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A Highly Conserved 3-Methylhistidine Modification Is Absent in Yeast Actin

Hamid R. Kalhor,* Agnieszka Niewmierzycka,* Kym F. Faull,† Xiaoyi Yao,‡ Stephanie Grade,‡ Steven Clarke,*,[1] and Peter A. Rubenstein‡,[2]

*Department of Chemistry and Biochemistry and the Molecular Biology Institute, and †Department of Psychiatry and Behavioral Sciences and the Neuropsychiatric Institute, University of California, Los Angeles, California 90095; and ‡Department of Biochemistry, University of Iowa College of Medicine, Iowa City, Iowa 52242

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To identify a protein histidine methyltransferase from *Saccharomyces cerevisiae,* we examined purified actin for the presence of the highly conserved 3-methylhistidine residue at position 73 by amino acid analysis of the whole protein and by amino acid analysis and mass spectrometry of the corresponding tryptic fragment. Surprisingly, we found that His-73 is not modified. A similar lack of modification was also found in actin from the yeast *Candida albicans,* while rabbit muscle actin revealed the expected 3-methylhistidine residue. Phylogenetic analysis of actin sequences suggests that this modification was introduced in evolution after the divergence of yeast from higher eukaryotic organisms, including unicellular eukaryotes such as *Acanthamoeba, Dictyostelium,* and *Physarum,* whose actins contain 3-methylhistidine. Our methodology for the analytical determination of 3-methylhistidine in actin offers an improved approach for investigating histidine methylation in proteins. © 1999 Academic Press

A histidine residue at position 73 is conserved in all known actins. With the single exception of the amoeba *Naegleria gruberi* actin, this histidine residue is post-translationally modified to 3-methylhistidine (1–3). This unusual modification is also found in some striated myosin isoforms (4, 5). The functional significance of this modification of actin is unknown. Clearly, it is not required for *Naegleria*. When actin His-73 is replaced using site-directed mutagenesis, the isotopically labeled mutant proteins readily copolymerize with carrier wild-type actin (6). However, in this system, it is possible that defects in the small amount of labeled mutant actin could be compensated for by the much larger amount of unlabeled wild-type actin used in the copolymerization experiment. Recently, we have replaced this histidine residue in yeast actin with five other residues and have shown that the resulting actins are compatible with yeast viability (P. A. Rubenstein, unpublished data). The availability of these actin mutants will allow us to test the necessity of histidine at the position 73 in a homogeneous preparation, but it will not allow us to assess the contribution of the methyl group per se.

Although neither the methylation nor the histidine itself is required for viability, at least in yeast, the presence of this modified residue may still confer some quantitative advantage on actin function. The imidazole of this residue lies in actin's subdomain 2 near the subdomain 1/2 interface on the exterior of the protein blocking a channel that allows water to enter the interior of the protein (7). From these data it has been suggested that the methylhistidine side chain, by interacting with phosphate ion released following hydrolysis of the actin-bound ATP during polymerization, may play an important role in preventing the facile exit of the phosphate through this channel (8). Since the stability of the actin filament is controlled by the state of the nucleotide bound to the monomer in the filament, such a role for the methylhistidine would maintain the actin filament for an extended period of time. This role for the methylhistidine, if true, would thus have a major impact on actin cytoskeletal dynamics within the cell.

[1] To whom correspondence may be addressed at UCLA Molecular Biology Institute, Box 951570, Los Angeles, CA 90095-1570. Fax: (310) 825-1968. E-mail: Clarke@mbi.ucla.edu.

[2] To whom correspondence may be addressed. Fax: (319) 335-9570. E-mail: pruben@blue.weeg.uiowa.edu.

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Despite the widespread occurrence of this modified residue in almost all actins, little if anything is known about the manner in which the methylation occurs. This is due in large part to the lack of a convenient assay and to the absence of an experimental system in which actin methylation can be prevented. Although Raghavan *et al.* (9) demonstrated a His-methylating enzyme in a mammalian cell extract using a peptide substrate, it is not clear that this activity can methylate the conserved histidine residue in actin.

