosynthesis.

The post-splicing processing factor Y14 is involved in multiple steps of mRNA biogenesis (1–5). Y14 forms a heterodimer with Mago (6, 7). Y14/Mago genes have coevolved in a wide range of species except for yeast and are essential for germ cell determination during gametogenesis (8). Drosophila Y14/Mago participates in the transport and translational control of posterior mRNAs during oogenesis (6, 9, 10). In vertebrates, Y14/Mago acts as a core component of the exon junction complex (EJC), which is deposited immediately upstream of every ligated exon during precursor mRNA (pre-mRNA) splicing, and is thus involved in mRNA export, nonsense-mediated mRNA decay (NMD), and translation control (3, 5, 7, 11).

Y14 is present in ribosome-associated mRNA ribonucleoprotein (mRNP) fractions (12), and depletion of Y14 inhibits splicing-dependent translational activation (5). In general, the EJC factors act in concert to promote the pioneer round of translation. The Y14/Mago interacting partner PYM interacts with ribosomal proteins and thus enhances the translation of EJC-bound spliced mRNAs (13). This observation further underscores the importance of Y14 in EJC-mediated translational control. When tethered to a reporter mRNA, Y14 enhances translation, as has been observed with other EJC and NMD factors (11). Y14 may function early during translation, whereas another EJC core factor, elf4AIII, activates translation after 80S ribosome complex formation (5). Hence, perhaps individual EJC factors modulate productive translation via different mechanisms and in a gene-specific manner.

Eukaryotic mRNA decay involves deadenylation-triggered decapping followed by 5′ to 3′ exonuclease activity (14, 15). Decapping is catalyzed by Dcp2 and is positively and negatively regulated by decapping activators and translation factors, respectively (16–21). We previously reported that human Y14 interacts with the decapping complex and inhibits the activity of Dcp2 (22). The eukaryotic translation initiation factor 4E (eIF4E) competes with Dcp1 for binding to the cap structure and inhibits the decapping activity of Dcp2, whereby it prevents mRNA decay (19). Moreover, the cytoplasmic poly(A)-binding protein also inhibits Dcp2, suggesting that translation competes with mRNA decay (18). We also previously showed that overexpression of Y14 prolongs the half-life of reporter mRNAs, implying a role for Y14 in mRNA protection (22); this may be in line with its function in promoting translation.

We have also reported that Y14 directly interacts with the mRNA cap structure (22), but whether cap binding is necessary for the function of Y14 in mRNA biogenesis remains unclear. In the present study, we attempted to understand the biochemical features and biological relevance of the cap-binding activity of Y14. We identified mutations that disrupted the cap-binding ability of Y14 and characterized one mutant with respect to its role(s) in mRNA metabolism.

Experimental Procedures

Plasmid Construction—The bacterial expression vectors encoding His-tagged human Y14 and Dcp2, and glutathione S-transferase (GST)-Y14 were described previously (22–24). The Y14 mutants (W73V, F76V, F93V, Y116V, Y121V, E122V, W148V, and F150V) were generated from pGEX-GST-Y14 by site-directed mutagenesis and were verified by DNA sequencing. The mammalian expression vectors for expression of FLAG-tagged Y14 and CBP80 and of MS2 coat protein (MCP)-fused Y14 were described previously (22). The Y14 mutants W73V and L118R encoded in mammalian expression vectors...
were also generated by site-directed mutagenesis. The reporter vectors and probes used for RNA degradation assays and translation assays were as described (22, 25–27).

Purification of Recombinant Proteins—Recombinant GST-fusion and His-tagged proteins were expressed and induced in Escherichia coli strain BLR and purified using glutathione-Sepharose (GE Healthcare) and His-Link Resin (Novagen), respectively, as described (22, 23, 28).

Cell Culture, Transfection, Immunoprecipitation, Cap-affinity Chromatography, and RT-PCR—Cell culture and transient transfection of human HEK293 cells and HeLa tet-off cells were carried out essentially as described (22, 27). Immunoprecipitation and cap-affinity chromatography were performed as described (22). Immunoprecipitated RNAs were treated with RNase-free T1 (Promega) and subjected to reverse transcription–PCR (RT-PCR) (27) using specific primers: βG forward: 5'-GAATGGTGCAATGTCGAC-3'; βG reverse: 5'-TTCAGATCC-AGCTGTTGAGCCGACG-3'; U4 forward: 5'-TGCGGATCT-CTAGCCGAAATGCGATATG; U4 reverse: 5'-CTGTGAAAAATGGCCAA-TGC; GAPDH forward: 5'-GGAGGTACAGGAATTGTGCTGATG-3'; GAPDH reverse: 5'-AGCCCTCTCCATGTTGTGGAAGAC-3'.

