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The *Saccharomyces cerevisiae* poly(A)-binding protein is subject to multiple post-translational modifications, including the methylation of glutamic acid

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ABSTRACT

Poly(A)-binding protein in mouse and man was recently found to be highly post-translationally modified. Here we analysed an ortholog of this protein, Pab1 from *Saccharomyces cerevisiae*, to assess the conservation and thus likely importance of these modifications. Pab1 showed the presence of six sites of methylated glutamate, five sites of lysine acetylation, and one phosphorylation of serine. Many modifications on Pab1 showed either complete conservation with those on human or mouse PABPC1, were present on nearby residues and/or were present in the same domain(s). The conservation of methylated glutamate, an unusual modification, was of particular note and suggests a conserved function. Comparison of methylated glutamate sites in human, mouse and yeast poly(A)-binding protein, along with methylation sites catalysed by CheR L-glutamyl protein methyltransferase from *Salmonella typhimurium*, revealed that the methylation of glutamate preferentially occurs in EE and DE motifs or other small regions of acidic amino acids. The conservation of methylated glutamate in the same protein between mouse, man and yeast suggests the presence of a eukaryotic L-glutamyl protein methyltransferase and that the modification is of functional significance.

1. Introduction

The poly(A)-binding protein has multiple functions. Known as Pab1 in *Saccharomyces cerevisiae* and PABPC1 in mammalian systems, it serves as a central regulator of mRNA stability and translation (reviewed in [1,2]). It is comprised of four RNA-recognition (RRM) domains, which are not identical, followed by a PABC (PABP C-terminal) domain. Protein–RNA interactions are mediated exclusively by the RRM domains. In vertebrates, RRM domains 1 and 2 provide the highest binding affinity to poly(A) while RRM domains 3 and 4 exhibit relaxed sequence specificity [3]. In yeast, RRM2 is most important for poly(A) binding while RRM4 is the most important for non-specific polypyrimidine RNA binding [4]. Poly(A)-binding protein has many interactions with other proteins; these include the eukaryotic elongation initiation factors eIF4E, eIF4G and eIF4A (reviewed in [1,2]), and the PABP-interacting proteins PAIP1 and PAIP2 [5,6]. Of these protein–protein interactions, the interaction with eIF4G has been most studied as it circularises the bound mRNA and stimulates translation of the mRNA [1,2,7]. In vertebrates, an interaction with paxillin mediates the nuclear to cytoplasmic shuttling of PABPC1 [8]. In *S. cerevisiae*, the nuclear export is mainly Crm1-dependent with the export signal localised to between residues 9–61, with Leu12 and Leu15 being essential. Nuclear import is via the importin Sxm1 with the import signal localised to between residues 281–338 [9,10]. Pab1 is highly conserved in all eukaryotes and is known to be essential in *S. cerevisiae* [11].

Recently, PABPC1 was analysed for the presence of post-translational modifications [12]. This revealed the presence of 14 modifications throughout the various functional domains of the human protein; all but one of these were also conserved in the murine version. The modifications included lysine acetylation, lysine methylation, arginine methylation and the unusual methylation of aspartic and glutamic acids. PABPC1 is also known, from large-scale screens to be phosphorylated [13,14]. While the precise functions of all these modifications remain unknown, initial modelling-based data suggests interplay between methyl- and acetyllysines to regulate protein–protein interactions of PABPC1. Intriguingly, it has also been suggested that the acetyllysines of PABPC1 are cell-cycle dependent and play a major role in affecting the pI of PABPC1, possibly in association with phosphorylation [12]. The complexity of PABPC1 modifications is not dissimilar to aspects of the histone code [15] and the recently proposed chaperone and transcription factor interaction codes [16,17] where a diversity of modifications, alone or in combination, controls the specificity of interactions. To help understand the importance of the PABPC1 post-translational modifications, here we analysed

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Pab1 from *S. cerevisiae*. We hypothesized that similar modifications would also be present in Pab1 if they are of functional significance.

2. Materials and methods

2.1. Yeast strains, Pab1 overexpression and purification

S. cerevisiae BY4741 was maintained according to [18]. Pab1 was overexpressed in BY4741 from a plasmid from the Movable Open Reading Frame (MORF) library [19] and purified according to [18].