We have been interested in identifying novel *S*-adenosylmethionine (AdoMet)[3]-dependent methyltransferases in *Saccharomyces cerevisiae,* particularly enzymes that are involved in posttranslational modifications of proteins (10). Yeast is an ideal organism for such a search. The complete elucidation of its genome sequence allows one to identify potential candidates for such enzymes by searching for open reading frames with sequence motifs similar to those found in AdoMet-utilizing enzymes. Yeast is genetically manipulatable and amenable to use for enzyme purification, and its intact cells take up [*methyl*-^{3}H]AdoMet so that methyl-acceptors can be radiolabeled. In this work, we focused on 3-methylhistidine formation in yeast actin. However, we found that this modification was absent in both *S. cerevisiae* actin as well as actin from the unrelated yeast *Candida albicans.*

MATERIALS AND METHODS

Actin purification. Rabbit muscle actin was a generous gift from Dr. Emil Reisler at UCLA. The site-directed mutagenesis procedure of Feng *et al.* (11) was used to generate a mutant *S. cerevisiae* strain carrying only the H73A actin. Details of the cell's phenotype and the behavior of the actin *in vitro* will be described elsewhere. Actin was isolated from extracts of mutant and wild-type *S. cerevisiae* and from *C. albicans* by a combination of DNase I affinity chromatography, DEAE-cellulose chromatography, and a cycle of actin polymerization/depolymerization as described previously (12).

Amino acid analysis of in vivo labeled yeast extracts. Intact yeast cells were labeled with [*methyl*-^{3}H]AdoMet and protein hydrolysates were analyzed on a column of Beckman AA-15 sulfonated polystyrene resin as described previously (10).

Fractionation of tryptic peptides of actin by HPLC. Purified actin (about 500 μg, 12 nmol) was denatured in 500 μl of 8 M urea for 1 h at 37°C. The solution was then diluted to give final concentrations of 2 M urea, 1.5 mM Tris–HCl pH 7.5, and 1 mM $CaCl_2$. Trypsin (25 μg, Sigma type XIII, *N*-tosyl-L-phenylalanine chloromethyl ketone-treated) was added and the mixture was incubated at 37°C for 4 h. The entire digest was injected onto a C18 reverse-phase column (Microsorb-MV, 5 μm, 4.6-mm inside diameter, 250-mm length, Rainin) that was equilibrated in solvent A (0.1% triflouroacetic acid in water) and eluted at room temperature at a flow rate of 1 ml/min with a linear gradient of solvent B (0–100%, 0.1% triflouroacetic acid in acetonitrile) over 60 min. Eluant absorbance was monitored at 214 and 295 nm. Fractions were collected manually.

Amino acid analysis. Either 1 μg of purified actin or 200 μl of a collected HPLC fraction (containing about 1.4 nmol of each tryptic fragment) was placed into a 6 × 50-mm glass vial, dried under vacuum, and acid-hydrolyzed in the vapor phase with 200 μl of 6 M HCl at 110°C for 20 h in a Waters Pico-Tag apparatus. Hydrolysates were dissolved in 200 μl of water, and 10 μl was mixed with 5 μl of *o*-phthalaldehyde reagent (0.4% (w/v) *o*-phthalaldehyde, 10% (v/v) methanol, 0.4% β-mercaptoethanol, and 0.8 mg/mL Brij-35 (Sigma) in 0.4 M potassium borate, pH 10.4). After 2 min, the reaction mixture was injected onto a C18 reverse-phase column (Alltech Econosphere, 5 μm, 4.6-mm inside diameter, 250-mm length) equilibrated in solvent C (895 ml 0.1 M sodium acetate, pH 7.2, 10 ml tetrahydrofuran, 95 ml methanol) and eluted at 1ml/min at room temperature with increasing methanol (solvent D) concentration (time, % C, % D: 0, 100, 0; 15, 90, 10; 20, 0, 100; 25, 100, 0, respectively). The fluorescent amino acid derivatives were detected using a Gilson Model 121 fluorimeter with an excitation filter at 305–395 nm and an emission filter at 430–470 nm at a setting of 0.1 RFU. Quantitation of derivatized amino acids was calculated based on the fluorescence of derivatized amino acid standards (Pierce Chemical Co., Standard H; 100–150 pmol) using an AD Instruments apparatus with PowerChrom software.

