Phosphorylation of Y14 Modulates Its Interaction with Proteins Involved in mRNA Metabolism and Influences Its Methylation*

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The multicomponent exon junction complex (EJC) is deposited on the spliced mRNA during pre-mRNA splicing and is implicated in several post-splicing events, including mRNA export, nonsense-mediated mRNA decay (NMD), and translation control. This report is the first to identify potential post-translational modifications of the EJC core component Y14. We demonstrate that Y14 is phosphorylated at its repeated arginine-serine (RS) dipeptides, likely by SR protein-specific kinases. Phosphorylation of Y14 abolished its interaction with EJC components as well as factors that function downstream of the EJC. A non-phosphorylatable Y14 mutant was equivalent to the wild-type protein with respect to its association with spliced mRNA and its ability in NMD activation, but the mutant sequestered EJC and NMD factors on ribosome-containing mRNA ribonucleoprotein (mRNPs). Moreover, we found that Y14 is possibly methylated at its repeated arginine residues in the carboxyl-terminal domain and that methylation of Y14 was antagonized by phosphorylation of RS dipeptides. This study reveals antagonistic post-translational modifications of Y14 that may be involved in the remodeling of Y14-containing mRNPs.

Eukaryotic mRNAs undergo several processing steps before export to the cytoplasm for translation. The splicing reaction removes introns from precursor mRNAs (pre-mRNAs) and positions the exon junction complex (EJC) on spliced mRNA in a sequence-independent manner (1, 2). The EJC is a dynamic multicomponent complex consisting of a heterodimer of Y14 with Mago and a number of associated factors (1, 2). The EJC may be functionally connected to transcription and acts as an adaptor for recruiting factors involved in the RNA metabolism steps downstream of splicing (3).

Previous work suggests that the EJC functions for the nuclear export of spliced mRNAs via the interaction of Y14/Mago, as well as other components, with the mRNA export receptor TAP (4–6). However, depletion of EJC components only marginally affects bulk poly(A)+ RNA export in cultured Drosophila cells (7), suggesting that the EJC may be an accessory factor for mRNA export. On the other hand, Y14/Mago and RNPS1, another EJC component, directly promote NMD-mediated mRNA degradation (8, 9). Y14 interacts with the NMD initiator Upf3 in the nucleus, which subsequently recruits other Upf proteins to yield the active NMD complex (1, 2). Depletion of Y14 abolishes NMD, indicating its essential role in this pathway (10). Recent reports show that the EJC promotes efficient translation by enhancing polysome association with mRNAs (11, 12). In particular, Y14/Mago, as well as RNPS1, is implicated in this translation enhancement (11–13). Thus, the EJC participates in several post-splicing events, including mRNA export/surveillance and translation control (1, 2, 14). The Y14/Mago heterodimer acts as a core that interacts with several key factors involved in the post-splicing mRNA metabolism pathways (15–19) and, indeed, plays a critical role in some steps of mRNP biogenesis (10). The central region of Y14 contains a phylogenetically conserved RNA recognition motif in the central region that directly interacts with Mago (20, 21). The carboxyl terminus of Y14 is rich in positively charged residues, and its function is not yet characterized. In particular, vertebrate Y14 proteins contain two consecutive arginine/serine (RS) dipeptides. Domains containing repeated RS dipeptides are characteristic of SR proteins that are primarily involved in pre-mRNA splicing (22). The RS domain is primarily involved in protein–protein interactions (22), which are directly impacted by the phosphorylation of this domain; hence, the activity, cellular localization, or stability of SR proteins can be modulated in this manner (22–24). Recent evidence shows that the phosphorylation level of shuttling SR proteins changes during mRNP maturation (25, 26), suggesting that RS domain phosphorylation/dephosphorylation governs mRNA export and perhaps subsequent events.

In addition to the duplicated RS dipeptide, the carboxyl-terminal region of Y14 contains several arginine residues adjacent to glycines, reminiscent of RGG motif-containing hnRNP proteins (27). Arginine methylation of these hnRNP proteins links transcription with mRNP formation and export (28). Methylation facilitates nuclear export by altering the interactions between factors involved in mRNA processing and export (29–31). Therefore, the two types of post-translational modification, namely phosphorylation and methylation, may coordinate to modulate mRNP rearrangement during mRNA export. In this study we examined post-translational modifications of Y14. We found that Y14 can be phosphorylated and methylated in the carboxyl-terminal domain. Moreover, we examined how the phosphorylation of Y14 influences its protein-protein interactions, post-splicing functions, and methylation.