2.2. Mass spectrometry and identification of modifications

Pab1 was separated by SDS–PAGE, and digested to peptides with trypsin according to [18]. Methanol was omitted in all analysis steps to prevent the introduction of artefactual methyl esters of aspartic or glutamic acid. Tandem mass spectrometry of peptides was undertaken using CID, according to [20]. Initial site assignment was undertaken through the analysis of MS/MS spectra with the database search program Mascot (version 2.3), according to [18,20], with the following variable modifications: mono-, di- and tri-methyllysine (K), acetyllysine (K), phosphoserine (S), phosphothreonine (T), phosphotyrosine (Y), methylglumate (E) and methylaspartate (D). Peptides that showed scores and/or expect values of significance were subsequently verified by manual inspection of the spectra. This involved the full annotation of spectra and a search for the presence of neutral losses associated with labile PTMs (e.g. phosphorylation). Due to the possibility of modification misassignment from amino acid polymorphisms, especially for methylated aspartic acid (which is isobaric with glutamic acid), we undertook two tests. First, we searched the Uniprot database to check that our identified modified residues were not subject to allelic polymorphisms. Second, because PTMs are usually substoichiometric, we identified and compared the MS/MS spectra for the modified and unmodified peptides. The MS/MS spectra for the unmodified peptides were also subject to rigorous manual verification.

2.3. Sequence analysis and comparison

Amino acid sequences for *S. cerevisiae* Pab1 (UniProt: P04147), along with human (UniProt: P11940) and mouse PABPC1 (UniProt: P29341), were from the UniProt database. Known post-translational modifications were as documented in the literature, some of which were also referenced in UniProt. Multiple sequence alignment was undertaken with the ClustalW tool using default parameters [21].

3. Results and discussion

3.1. Yeast Pab1 is highly post-translationally modified

Yeast Pab1 shows ~60% sequence identity to human PABPC1 across the four RRM domains, a length of 360 amino acids, and also shows high sequence similarity in the C-terminal PABC domain. This striking degree of sequence conservation in these unrelated species, and the fundamental role of the poly(A)-binding protein in eukaryotic cells, raised the question of whether the multiple post-translational modifications of mammalian PABPC1 are also found in the yeast Pab1 ortholog. To investigate this, Pab1 was overexpressed, purified and analysed by tandem mass spectrometry.

The yeast Pab1 protein was found to be highly modified. A total of 12 modified amino acid residues were present, including six methylglutamate sites, five acetyllysine sites, and one phosphoserine site (Table 1). Unmodified versions of all peptides were also observed, facilitating the high confidence assignment of modifications to specific sites on each peptide. Representative, annotated spectra for these three types of modification are given in Fig. 1 and annotated spectra for all other modified and matching unmodified peptides are included in the Supplementary Materials. It should be noted that a further modification of Pabl is also known; ETD-based LC-MS/MS undertaken by us elsewhere has identified the methylation of arginine 107 [18].

3.2. Protein modifications of Pab1 show evolutionary conservation

The four types of modifications found in yeast Pab1 are known to be present on mammalian PABPC1 [12,14]. PABPC1 is also reported to carry methylaspartate and methyllysine. To facilitate the comparison between the poly(A)-binding protein modifications in yeast and mammals, all known modified residues were mapped onto a multiple sequence alignment (Fig. 2).

The methylation of glutamic acid residues is a rare and unusual modification. It has been previously reported only once in yeast [22]. It was therefore particularly striking that the RRM2-3 spacer and RRM3 domains of Pab1 carried methylglutamate (Table 1) and that this corresponded to that seen in the same domains of mouse and human PABPC1 [12]. In Pab1, two methylated glutamic acid residues were present in the RRM2/RRM3 spacer on peptides ERDSQLE(met)ETK and ERDSQLEE(met)TK; in human/mouse PABPC1, methylated glutamate was reported at −2 to −5 positions relative to the corresponding residues in yeast (Fig. 2). In RRM3, residue Glu233 in Pab1 was methylated in peptide NINSETTDE(met)QFQELFAK; the equivalent residue, Glu205, was methylated in the mouse PABPC1 peptide NFGEDMDDE(met)R. We found the PABC domain of yeast Pab1 carried three further methylated glutamate modifications (Table 1). This modification was also reported in the same domain in mouse PABPC1 (Fig. 2), although in a more N-terminal position [12]. In the future, it will be fascinating to see whether RRM domains in other proteins carry methylglutamate(s) or if it is confined to the poly(A)-binding proteins.