Electrospray mass spectrometry. A Perkin–Elmer Sciex (Thronhill, Canada) API III triple quadrupole mass spectrometer was tuned and calibrated as previously described (13). HPLC fractions containing actin tryptic fragments were analyzed by direct injection (10–20 μl/injection) in a stream of water/acetonitrile/formic acid (50/50/0.1, by vol; 10 μl/min) entering the Ionspray ion source. Normal spectra were obtained by scanning from *m*/*z* 100–2300 (0.3 Da step size; 1-ms dwell time; 7.72 sec/scan; orifice voltage, 50). Averaging of all the scans accrued from each sample injection, calculation of molecular weights from the series of multiply charged ions found in the spectra, and deconvoultion of the ion series into molecular weight spectra were achieved with the MacSpec computer program (version 3.3, PE Sciex, Ontario, Canada). For MS/MS spectra, positive fragment ion spectra of Q1 preselected parent ions were produced by collisionally induced dissociation (collision gas (10% nitrogen in argon) thickness instrument setting (CGT) of 200, R_0-R_2 offset of 30 V) by scanning Q3 from *m*/*z* 50–2000 (step size, 0.3 Da; dwell time, 1 ms; 6.84 s/scan; orfice voltage, 75). Averaging of all the scans accrued from each sample injection was achieved with the MacSpec computer program. Prediction of fragment ion *m*/*z* values (a, b, and c for N-terminal-containing fragments and x, y, and z for the C-terminal-containing fragments) from theoretical amino acid sequences was achieved with the MacBiospec computer program (version 1.01, PE Sciex, Ontario, Canada, based on the MacProMass computer of Lee and Vermuri (14)).

RESULTS

To study actin methylation in yeast, we incubated intact *S. cerevisiae* cells with [*methyl*-^{3}H]AdoMet to label methylated cell components. Total cellular protein hydrolysates were analyzed by amino acid analysis to compare the methylated amino acid residues of wild-type yeast and a strain in which the actin gene is mutated to convert the conserved histidine residue at position 73 to an alanine residue to prevent methylation (Fig. 1). There was no reduction in the amount of radioactivity that eluted at or near the position of 3-methylhistidine in the hydrolysate of the mutant which contained Ala-73 actin. These results suggest that either the fraction of the total yeast 3-methylhistidine in actin is minimal or that yeast actin is not methylated at the conserved histidine.

[2] Abbreviations used: AdoMet, *S*-adenosyl-L-methionine; [*methyl*-^{3}H]AdoMet, *S*-adenosyl-L-[*methyl*-^{3}H]methionine.