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∥ The abbreviations used are: pre-mRNA, precursor mRNA; EJC, exon junction complex; HA, hemagglutinin; hnRNP, heterogeneous nuclear ribonucleoprotein; mRNP, mRNA ribonucleoprotein; MCP, MS2 coat protein; NMD, nonsense-mediated mRNA decay; PRMT1, protein arginine methyltransferase 1; SA, S166A/S168A; SRPK1, SR protein kinase 1.
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EXPERIMENTAL PROCEDURES

Plasmids—The expression vector pcDNA-FLAG-Y14 was provided by Jens Lykke-Andersen (University of Colorado, Boulder, CO). Truncation of the carboxyl-terminal 23 amino acids of Y14 was made by PCR, and site-specific mutagenesis of Y14 was achieved using the QuikChange system (Stratagene). The cDNAs encoding wild-type and mutants of Y14 were each subcloned into pGEX-5X (Amersham Biosciences) to create vectors for expressing GST fusions. The FLAG-tagged Y14 SA mutant was directly generated from pcDNA-FLAG-Y14 by site-directed mutagenesis. Plasmid pMPC was modified from pEGFP-N3 (BD Biosciences) in which the enhanced green fluorescent protein (EGFP) coding fragment was replaced by the sequence encoding the MS2 coat protein (MCP) (a gift of Kerry Bloom, University of North Carolina, Chapel Hill, NC). The cDNAs encoding Y14 or Upf3b were each inserted into pMPC to produce vectors for expressing MCP fusions. The TAP and Upf3b cDNA fragments were each cloned in-frame with the HA tag into a modified pCEP4 vector (Invitrogen). The Y14 and Mago coding sequences were each inserted into pET15b and pET29b (Novagen), respectively, to create bacterial expression vectors for His-tagged proteins. The pGEX-SRPK1 vector was described previously (30), and an analogous vector was constructed for expressing GST-Clk. In addition, the SRPK1 cDNA was subcloned into pET29b, yielding a plasmid used for Y14 phosphorylation in Escherichia coli. The vector for expressing GST fusion to rat PRMT1 was obtained from Wey-Jing Lin (Yang-Ming University, Taipei, Taiwan).

Protein Purification and in Vitro Pull-down—GST fusion proteins, including Y14, SRPK1, Clk1, PRMT1, hnRNPA1, and the ASF RS domain, were overexpressed in E. coli strain BLR(DE3) and purified using glutathione-Sepharose 4B (Amersham Biosciences). To produce the recombinant Y14/Mago heterodimer, E. coli lysates containing either GST-Y14 or His-tagged Mago were combined and incubated at 4 °C for 2 h before sequential purification through glutathione-Sepharose 4B and HisBind resin (Novagen). Purified GST-Y14 or a GST-Y14/His-Mago heterodimer was dialyzed against a buffer containing 20 mM HEPES (pH 7.4), 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, and 1 mM EGTA. To obtain in vivo phosphorylated GST-Y14, the expression vector encoding His-tagged SRPK1 was transformed into BL21(DE3) carrying pGEX-Y14. GST-Y14 was phosphorylated in vivo and purified as described above. For in vitro pull-down assays, prey proteins (32) were in vitro translated and [35S]-labeled using the Tnt-coupled transcription/translation system (Promega).

Antibodies—To raise anti-Y14 serum, His-tagged Y14 was overproduced in E. coli, recovered from inclusion bodies, and then used as antigen. Anti-phosphorylated Y14 serum was raised against eight copies of peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple anti-peptide Gly-Arg-Arg-Ser(P)-Arg-Ser(P)-Pro-Asp-Arg-Arg (where Ser(P) represents phosphoserine) that were linked by the multiple

RESULTS

Phosphorylation of Y14 at the Duplicated RS Dipeptides—We first examined whether Y14 is phosphorylated at its duplicated RS dipeptides. FLAG-tagged Y14, either wild-type or double mutant S166A/S168A (abbreviated herein as SA; Fig. 1A), was transiently expressed in HeLa cells and metabolically labeled with [32P]orthophosphate (Amersham Biosciences) for 2.5 h. In vivo methylation was performed using five plates of HEK 293 cells that transiently expressed FLAG-Y14. Cells were cultured in Dulbecco’s modified Eagle’s medium containing 100 μg/ml cycloheximide and 40 μg/ml chloramphenicol for 30 min and subsequently labeled with 10 μCi/ml l-[methyl-3H]methionine (Amersham Biosciences) in methionine-lacking Dulbecco’s modified Eagle’s medium for 3 h. FLAG-tagged Y14 was immunoprecipitated from the cell lysates using anti-FLAG M2 agarose (Sigma) in a buffer containing 10 mM Tris-HCl (pH 7.4), 100 mM NaCl, 2.5 mM MgCl2, 0.5% Triton X-100, 0.1 mM dithiothreitol, and a protease inhibitor mixture (Roche Applied Science) used as recommended by the supplier.