Acetylation was found to be present on five lysine residues in five different yeast Pab1 peptides (Table 1), predominantly on RRM or RRM spacer domains. In all cases, the modification prevented tryptic cleavage C-terminal to the acetyllysine residue; this was expected to occur [23]. Two acetylation sites showed complete conservation between Pab1 and mouse PABPC1 [12]. This was at Lys122 and Lys390 in Pab1, in peptides K(ac)KGSGNIFIK and NQQIVAGK(ac)PLYVAIAQR, where the equivalent acetylated lysines in mouse PABPC1 were Lys95 and Lys361 in peptides K(ac)SGVGNIFIK and IVATK(ac)PLYVALAQR, respectively (Fig. 2). Acetylated Lys95, in mouse, was also acetylated in human PABPC1. The yeast to man conservation of acetylation is striking given the evolutionary distance between these species. The presence of the modification in RRM domains also suggests a possible role of these acetyllysines in the protein–RNA interactions of poly(A)-binding protein. This has not been investigated to date.

Our analysis showed Ser125 to be phosphorylated in Pab1, in peptide KGS(p)GNIFIK (Table 1, Figs. 1 and 2). This confirms previous reports of this site from large-scale yeast phosphoproteomics screens [24,25]. Other sites have also been reported for Pab1, including Tyr393 and Ser405 [25]. Although not found in [12], PABPC1 is also reported to be phosphorylated at a total of 12 sites. Of note is that the phosphorylation of Tyr364 in PABC1 is conserved on the equivalent residue in yeast Pab1, Tyr393 [13,26,27].

3.3. Methylation of glutamate residues is enriched in acidic motifs

The sequences or motifs in which methylation of glutamic acid occurs are not well characterised. Here we report that methylation

Table 1
Poly(A)-binding protein Pab1 peptides carrying post-translational modifications.

Peptide sequence[a]	Residues (yeast)	Observed m/z (charge)	Score (E-value)	Domain	Conservation and observations
Methyl (E)					
ERDSQLEETK	206–215	624.8077 (+2)	55 (2.3×10^{-3})	RRM2/RRM3 spacer region	Residue not conserved. Methyl (E) at −3 and −5 in human and mouse
DSQLEETK	208–215	482.2351 (+2)	40 (2.2×10^{-2})	RRM2/RRM3 spacer region	Residue not conserved. Methyl (E) at −2 and −4 in human and mouse
DSQLEETK	208–215	482.2349 (+2)	42 (1.4×10^{-2})	RRM2/RRM3 spacer region	Residue not conserved. Methyl (E) at −3 and −5 in human and mouse
NINSETTDEQFQELFAK	225–241	1014.4810 (+2)	69 (5.5×10^{-6})	RRM3 domain	Conserved residue. Conserved modification in mouse
TSNEEAAGK	521–529	460.7195 (+2)	24 (1.5×10^{-1})[b]	PABC domain	Region not aligned
TSNEEAAGK	521–529	460.7196 (+2)	55 (3.0×10^{-4})	PABC domain	Region not aligned
EASAAYESFK	558–567	558.7646 (+2)	31 (4.8×10^{-3})	PABC domain	Residue not conserved
Acetyl (K)					
ADITDKTAEQLENLNIQDDQK	2–22	1222.5930 (+2)	78 (3.2×10^{-7})	Close to nuclear export signal	Region not aligned
KAIEQLNYTPIK	94–105	730.4116 (+2)	56 (1.0×10^{-3})	RRM1 domain	Residue not conserved
KKGSGNIFIK	122–131	378.5609 (+3)	45 (4.9×10^{-3})	RRM1/RRM2 spacer region	Conserved residue. Conserved modification in human and mouse
AQKKNER	294–300	458.2541 (+2)	32 (9.4×10^{-2})[b]	RRM3 domain	Conserved residue. Modification unconfirmed
NQQIVAGKPLYVAIAQR	383–399	956.0446 (+2)	36 (5.8×10^{-3})	RRM4 domain	Conserved residue. Conserved modification in mouse
Phospho (S)					
KGSGNIFIK	123–131	522.2687 (+2)	21 (5.7×10^{-2})[c]	RRM1/RRM2 spacer region	Residue not conserved. Phospho (S) at −2 in human

[a] Modification sites are underlined.
[b] Of low score however high confidence assignment possible through comparison with unmodified spectra.
[c] Neutral losses present in spectra and comparison with unmodified spectra confirmed the presence of phosphorylation.