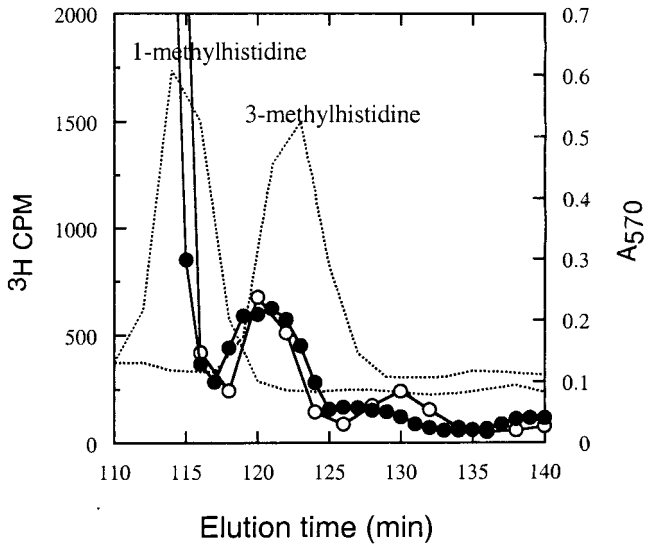

FIG. 1. Amino acid analysis of 3-methylhistidine in intact *S. cerevisiae.* Yeast cells containing a wild-type actin gene (strain AN12 (10)) and cells containing a mutant actin gene where the His-73 residue is replaced by alanine (H73A) were labeled with [*methyl*-^{3}H]AdoMet and the acid-hydrolyzed protein fraction analyzed by cation exchange chromatography as described under Materials and Methods. The radioactivity in the AN12 strain is shown by open circles and that of the H73A strain with closed circles. The intensity of the ninhydrin color (570 nm) of 1-methylhistidine and 3-methylhistidine standards (1 μmol) is shown as dotted lines. The 3-methylhistidine standard was cochromatographed with the H73A sample; the 1-methylhistidine standard was chromatographed in a parallel experiment.

To directly examine the methylation of this histidine residue in yeast actin, we carried out amino acid analyses of the purified actin from *S. cerevisiae* and from rabbit muscle actin as a control. We optimized the chromatographic conditions to separate the *o*-phthaladehyde derivatives of 3-methylhistidine from those of the other amino acids present in the hydrolysate. An elution system was developed in which the fluorescent derivative of 3-methylhistidine elutes as a single symmetrical peak at 9.5 min, baseline resolved from the histidine peak at 8.0 min and from the arginine peak at 13.0 min (Fig. 2A). The hydrolysate of purified rabbit actin showed a peak at 9.5 min retention time, corresponding to 3-methylhistidine as expected (Figs. 2A and 2B, trace A), but no peak was evident in the *S. cerevisiae* actin hydrolysate (Fig. 2B, trace B). The hydrolysate of purified actin from *C. albicans* also did not contain a peak eluting at the retention time corresponding to 3-methylhistidine (Fig. 2B, trace C). These results strongly suggest that actins from these fungi are not modified by histidine methylation.

To confirm unambiguously that His-73 is not methylated in actins of *S. cerevisiae* and *C. albicans,* we isolated the actin tryptic fragments containing His-73 from the purified proteins. Although the HPLC separation of trypsinized actin from mammalian brain cells has been reported in the literature (15), the assignment of the HPLC retention time for each tryptic fragment has not been done. Therefore, we exploited the presence of a single conserved Trp residue in the predicted 69–84 tryptic peptide containing the His-73 residue from each of the species. The amino acid sequences of actins from yeast and rabbit predict a total of only three Trp-containing tryptic peptides; one is expected to elute earlier than the His-73 containing peptide, and the third fragment is expected to elute later (16). Using an absorbance detector at 295 nm to specifically monitor tryptophan-containing peptides, we were able to detect two main peaks for each trypsinized actin (Fig. 3). The predicted third fragment was not detected under these conditions. The second peak had the expected elution time for the 69–84 fragment, and it was collected and analyzed by mass spectroscopy and amino acid analysis as described below.

The electospray mass spectra of the HPLC-purified putative actin 69–84 fragments from rabbit, *C. albicans,* and *S. cerevisiae* all revealed signals for the doubly and triply charged molecules (data not shown). The molecular weight calculation based on these signals for the rabbit peptide agreed within experimental error with that calculated for the 69–84 tryptic fragment in which His-73 is substituted with methylhistidine (Table I). In contrast, the spectra of the *S. cerevisiae* and the *C. albicans* fractions were consistent with peptides containing an unmodified histidine residue (Table I). For rabbit actin, we saw no signal for the corresponding nonmethylated tryptic fragments, and the yeast samples gave no signal for a methylated fragment. The limits of detection were estimated at less than 5% of the main signals.