Antibodies—Monoclonal antibodies used were against tubulin (MS-581, NeoMarkers), GAPDH (60004-1-Ig, ProteinTech), eIF3 (EMCV) internal ribosome entry site sequence (pFL-iresRL) and.FLAG-Y14 expression vector (wild-type or mutants). After transfection for 48 h, cells were lysed in lysis buffer (Promega), and FLAG-Y14 expression vector (wild-type or mutants). After transfection for 48 h, cells were lysed in lysis buffer (Promega), and the reporter transcripts were examined by RT-qPCR using specific primers: pFL forward: 5'-GGATATATAGTTGTGAATCCCTT-3'; pFL reverse: 5'-CTGTGAAAAATGGCCAA-TGC; pFL-iresRL forward: 5'-GGAGGTACAGGAATTGTGCTGATG-3'; pFL-iresRL reverse: 5'-CTAGCTAGCGAAGTTCAATTTG-3'; pFL-iresRL reverse: 5'-CTAGCTAGCGAAGTTCAATTTG-3'; pFL-iresRL reverse: 5'-CTAGCTAGCGAAGTTCAATTTG-3'. The activities of firefly luciferase and Renilla luciferase were measured using the Renilla-Glo Luciferase Assay system (Promega) or the Dual-Luciferase Reporter Assay system (Promega) with the SpectraMax Luminescence Microplate Reader System (Molecular Devices).

In Vivo Translation Assay—In vivo translation was performed essentially as described (25, 26). HeLa cells were cotransfected with a Renilla luciferase reporter (pRL) or the dicistronic reporter containing the encephalomyocarditis virus (EMCV) internal ribosome entry site sequence (pFL-iresRL) and FLAG-Y14 expression vector (wild-type or mutants). After transfection for 48 h, cells were lysed in lysis buffer (Promega), and the reporter transcripts were examined by RT-qPCR using specific primers: pRL forward: 5'-GGATATATAGTTGTGAATCCCTT-3'; pRL reverse: 5'-GGATATATAGTTGTGAATCCCTT-3'; pRL reverse: 5'-GGATATATAGTTGTGAATCCCTT-3'; pRL reverse: 5'-GGATATATAGTTGTGAATCCCTT-3'. The activities of firefly luciferase and Renilla luciferase were measured using the Renilla-Glo Luciferase Assay system (Promega) or the Dual-Luciferase Reporter Assay system (Promega) with the SpectraMax Luminescence Microplate Reader System (Molecular Devices).

In Vitro Translation Assay—In vitro translation was performed essentially as described (25, 26). The Renilla luciferase reporter (pRL) was linearized and used as a template for in vitro transcription. Different amounts of recombinant His-tagged Y14 or 2 μg (~100 pmol) of His-Y14 (wild-type or W73V mutant) were incubated with 5 μl of rabbit reticulocyte lysate (Promega) and 50 ng of in vitro transcribed Renilla luciferase reporter mRNA in a 10-μl mixture at 30 °C for 3 h. Renilla luciferase activity was measured as described above. The Renilla luciferase transcripts were examined by RT-qPCR using primers as described above.

Affinity Purification of Biotinylated 1-Azidohomoalanine-labeled Proteins—HEK293 cells were transiently transfected with the empty or FLAG-Y14 (wild-type or W73V vector). After transfection for 48 h, cells were cultured in methionine-free medium for 30 min to deplete endogenous methionine, followed by incubation with 50 μM 1-azidohomoalanine (Invitrogen) for 4 h. Cells were collected, suspended in lysis buffer containing 1% SDS, 50 mM Tris, pH 8.0, and 1× protease inhibitor mixture (Roche), sonicated for two cycles of 30 s each and centrifuged for 5 min at 13,000 × g at 4 °C to pellet cellular debris. Cell lysates were subjected to Click reactions overnight at 4 °C with 40 μM biotin (Invitrogen) using the Click-IT™ Protein Reaction Buffer kit (Invitrogen) according to the instructions. Biotin-tagged proteins were then incubated with 20 μl of Dynabeads M-280 Streptavidin (Invitrogen) overnight at 4 °C followed by washing five times with phosphate-buffered saline.
containing 0.5% SDS. Purified proteins were subjected to SDS-PAGE (12% acrylamide) and immunoblotting. 