of glutamic acid tends to be found in small regions of acidic residues. Of the six sites we found on Pab1, four showed the modification at adjacent glutamic acids – in peptides DSQLE(met)ETK and DSQLEE(met)TK, and in peptides TSNE(met)EAAGK and TSNEE(met)AAGK. Interestingly, the only other protein in yeast currently reported to have methylglutamates, Tdh3, has adjacent methylglutamates – in peptide VINDAFGIE(met)E(met)GLMTT VHSLTATQK [22]. Human PABPC1 showed methylation of near-adjacent glutamic acid residues – in peptides E(met)AELGAR and EAE(met)LGAR [12]; these sites were also reported in mouse. Methylglutamate sites exist adjacent to aspartic acid residues in other cases. In yeast Pab1, one site was adjacent to an aspartic acid – in peptide NINSETTDE(met)QFQELFAK. A similar patterns was also seen in mouse PABPC1 which showed methylation in peptides NFGEDMDDE(met)R, NLDDGIDDE(met)R and VVCDE (met)NGSK. Overall, 7 out of the 10 methylglutamate sites identified on the murine PABPC1 were found adjacent to, or one residue away from, acidic residue(s). If acidic residues up to 2 residues away from the methylglutamate site were considered, this figure increased to a striking 9 out of 10.

Methyltransferases involved in bacterial chemotaxis have been previously described. Of these, the best understood is the CheR L-glutamyl protein methyltransferase from *Salmonella typhimurium* [28,29]. In line with our observations, above, it was intriguing to observe that CheR also tends to methylate within small acidic motifs. Six out of the 8 known CheR methylation sites are in Glu–Glu sequences [28].

3.4. L-Glutamyl methyltransferases in yeast and man

Methyltransferases that catalyse the methylation of lysine and arginine residues are well characterised in yeast and higher organisms [30,31]. However, in eukaryotes, there are no methyltransferases that are known to methylate L-glutamic or L-aspartic acid residues. The conservation of methylglutamates in Pab1 and PABPC1, shown here, and the existence of methylglutamate on other proteins in yeast and HeLa cells [22], strongly suggests the existence of one or more L-glutamyl protein methyltransferases. There are numerous putative methyltransferases in *S. cerevisiae* which are currently of unknown specificity or function [32]. While none of these show sequence similarity to bacterial CheR (data not shown), these will be of immediate interest as possible L-glutamyl protein methyltransferases. Given the conservation of acidic motif in yeast and man, it is likely that any Pab1/PABPC1 methyltransferase will be found in all eukaryotes.

3.5. Functional implications and concluding remarks

The function of protein phosphorylation and acetylation are well documented, including their role in the modulation of protein–protein interactions [33–35]. In both these cases, the change in charge of these residues (gain of negative charge by phosphorylation, loss of positive charge by acetylation) affect a protein's structure and thus function. The methyl esterification of glutamate, and for that matter aspartate, is likely to be of importance as it removes the capacity of these amino acids in proteins to ionise. This results in the loss of a negative charge. There are several functional implications of this. The formation of salt bridges, important for stabilising tertiary structures of proteins, requires either glutamic or aspartic acids [36]. In enzyme catalysis, aspartate and glutamate residues are the 2nd and 4th most common residue in catalytic cores, constituting 15% and 11% of all residues directly involved in catalysis, respectively [37]. The methyl esterification of glutamate or aspartate may also modulate protein–protein interactions. By comparison, it is interesting to note that the methylation of arginine and lysine, which only changes the hydrophobicity of these amino acids but not their charge, affects the interactions of many histone and non-histone proteins [38,39]. Hence, while the function of glutamate methylation currently remains obscure, the conservation of this modification that we report here – and the large evolutionary distance