We next confirmed the presence or absence of methylhistidine in the purified tryptic fragments by the amino acid analysis system described in the legend to Fig. 2. As expected, a symmetrical peak with elution time of 9.5 min corresponding to 3-methylhistidine was detected only from the purified rabbit tryptic fragment (Fig. 4). For each eluted tryptic fragment, the amount of 3-methylhistidine was calculated with respect to the peak for aspartate and glutamate (not shown). We found 1.05 mol of 3-methylhistidine per mole of purified rabbit tryptic fragment, but less than 0.007 mol of 3-methylhistidine per mole of the *S. cerevisiae* and *C. albicans* tryptic fragments.

The tryptic fragments were further analyzed by MS/MS to prove that the observed molecular mass difference between the rabbit tryptic fragment and those of the yeast was the result of an extra methyl group on the histidine residue at position 73. The MS/MS spectra collected from collisionally activated dissociation of each doubly protonated molecule, revealed a series of fragment ions (Fig. 5). The majority

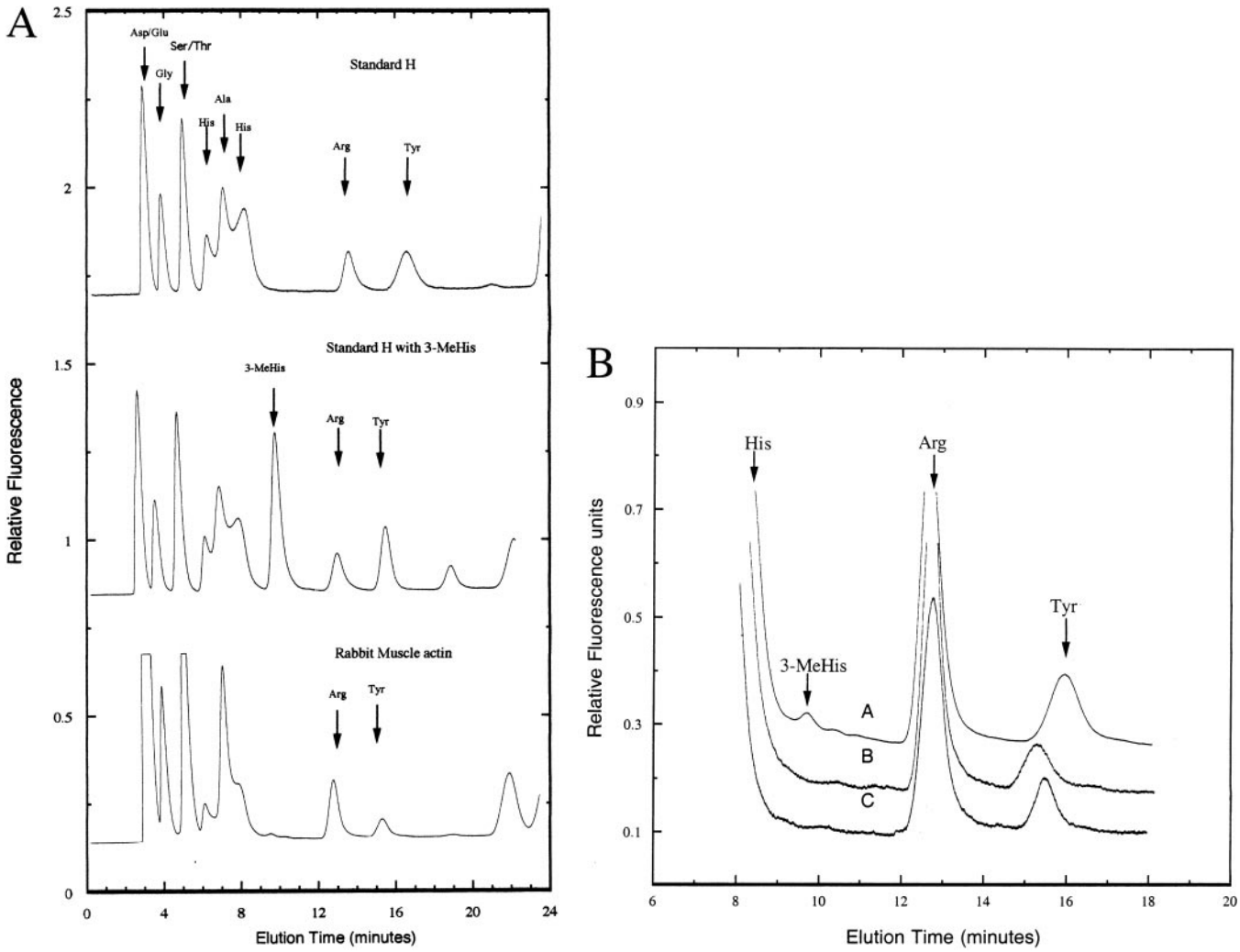

FIG. 2. Amino acid analysis of 3-methylhistidine in purified actin hydrolysates. (A) *o*-Phthaladehyde-derivatized standard amino acids (Pierce Standard H, 100 pmol) with or without 3-methylhistidine (150 pmol) and the acid hydrolysate of rabbit muscle actin were chromatographed on a reverse-phase HPLC column as described under Materials and Methods. The elution positions for the derivatized amino acids are indicated by arrows and 3-methylhistidine is labeled as 3-MeHis. In this system, aspartate and glutamate coelute, serine and threonine coelute, histidine gives two peaks, and the hydrophobic amino acids are retained. (B) Enlarged portions of the chromatographs for derivatized hydrolysates of purified actin from rabbit (trace A), *S. cerevisiae* (trace B), and *C. albicans* (trace C).