Sucrose Gradient Centrifugation and Polysones Profile Analysis—Cytoplasmic extracts of HeLa cells were fractionated on linear 15–40% sucrose gradients as described (25). Centrifugation was performed with an SW41 rotor (Beckman) at 38,000 rpm for 3 h at 4 °C. After centrifugation, 20 fractions of 500 µl each were taken sequentially from the top of each gradient, and RNAs were recovered for RT-PCR using specific primers: 18S forward: 5’-TACCACATCCAAGGAGGAGCA; 18S reverse: 5’-TGGAAATTAGCCGCGTGTGGCA; 28S forward: 5’-AACGGAGTTTCCCAGTCTCC; 28S reverse: 5’-CTTACCGGTCCAGACTGAG; Tce2b forward: 5’-TGGCAACCCAGTCAACAG; Tce2b reverse: 5’-AAACATGGCTTGGGTCTCAG; Rrp41 forward: 5’-TACATTGACGGCACAAC; Rrp41 reverse: 5’-ATGGCTGCTCTGCAGATCTG; NdubB forward: 5’-GTCGCGGTTAGGACTAGG; NdubB reverse: 5’-GTAGTCGCGGCGGAGGT; H3 forward: 5’-CTGGCTTTTCAAGGCGTGTG; H3 reverse: 5’-GCAAGGGTTGCTTCCAAA.

In Vitro Pull Down Assay—In vitro pull down was performed essentially as described (23, 24). PYM was in vitro translated and 33S-labeled using the TNT-coupled transcription/translation system (Promega) and was incubated with 2 µg of individual GST-fusion proteins (~40 pmol) in a 50-µl mixture for 30 min at 30 °C followed by affinity selection with glutathione-Sepharose (GE Healthcare).

Electrophoretic Mobility Shift Assay—For electrophoretic mobility shift assay, 5 × 10⁵ cpm (~75 fmol) of in vitro-transcribed 33P-labeled PIP85αΔi RNA was incubated with 1 µg of one of the GST-Y14 fusions (~20 pmol) in a 25-µl mixture for 30 min at 30 °C. The reaction mixture was then analyzed by electrophoresis on a non-denaturing 6% polyacrylamide gel in 0.5 × TBE buffer (90 mM Tris, 64.6 mM boric acid, 2.5 mM EDTA, pH 8.3).

Results

Identification of Residues in Y14 that Bind the mRNA Cap—To identify residues required for cap-binding of Y14, we first compared Y14 with that of two other cap-binding proteins, namely eIF4E and CBP20. Basic local alignment search revealed that Y14 has 25.3% and 27.5% overall amino acid sequence similarity with eIF4E and CBP20, respectively (Fig. 1A). The similarity between Y14 and CBP20 is essentially confined to the RNA recognition motif. Molecular modeling and site-directed mutagenesis have revealed that four residues (Tyr-20, Tyr-43, Phe-83, and Phe-85) in the RNA recognition motif of CBP20 are critical for cap binding (29, 30); among those, Tyr-43 and Phe-83 associate with mRNA substrates. The result showed that W73V mutant had equivalent capacity as wild-type to co-precipitate the mRNA decapping and degradation factors including Dcp2 (Fig. 2A). We also performed the decapping assay by incubating recombinant His-tagged Dcp2 with the RNA fragment containing 32P-labeled cap (m7G’pppG) as used above. Dcp2 generated decapped RNA and m7G’ppp (Fig. 2B, lane 2), as previously reported (22). The W73V mutant was able to inhibit the decapping activity of Dcp2 the same as the wild-type (lanes 4 and 5), suggesting that Y14-mediated inhibition of decapping is independent of cap binding.