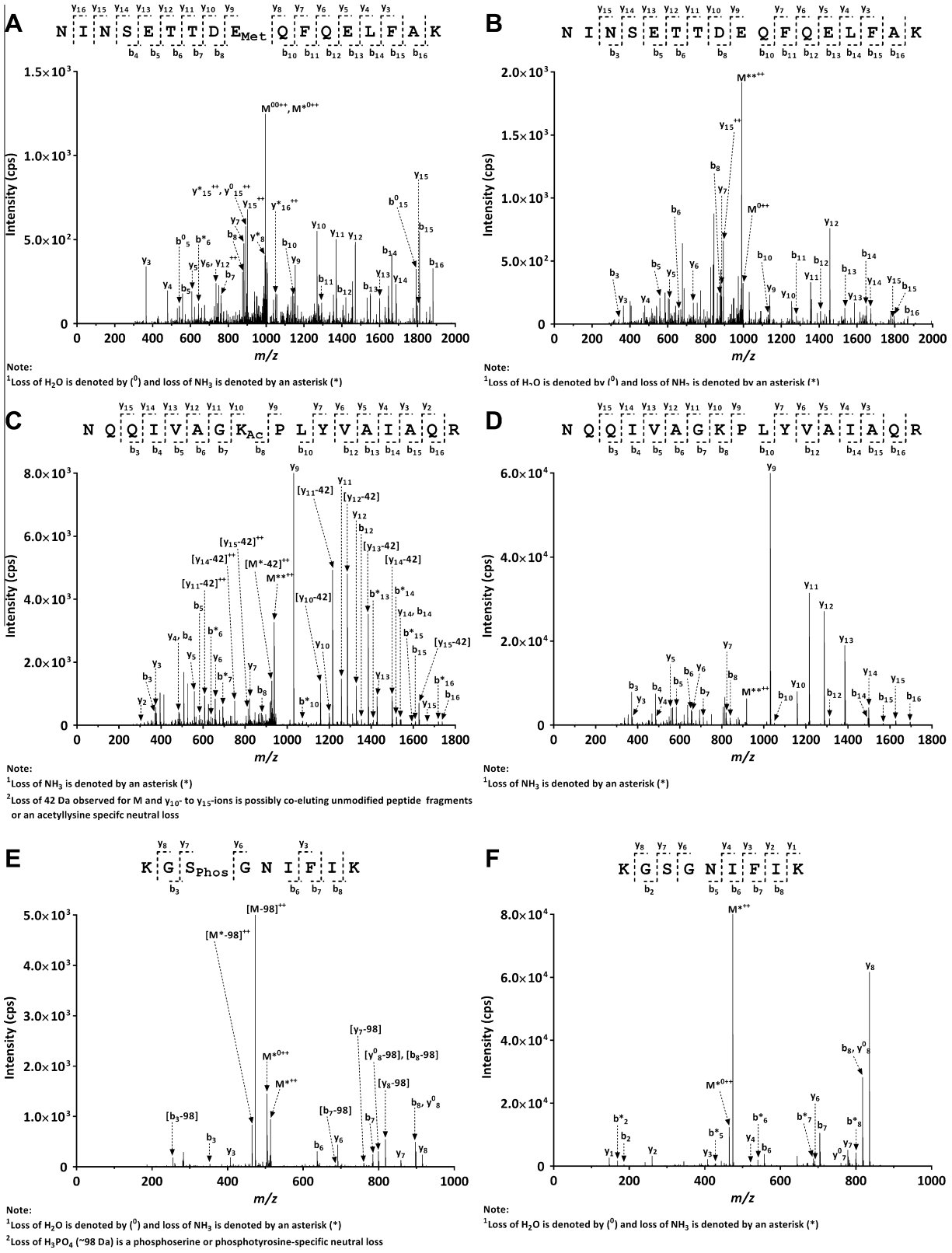

Fig. 1. MS/MS spectra of representative methylated, acetylated and phosphorylated Pab1 peptides and corresponding unmodified peptides. Included in each panel is the summarised fragment-ion coverage where observed *b*- and *y*-ions and their derivatives are shown. In each instance, near complete fragment-ion series were observed. Diagnostic phosphorylation-specific neutral losses were also observed for Panel E. Precursor ions, diagnostic neutral losses, *b*-, *y*-ions and their derivatives are labelled in the spectra. (A) Annotated CID-MS/MS spectrum obtained for the doubly-charged tryptic Pab1 peptide NINSETTDE(methyl)QFQELFAK observed at 1014.4810 *m*/*z*. (B) Annotated CID-MS/MS spectrum obtained for the corresponding doubly-charged unmodified peptide NINSETTDEQFQELFAK observed at 1007.4730 *m*/*z*. (C) Annotated CID-MS/MS spectrum obtained for the doubly-charged tryptic Pab1 peptide NQQIVAGK(acetyl)PLYVAIAQR observed at 956.0446 *m*/*z*. (D) Annotated CID-MS/MS spectrum obtained for the corresponding doubly-charged unmodified peptide NQQIVAGKPLYVAIAQR observed at 935.0397 *m*/*z*. (E) Annotated CID-MS/MS spectrum obtained for the doubly-charged tryptic Pab1 peptide KGS(phospho)GNIFIK observed at 522.2687 *m*/*z*. (F) Annotated CID-MS/MS spectrum obtained for the corresponding doubly-charged unmodified peptide KGSGNIFIK observed at 482.2846 *m*/*z*.