of these could be assigned as b- or y-type ions on the basis of their observed *m/z* values by comparison to the predicted array of fragments for each putative peptide. Both y- and b-type ions are formed by cleavage of the amide bonds; charge retention on the N-terminal fragment gives rise to b-type ions, and charge retention on the C-terminal fragment gives rise to y-type ions. In particular, each spectrum unequivocally revealed b4/b5 and y11/12 ion pairs which spanned the putative His-73 residue in *S. cerevisiae* and *C. albicans* and the methylhistidine in rabbit (Fig. 5). These results clearly demonstrate that His-73 is methylated in the rabbit tryptic fragment and that it is not methylated in that of either *S. cerevisiae* or *C. albicans.*

We were initially puzzled that some actins from unicellular eukaryotes are methylated (i.e., *Dictyostelium discoideum* (17), *Physarum polycephalum* (18), and *Acanthamoeba castellanii* (19)) and that some are not (i.e., *Naegleria gruberi* (20), *S. cerevisiae,* and *C. albicans* (this work)). To determine if there might be an evolutionary basis for the difference in the methylation between these two groups of actins, a phylogenetic tree was constructed using the primary sequences of actins from several species in which absence or presence of methylation of His-73 has been reported. The results, shown in Fig. 6, demonstrate a clear phylogenetic demarcation between those organisms that are methylated and