Next, we evaluated the capability of W73V to protect mRNA from degradation. We used a tetracycline-inducible chimeric β-globin gene as a reporter, of which the 3’-UTR was derived from the TNF gene (Fig. 3A, diagram). The TNF-UTR contains AU-rich elements, which lead to rapid degradation of mRNA (32). As compared with the mock-treated samples, overexpression of Y14 prolonged the half-life of the reporter mRNA (Fig. 3A, lanes 1–6). W73V failed to stabilize this AU-rich element-containing reporter mRNA (lanes 7–9), although it could inhibit decapping in vitro (Fig. 2). We then performed immunoprecipitation-coupled RT-PCR to evaluate whether Y14 associates with mRNA substrates. The result showed that W73V largely lost its mRNA-binding capacity as compared with wild-type (Fig. 3B). Moreover, the electromobility shift assay also revealed that W73V failed to form stable complexes with an RNA substrate (Fig. 3C). Therefore, W73V may be unable to protect mRNAs.

W73V Retains Partial NMD Activity—The EJC is loaded onto the spliced mRNA during pre-mRNA splicing and plays an important role in NMD (1, 3, 4). We next examined whether W73V retained the NMD activity of Y14. Using immunoprecipitation and immunoblotting, we observed that the W73V mutant was able to interact with other EJC core components
A Y14 Mutant Defective in Cap Binding and Translation Enhancement

A

Y14  EDYDSVEQDGDEPGP-QRSVEQWILYTGVHEEATEDIHDKPAEYEIKNIHNLNDRT
CBP20 SQYRDQHRGDNSEGEKLLKSCTLYGVLNYFYTEEQIYELFSKSGDIKKIMGDLDMK

β1  β2

α1  α2

β3  β4

PVM  116  121122  148190

Y14  GYLKQELTVIVETYKEAQAAMEGLNGQDLMQPISSVDRCFV--RPPPKGKRGRGRRSRS
CBP20  KTACGFCYEYSSRADAENAMYINGTRLDDRIIIRTDAAGFEKGRQYGRSGGO---V

β1  α1  β2

PVM  116  121122

Y14  ECHLILFVTGVHEEATEDIHDKPAEYEIKNIHNLNDRTGYLKGYTLYVETYKEAQQAA
eIF4E  KTIGANLRLIKSFDTVEDFWALYNIHQLSNNLMPGCDYSLFKDGIPEM---WDEKNNRRGG

β1  α1  β2

PVM  116  121122

Y14  MEGLNG
eIF4E  RWLITL.

B

F150
W73
F76
Y116

Y124
W148

D

mock  Y14  W75V  L178R  CBP80

- Y14

- Y14

- CBP80

- CBP80

mGTP Sepharose

10% input

C

GST  Y14  W75V  F150V

GST  Y14  W75V  F150V

Auto-radiography

Coomassie blue

Y14

Y14

1  2  3  4  5

Relative cap-binding activity

kDa

47  32

Y14  W75V  F76V  P33V  Y116V  Y121V  E122V  W148V  F150V

GST-Y14

1  2  3  4  5  6  7  8  9

8568  JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 291 • NUMBER 16 • APRIL 15, 2016
FIGURE 1. Identification of Y14 residues that are essential for cap binding. A, sequence alignment was performed using Clustal Omega and Vector NTI AlignX software (Invitrogen). The RNA recognition motif of Y14 and CBP20 is underlined. The secondary structure elements of Y14 are shown above the sequences. Red: cap-binding residues of CBP20 and eIF4E. Blue: residues of Y14 mutated in this study. Yellow highlighting: mutated Y14 residues that are conserved with CBP20 and eIF4E. Gly-111 and Tyr-112 of Y14 interact with PYM. B, predicted structure of the Y14 and m^3GppG interaction shows that Trp-73, Phe-76, Tyr-116, and Phe-150 on the surface are engaged in cap interaction.

C, for UV cross-linking, GST or GST-Y14 was incubated with 32P-labeled capped PIP85aΔi RNA. After UV radiation and RNase digestion, proteins were resolved by SDS-PAGE and detected with autoradiography and Coomassie Blue staining. The bar graph shows relative cap-binding ability of different Y14 mutants compared with wild-type Y14; the averages and standard deviations were obtained from three independent experiments. Below the bar graph are recombinant GST-Y14 (wild-type and mutants).