In vitro phosphorylation was performed as described (26). For in vitro methylation, each 20-μl reaction contained 5 μg of substrate, 2.5 μg of GST-PRMT1, and 2 μCi of S-adenosyl-l-[methyl-3H]methionine. The reaction was incubated at 30 °C for 1 h.

Oocyte Injection—Internally [32P]-labeled PIP85 RNA was prepared by in vitro transcription. Xenopus laevis oocytes were maintained in Barth’s solution containing 15 mM HEPES (pH 7.6), 88 mM NaCl, 2.3 mM NaHCO3, 1 mM KCl, 0.3 mM Ca(NO3)2, 0.4 mM CaCl2, 0.8 mM MgSO4, and 10 μg/ml gentamycin at 18 °C. For each oocyte, 60 ng of GST-Y14/His-Mago and 1 × 106 cpm (≈0.6 fmol) pre-mRNA were injected into the nucleus. After 45 min of incubation, lysates were prepared from 10–15 nuclei in radioimmune precipitation assay buffer containing 0.1% Nonidet P-40. Pull-down of mRNPs from the lysates was performed using glutathione-Sepharose, and radiolabeled RNA was recovered from bound fractions for gel electrophoresis.

NMD Assay—For the tethering NMD assay, HeLa cells in 35-mm dishes were transfected with 1.5 μg each of pCPwtb2–6MS2 and pCP-wtGAP3-UAC (gifts of Jens Lykke-Andersen) and 3 μg of pMCP-efector protein. At 24 h post-transfection, total RNA was prepared for Northern blotting analysis (9).

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FIGURE 1. Y14 is phosphorylated at the RS dipeptides. A, domain structure and the carboxyl-terminal 23 residues of Y14. RS dipeptides and potential methylation sites are shown in enlarged boldfaced letters and in gray boxes, respectively. B, HeLa cells were transiently transfected with an empty vector (mock) or a vector encoding the FLAG-tagged wild-type (WT) Y14 or the Y14 SA mutant. Metabolic labeling was performed using [32P]orthophosphate for 2.5 h. FLAG-Y14 was immunoprecipitated and analyzed by autoradiography. C, GST-Y14 and GST-ASF RS were subjected to in vitro phosphorylation in the presence of [γ-32P]ATP using recombinant SRPK1 (lane 1) or Clk1 (lane 2). D, the GST-Y14/His-Mago heterodimer containing either wild-type (WT) (lane 1) or the SA mutant (lane 2) of Y14 was in vitro phosphorylated by SRPK1 (lanes 3 and 4) or HeLa cell nuclear (N) (lanes 5 and 7) or cytoplasmic (C) extract (lanes 6 and 8).

Post-translational modification of Y14 affects its interactions with known partners. Therefore, phosphorylation of the carboxyl terminus of Y14 likely impaired its interactions with the EJC and EJC-associated factors.

FIGURE 2. Phosphorylation of Y14 modulates its protein-protein interactions. Recombinant GST or GST-Y14/His-Mago containing wild-type (WT), the SA mutant, or phosphorylated (phos) Y14 (section labeled Coomassie blue) was used as bait in the pull-down experiment. Western blotting was performed using anti-Y14 and anti-phosphorylated Y14 (section labeled Immunoblotting). For the pull-down experiment, prey proteins were in vitro translated and EJC associated proteins were in vitro translated and 35S-labeled. Bound fractions were analyzed by SDS-PAGE followed by autoradiography (section labeled Autoradiogram).

The Non-phosphorylatable Y14 Mutant Is Able to Incorporate into Post-splicing mRNPs and Activate NMD—Although the SA mutant of Y14 was not distinguishable from the wild-type with regard to interactions with other EJC factors, it was not clear whether the mutant has any post-translational function. To evaluate the association of Y14 with spliced mRNA, the recombinant GST-Y14/His-Mago heterodimer was co-immunoprecipitated with radiolabeled PIP85 pre-mRNA into Xenopus oocyte nuclei. At 45 min post-injection, tagged Y14/Mago was pulled down from nuclear extracts using glutathione-Sepharose. Y14/Mago associated primarily with the spliced mRNA (Fig. 3A, lanes 3 and 4) as reported (4). The SA mutant also bound to the spliced RNA as did the wild-type (Fig. 3A, lanes 5 and 6). Moreover, the levels of spliced PIP85 mRNA in the cytoplasm were examined over a 2-h period post-injection. No significant difference was observed between the injection of GST and either form of Y14 (Fig. 3A, lower section, part labeled mRNA export). Therefore, the serine-to-alanine mutations within the RS repeat of Y14 affected neither the association of the EJC (containing Y14) with mRNA nor mRNA export.