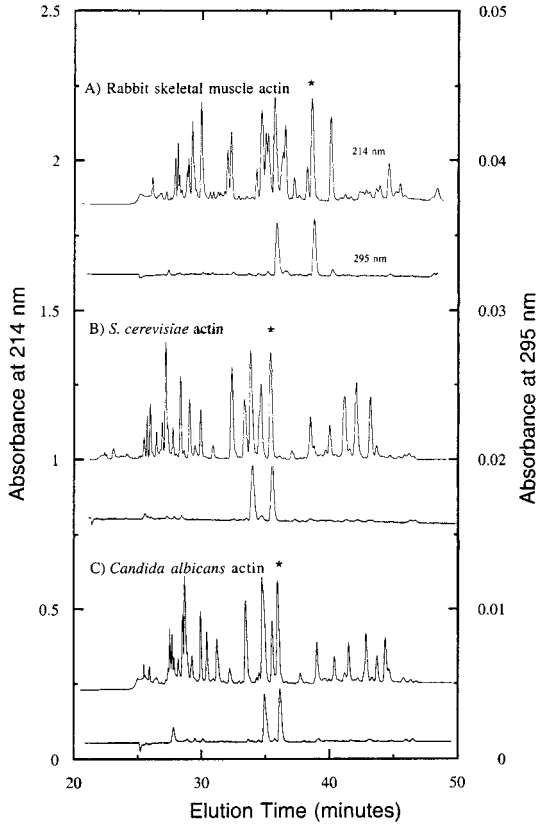

FIG. 3. HPLC fractionation of the trypsin digest of purified actins. Actins from rabbit skeletal muscle, *S. cerevisiae*, and *C. albicans* were digested with trypsin, and the resulting peptides fractionated using reverse-phase HPLC as described under Materials and Methods. The HPLC eluate was monitored at 214 and 295 nm. The tryptic fragment 69–84, identified by electrospray mass spectrometry (Table 1) and amino acid analysis (Fig. 4), is indicated by an asterisk.

those that are not. This tree is consistent with the idea that the methylation of His-73 appeared in evolution after the separation of yeast from *Dictyostelium, Physarum, Acanthamoeba,* and higher organisms. The tree also agrees with previous reports that *Dictyostelium* is more closely related to mammalian cells than is yeast (21).

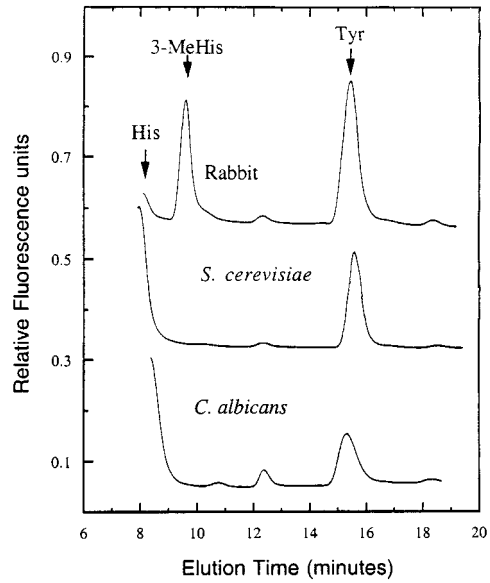

FIG. 4. Amino acid analysis of 3-methylhistidine in purified tryptic peptides. HPLC-purified tryptic peptide 69–84 from rabbit, *S. cerevisiae*, and *C. albicans* was acid hydrolyzed, *o*-phthaladehyde derivatized, and eluted on a reverse-phase HPLC column as shown in Fig. 2. 3-Methylhistidine is labeled as 3-MeHis.

DISCUSSION

Despite the high conservation of the uncommon 3-methylhistidine residue at position 73 in actin, we have documented the absence of this posttranslational modification in two distantly related yeast species by amino acid analysis and mass spectrometry. We have shown that there appears to be a clear evolutionary demarcation among species that do or do not methylate actin His-73 residues, suggesting that this modification was introduced after the appearance of unicellular eukaryotes. Our earlier work demonstrated another type of posttranslational processing in actins from cells ranging from *Dictyostelium* to mammals. This modification involves N-terminal processing in which one or two amino acids are removed singly from the N-terminus following acetylation as N-acetylamino acids until an acidic residue is reached. Acetylation of this residue

TABLE I

Molecular Weights of Actin Tryptic Peptide 69–84 from Various Sources Determined by Electrospray Mass Spectrometry

Species (predicted sequence)	Observed molecular weight[a]	Calculated molecular weight for unmethylated peptide[b]	Calculated molecular weight for methylated peptide[b]
Rabbit (YPIEHGIITNWDDMEK)	1974.9 ± 0.6	1961.2	1975.2
S. cerevisiae (YPIEHGIVTNWDDMEK)	1946.8 ± 0.5	1947.2	1961.2
C. albicans (YPIEHGIVSNWDDMEK)	1932.6 ± 0.2	1933.1	1947.1

[a] Each value is an average determined from the masses of doubly and triply charged ions from two separate experiments ± standard deviations.