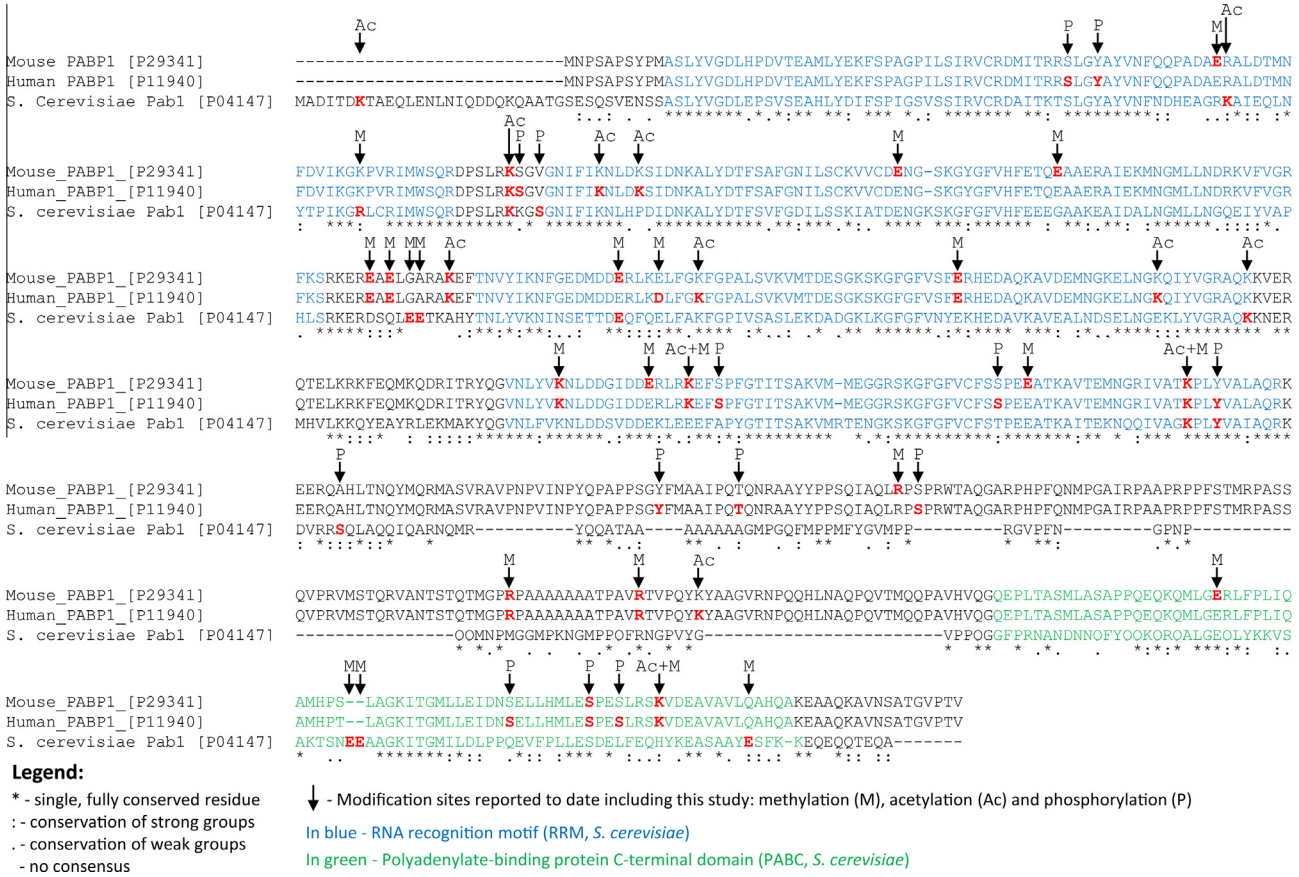

Fig. 2. ClustalW alignment of PABPC1 from human, mouse and *S. cerevisiae* Pab1, with modified amino acids highlighted in red. Mouse PABPC1 (UniProt: P29341), Human PABPC1 (UniProt: P11940) and *S. cereivisiae* Pab1 (UniProt: P04147) were aligned using ClustalW. The alignment showed ~60% sequence identity across the four RRM domains. Included here is all the post-translational modification (PTMs) sites identified to date. Some of these PTMs show conservation despite the large evolutionary gap. This suggests conserved function for the PTM involved. The *S. cerevisiae* RRM domains are shown in blue while the PABC domain in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between yeast and man – suggests an important role for methyl glutamate in the poly(A)-binding protein and in the cell.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.12.009.

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