FIGURE 2. The cap binding-deficient W73V is capable of interacting with the decapping complex and inhibiting decapping in vitro. A, the empty (mock) vector or expression vector encoding FLAG-Y14 (wild-type or W73V) was transfected into HEK293 cells. Immunoprecipitation (IP) was performed with anti-FLAG agarose in the absence (−) or presence (+) of RNase A. Input (2%) and IP were subjected to immunoblotting using antibodies against Edc4, Xm2, Dcp1a, Dcp2, GAPDH, and FLAG. Unbound lysates (2%) were analyzed only by using anti-FLAG. B, decapping reaction was performed by incubating His-Dcp2 with 32P-labeled capped PIP85aΔi RNA as substrate without (−) or with addition of GST or GST-Y14 (wild-type or W73V); as shown in Fig. 1C; the products were analyzed by thin-layer chromatography (Ori: origin of the analyte). Lane 1 shows RNA only. Asterisk represents 32P. Dot indicates an unidentified nucleotide, whose level varied in different batches of recombinant Y14 proteins (22). Decapping efficiency was determined by the ratio of m^7Gpp to total (sum of all spots). Relative decapping efficiency is shown at the bottom; the averages (Ave.) and standard deviations were obtained from three independent experiments.

FIGURE 3. The cap binding-deficient W73V fails to protect mRNA owing to loss of mRNA-binding activity. A, HeLa tet-off cells were co-transfected with vectors encoding the TNFα-3′-UTR-containing βG reporter (TNF-UTR), the reference GFP and the mock or FLAG-Y14 expression vector for 48 h. After Dox addition for 0, 2, or 4 h, total RNA was prepared from each transfectant and then subjected to RT-PCR using specific primers for GFP or βG. Immunoblotting (IB) shows FLAG-Y14, Y14, and tubulin proteins. A semi-logarithmic graph of the RT-PCR analysis shows relative βG levels that were normalized to the GFP level in each transfectant; the averages and standard deviations were obtained from three independent experiments. B, transient transfection was performed as in panel A (without the GFP vector). Cell lysates were subjected to immunoprecipitation (IP) using anti-FLAG-agarose, followed by RT-PCR using primers specific to βG mRNA, U4 small nuclear RNA and GAPDH. C, EMSA was performed using GST or GST-Y14 (wild-type or W73V) and in vitro transcribed 32P-labeled PIP85aΔi RNA as substrate. The reactions were analyzed on a 6% polyacrylamide non-denaturing gel.
A Y14 Mutant Defective in Cap Binding and Translation Enhancement

(A) Y14 Selectively Affects Protein Biosynthesis—We observed above that overexpression of W73V suppressed reporter translation (Fig. 5) but did not significantly affect global new protein synthesis (Fig. 6A). Because Y14 can inhibit the activity of Dcp2, we posited that Y14 may modulate the expression of Dcp2-specific targets (35). Upon overexpression of W73V, we did not detect any significant change in the steady-state mRNA level of selected Dcp2 targets (the Von Hippel-Lindau complex component Tceb2, the exosome component Rrp41 and the NADH dehydrogenase subunit NdufB7) using RT-PCR (Fig. 6B, RT-PCR). Therefore, we examined the synthesis of nascent proteins upon W73V overexpression. Using e-azidohomoalanine labeling followed by in vitro biotinylation, we selected newly synthesized proteins using streptavidin chromatography. Immunoblotting showed that the level of nascent Rrp41, Tceb2, and NdufB7 was significantly reduced in W73V-overexpressing HeLa cells, whereas the level of histone 3 (control) was only partially reduced (Fig. 6B, immunoblotting).

To determine whether Y14 is indeed involved in the translation of the above-mentioned transcripts, we depleted Y14 using a short interfering RNA and evaluated the resultant polysome profiles. Y14 knockdown did not cause a significant change in

not eIF4E (Fig. 5A, lane 7). This observation was consistent with a previous report that EJC factors prefer to associate with CBP80-bound transcripts (34). However, the interactions of Y14 with PYM and eIFs, except for eIF4B, were essentially dependent on RNA (lanes 8). W73V did not interact with the majority of those factors, except for eIF4B (lane 9). Using recombinant Y14 proteins, we further confirmed that W73V failed to pull down in vitro translated PYM (Fig. 5B). It has been reported that PYM interacts with Y14/Magoh and recruits the eIF complex to the EJC and promotes translation (13). Immunoprecipitation confirmed that excess PYM promoted the interaction between FLAG-Y14 and eIFs but failed to recruit eIFs to W73V (Fig. 5C).