We next examined the activity of the Y14 SA mutant in NMD. We adopted a mRNA tethering system in which the NMD activity of a fusion protein containing the MCP is measured upon binding to a reporter mRNA containing repeated MS2 sites downstream of the stop codon (9). An expression vector encoding MCP and HA doubly tagged Y14 was cotransfected into HeLa cells along with two vectors, one for the MS2-containing NMD reporter (βMS2) and another encoding a control mRNA (βG) (Fig. 3B, upper section). As observed with the NMD initiator protein Upf3b, tethering of either the wild-type or the SA mutant of Y14 significantly reduced the level of βMS2 RNA (Fig. 3B, lower section). Therefore, the SA mutation had no detectable effect on the capability of Y14 to activate NMD. Moreover, when overexpressed the mutant Y14 promoted NMD of a nonsense-containing mRNA as...
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Phosphorylation of Y14 Interferes with Its Arginine Methylation—Although Y14 appeared to be phosphorylated at the RS dipeptide, the non-phosphorylatable Y14 behaved similarly to the wild-type protein with regard to both its association with the EJC (which, in turn, binds to spliced mRNA) and participation in NMD activation (Figs. 2 and 3). Therefore, phosphorylation might occur after Y14, along with several EJC factors, is exported to the cytoplasm and accomplishes mRNA surveillance. Nevertheless, phosphorylation of Y14 prevented its interaction with the EJC and downstream factors in vitro (Fig. 2). We thus hypothesized that phosphorylation triggers dissociation of export or NMD factors from Y14 and/or removes Y14 from ribosome-containing mRNPs. If phosphorylation is blocked, Y14-containing complexes might accumulate. To test this hypothesis, a FLAG-tagged wild-type or SA mutant of Y14 was transiently co-expressed with the mRNA export factor TAP or the NMD factor Upf3b in HEK 293 cells. Immunoprecipitation with anti-FLAG showed that higher levels of TAP or Upf3b were co-precipitated with the SA mutant as compared with wild-type Y14 (Fig. 4, lanes 1–6). NMD takes place in polysomes containing the cap-binding protein CBP20/80 rather than eIF4E during a pioneer run of translation (35). Using specific antibodies, we detected CBP80 and ribosomal protein L22 (Fig. 4, lanes 7–12) but not eIF4E (data not shown) in the Y14 mutant immunoprecipitates. Consistent with our idea, this result indicated that non-phosphorylated Y14 bound more tightly with NMD factors and the pioneer translation machine than did phosphorylatable Y14.

Phosphorylation of Y14 Interferes with Its Arginine Methylation—The notion that Y14 contains potential arginine methylation sites within the carboxyl-terminal domain prompted us to examine its possibility. HEK 293 cells transiently expressing FLAG-Y14 were incubated with l-[methyl-3H]methionine or l-[35S]methionine in the presence of protein synthesis inhibitors. Immunoprecipitation using anti-FLAG showed that Y14 was labeled only with 3H (Fig. 5A) but not with 35S (data not shown), indicating that Y14 was post-translationally methylated in cells. Like hnRNP A1, Y14 can be methylated in vitro by recombinant rat PRMT1, a type I arginine methyltransferase (Fig. 5B, lanes 2 and 3). Truncated Y14 lacking the carboxyl-terminal 23 residues was poorly labeled, suggesting that methylation occurred primarily at the carboxyl-terminal arginines (Fig. 5B, lane 4). To more accurately determine the methylation sites, we made several arginine-to-lysine point mutations within the carboxyl-terminal domain of Y14. In vitro methylation using PRMT1 occurred primarily at six arginines, including four (Arg-159, Arg-160, Arg-163, and Arg-164) immediately adjacent to glycine residues and two (Arg-165 and Arg-167) within the RS dipeptide.
D. Methylation assay with recombinant rat PRMT1 and various mutants. 

FIGURE 5. Methylation of Y14 is antagonized by RS peptide phosphorylation. A, HEK 293 cells were transiently transfected with an empty (mock) or FLAG-Y14 expression vector. Cells were incubated with L-[methyl-13H]methionine in the presence of protein synthesis inhibitors. FLAG-Y14 (arrow) was immunoprecipitated and analyzed by autoradiography. B, GST or GST fusions to hnRNP A1, Y14, carboxyl-terminally truncated Y14 (Y14AC23), or the Y14/Mago heterodimer was subjected to in vitro methylation using recombinant rat PRMT1. lane C and D, as in panel B, in vitro methylation was performed with GST or GST fusions to wild-type (WT) or RK mutants of Y14 (panel C) and non-phosphorylated (non-phos) or phosphorylated (phos) Y14 (panel D). In panels B–D, Y14 and its various mutants used for the in vitro methylation assay are shown at the bottom.