Next, we assessed the effect of W73V on translation both in vivo and in vitro using translation reporter assays. For the in vivo translation assay, we used the single reporter, pRL, and a dicistronic reporter consisting of firefly luciferase followed by an internal ribosome entry site sequence and Renilla luciferase, pFL-iresRL. Overexpression of wild-type Y14 increased cap-dependent translation of both reporters by ~50% (Fig. 5D); this comparable translation enhancement indicated that Y14 functions in cap-dependent translation as reported (5). Furthermore, the translation assays showed that L118R also promoted translation, albeit to a lesser extent than the wild-type, whereas W73V suppressed the translation of both reporters by ~50% (Fig. 5D). Therefore, W73V had a dominant-negative effect as observed above.

The in vitro translation assay was performed in rabbit reticulocyte lysate using recombinant His-tagged Y14. His-tagged Y14 promoted luciferase reporter translation in a dose-dependent manner (Fig. 5E). While compared wild-type and W73V, we observed that W73V suppressed translation (Fig. 5F), consistent with the result of in vivo translation. Together, the results indicated that the W73V mutation disrupted the interaction between Y14 and PYM and several eIFs and further exerted a dominant-negative effect on translation.

We observed above that overexpression of W73V suppressed reporter translation (Fig. 5) but did not significantly affect global new protein synthesis (Fig. 6A). Because Y14 can inhibit the activity of Dcp2, we posited that Y14 may modulate the expression of Dcp2-specific targets (35). Upon overexpression of W73V, we did not detect any significant change in the steady-state mRNA level of selected Dcp2 targets (the Von Hippel-Lindau complex component Tceb2, the exosome component Rrp41 and the NADH dehydrogenase subunit NdufB7) using RT-PCR (Fig. 6B, RT-PCR). Therefore, we examined the synthesis of nascent proteins upon W73V overexpression. Using 1-azidohomoalanine labeling followed by in vitro biotinylation, we selected newly synthesized proteins using streptavidin chromatography. Immunoblotting showed that the level of nascent Rrp41, Tceb2, and NdufB7 was significantly reduced in W73V-overexpressing HeLa293 cells, whereas the level of histone 3 (control) was only partially reduced (Fig. 6B, immunoblotting).

To determine whether Y14 is indeed involved in the translation of the above-mentioned transcripts, we depleted Y14 using a short interfering RNA and evaluated the resultant polysome profiles. Y14 knockdown did not cause a significant change in...
polysomes (28S and 18S rRNAs) but affected the distribution of the examined transcripts to different extents (Fig. 6C, polysome profiles). To quantify the effects of Y14 depletion, we pooled light and heavy fractions and performed RT-PCR to detect the three above-mentioned transcripts. The result confirmed that the abundance of Tceb2 mRNA was increased in light fractions, whereas Rrp41 and NdufB7 had modest or minimal change (Fig. 6C, bar graphs). Nevertheless, the profile of histone 3 mRNA was not changed. Thus, Y14 may be differentially involved in the translation of these mRNAs. Finally, we performed immunoblotting analysis to evaluate their encoded proteins at the steady-state level. Fig. 6D shows that the level of Tceb2 protein was reduced up to 60%, whereas that of the other two was minimally or not affected. Therefore, Y14 may selec-
A Y14 Mutant Defective in Cap Binding and Translation Enhancement

A Y14 Mutant Defective in Cap Binding and Translation Enhancement

Discussion

Our previous report revealed that Y14 directly binds the cap structure of mRNAs (22). Based on sequence analysis and structural modeling, we performed mutagenesis and identified residues Trp-73 and Phe-150 of Y14 as being critical for cap binding (Fig. 1). Moreover, none of the other examined residues was important. Trp-73 and Phe-150 are evolutionarily conserved and are located at the N- and C-terminal ends of the RNA recognition motif, respectively (6). A model for cap binding by Y14 suggested that the cap forms base-stacking interactions with these residues, which may be analogous to Trp-56 and

FIGURE 6. Overexpression of W73V or depletion of Y14 impairs the translation of specific cellular mRNAs. A, HEK293 cells were transiently transfected with the empty or FLAG-Y14 (wild-type or W73V) vector. At 2 days post-transfection, L-azidohomoalanine was added into methionine-free medium for 4 h. Total protein was harvested and subjected to in vitro biotinylation and immunoblotting using anti-biotin. B, as in panel A, biotinylated cellular proteins were subjected to affinity purification (AP) using streptavidin. Input protein (1%) is shown. Immunoblotting was performed using antibodies against the indicated proteins (upper panel). Lower panel shows RT-PCR analysis of indicated mRNAs. C, HeLa cells were transfected with control short interfering RNA (siC) or Y14-targeting short interfering RNA (siY14) for 2 days. The cytoplasmic extract was subjected to sedimentation through a continuous 15–40% sucrose gradient. RNAs were recovered from odd-numbered fractions of 20 gradient fractions and analyzed by RT-PCR using specific primers as indicated. The positions of 40S, 60S, and 80S ribosomes and polysomes are indicated. Immunoblotting revealed the Y14 knockdown efficiency. Quantitative representation shows relative mRNA abundance in light fractions (1–9) (versus total) of mock and Y14-depleted HeLa cells. The averages and standard deviations were obtained from three independent experiments. D, immunoblotting was performed using total cell protein from mock or Y14-knockdown HeLa cells using antibodies against the indicated proteins.
Trp-102 of eIF4E (31). However, we previously showed that the RNA body of capped mRNAs also contributes to Y14 binding to the cap (22), and thus we cannot exclude the possibility that Trp-73 or Phe-150 directly contacts the RNA body, thereby enhancing the binding of Y14 to the cap. We indeed observed that W73V bound poorly to mRNAs in vivo and in vitro (Fig. 3). This might be a consequence of the loss of cap binding by the W73V mutant; alternatively, this may support the above assumption that Trp-73 interacts with the RNA body. Nevertheless, the proposed model for the Y14-cap interaction requires further experimental confirmation.

W73V could interact with the EJC and NMD factors as well as the decapping factors and mRNA degradation enzymes (Figs. 2 and 4), but it failed to interact with the translation initiation complex (Fig. 5). It is reasonable to assume that the latter results from the inability of W73V to interact with PYM, the factor that connects the EJC to the translation machinery (3). However, since Trp-73 is located in the B1 sheet of Y14 and is structurally distant from the previously identified PYM-interacting B2-B3 loop (36), its mutations may not directly disrupt the Y14-PYM interaction. We assume that the stable Y14-PYM interaction may require RNA and additional protein factors, and thus W73V, which cannot bind RNA, failed to stably interact with PYM. Nevertheless, since W73V retained the ability to interact with eIF4B, it may be able to sequester eIF4B and perhaps other yet unidentified eIFs to exert a dominant-negative effect on translation.

Our previous report indicated that Y14 can protect mRNAs from degradation independent of the EJC (22). Our present study shows that W73V could still interact with the decapping factors and inhibit Dcp2 activity, but it was unable to slow the degradation of a reporter bearing a signal for rapid mRNA degradation (Figs. 2, 3, and 7). We assumed that this was due to the loss of its ability to bind the cap or RNA. Moreover, our tethering assay showed that W73V retained partial ability to participate in NMD (Fig. 4). Perhaps the association of W73V with the NMD factors could somewhat compensate for this deficit in RNA binding and translation.

In this study, we found novel functions of Y14. We observed that Y14 interacts with the mRNA cap and two components of the cap binding complex (i.e. eIF4A1 and eIF4G) but does not interact with eIF4E, and that PYM could enhance the interaction between Y14 and eIF4A1/4G (Fig. 5). Therefore, it is intriguing whether Y14 promotes translation via directly interacting with the cap and certain eIFs and whether PYM promotes such a function of Y14 (Fig. 7). Moreover, we assessed the stability and translation capacity of previously reported Dcp2 target mRNAs (Tceb2, Rrp41, and NdufB7). Overexpression of W73V did not change the steady-state levels of these mRNA but impaired their translation (Figs. 6 and 7). Interestingly, we reproducibly observed that Y14 knockdown reduced the steady-state level of Tceb2 protein. Tceb2 protein appeared to be susceptible to proteasome degradation (data not shown). Perhaps some of the Y14 target mRNA-encoded proteins, e.g. those having a short half-life, are more vulnerable to Y14 depletion. Together, our results provide a clue to the functional role of Y14 in mRNA and protein metabolism beyond NMD.

**Acknowledgments**—We thank Dodda Dhananjaya and the Core Facility of Institute of Biomedical Sciences, Academia Sinica, for technical assistance.

**References**


Y14 Mutant Defective in Cap Binding and Translation Enhancement

in mammalian cells: an additional function of the exon junction complex. Genes Dev. 18, 210–222

8574 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 291 • NUMBER 16 • APRIL 15, 2016

ASBMB