FIGURE 6. Model for the functions of Y14 in mRNA metabolism modulated by post-translational modification. We hypothesize that phosphorylation and methylation of Y14 take place in the cytoplasm and in the nucleus, respectively. Hypophosphorylated Y14 in the EJC recruits export and NMD factors in the nucleus. Y14 is phosphorylated during the pioneering run of translation, leading to dissociation of the NMD complex from polysomes upon completion of mRNA surveillance. Perhaps methylation of Y14 facilitates its nuclear export, and improper phosphorylation of Y14 in the nucleus prevents its methylation.

DisCUSSION

In this study we provide evidence that Y14 is subject to phosphorylation and methylation at its carboxy-terminal domain. These post-translational modifications may provide a layer of regulation that controls the activity of Y14. At present, it is not known at which steps of mRNA biogenesis or subcellular locations these modifications take place. However, our present data allow the simple speculation that hypophosphorylated Y14 recruits the EJC and downstream-acting factors to the spliced mRNA in the nucleus and that phosphorylation of Y14 occurs after initial runs of translation of the mRNA in the cytoplasm to dissociate the NMD complex (Fig. 6). Perhaps, like hnRNP proteins, Y14 is methylated in the nucleus for remodeling or export of its associated mRNP. We discuss these possibilities below.

It is plausible that phosphorylation of Y14 stabilizes its activity; however such modification appears to be dispensable for Y14 with regard to its association with spliced mRNA and activation of NMD (Fig. 3). Moreover, no dominant negative effect was detected with the non-phosphorylatable mutant of Y14 either on mRNA export in Xenopus oocytes (Fig. 2) or on NMD of nonsense transcripts (data not shown). Nevertheless, the in vitro binding assay showed that phosphorylated Y14 does not interact with its partners that act downstream in the RNA metabolism pathway (Fig. 2). Accordingly, non-phosphorylatable Y14 appeared to be sequestered in ribosome-containing mRNP together with export- and NMD-related factors (Fig. 4). Y14 is present in ribosome-associated mRNP fractions as it functions in mRNA surveillance and is subsequently removed from mRNAs by active translation (36). Therefore, upon the completion of mRNA surveillance, phosphorylation of Y14 may trigger its dissociation from ribosome-containing mRNP and, hence, from other interacting partners (Fig. 6). Such RNP remodeling may be hampered if Y14 fails to be properly phosphorylated, leading to prolonged association of Y14 as well as NMD factors with polysomes (Fig. 4). Nevertheless, the exact mechanism by which the function of Y14 is regulated through phosphorylation needs further investigation.

The observation that the mRNA export adaptor TAP preferentially interacted with non-phosphorylated Y14 is reminiscent of shuttling SR proteins. SR proteins join pre-mRNAs for splicing probably in a hyper-phosphorylated form (23). Shuttling SR proteins that function as export adaptors for mRNAs bind directly to TAP (37). Recent evidence suggests that dephosphorylation of SR proteins takes place during pre-mRNA splicing or subsequent steps for recruiting TAP (25, 26). However, it is not yet known whether dephosphorylation of SR proteins is a prerequisite for nuclear export of mRNAs. Nevertheless, our present data indicated that not only authentic SR proteins but also the EJC component Y14 recruits TAP for export in a hypophosphorylated form. Therefore, dephosphorylation is probably common for RS repeat-containing export adaptors, thereby providing a signal to the export machinery for exit from the nucleus.

Finally, we found that Y14 can be methylated and that this methylation is abrogated by prior phosphorylation of the protein (Fig. 5). It is evident that arginine methylation of several yeast hnRNP proteins facilitates their export from the nucleus, probably by modulating the assem-
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bly of export complexes (29–31). However, methylation of hnRNP proteins may not necessitate hnRNP complex formation in higher eukaryotes (38), and thus the role of methylation in mRNA export remains elusive. Further experiments should address whether methylation modulates the interactions of Y14 with its partners, thereby further specifying Y14 functions. Interestingly, Y14, like the yeast RNA-binding protein Npl3p (39), shares properties with SR proteins and hnRNP proteins with regard to post-translational modifications. Thus, mRNP rearrangement and export in higher eukaryotes might be modulated through modification of Y14.

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