[b] Calculated average (chemical) molecular weights.

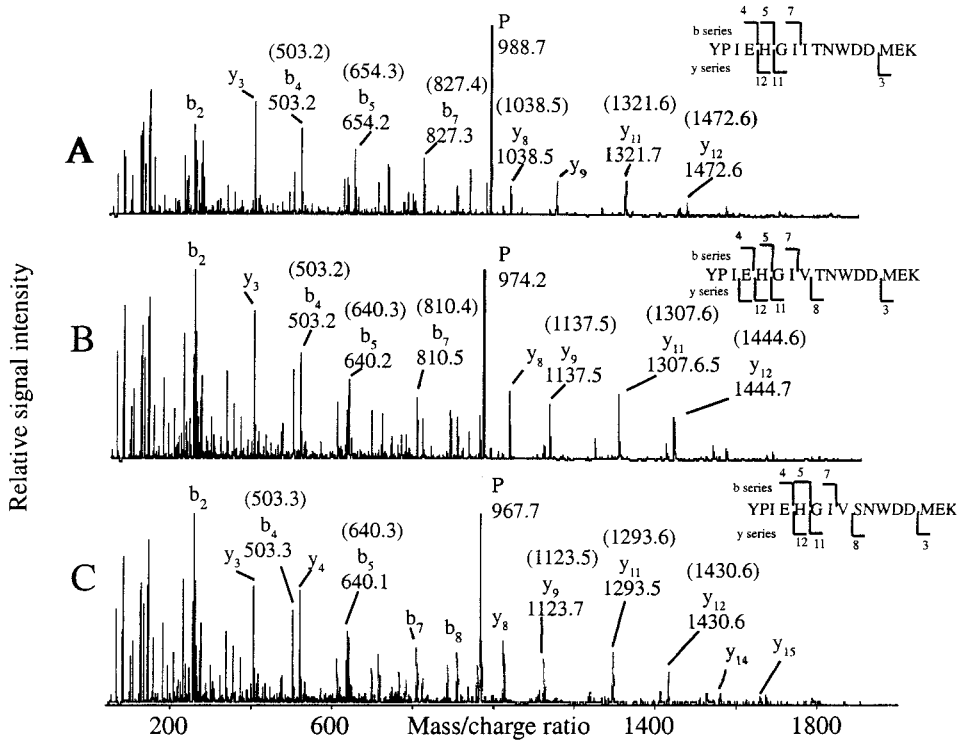

FIG. 5. MS/MS spectra of purified peptides. MS/MS spectra derived by collisionally activated dissociation of the doubly charged parent (P) for rabbit (A), *S. cerevisiae* (B), and *C. albicans* (C) purified tryptic fragment 69–84. Ions assignments (y and b) were made after comparison with the array of ions predicted for each peptide and are shown for a few crucial ions as observed *m/z* with calculated *m/z* in parentheses. The sequence for each tryptic fragment along with some of the b and y ion fragmentation is shown as an inset.

produces the final N-acetylated amino terminus of the protein (22–25). Interestingly, this modification also does not occur in the yeasts *S. cerevisiae* and *C. albicans*, nor the yeast *Schizosaccharomyces pombe* or the filamentous fungus *Aspergillus nidulans* (26). When we examined *Acanthamoeba* actin, we found that processing did not occur both because the pre-actin was not a substrate and because the organism itself did not possess processing activity (27). Although we did not study the processing in *Naegleria*, this particular type of processing cannot occur in this organism since the coding sequence for its N-terminus includes 10 additional amino acids which are not found in its mature form of the protein (27). Thus, our demonstration of the lack of histidine methylation would be the second post-translational modification that separates the actins of this older group of protists from their more evolutionary advanced counterparts. However, since *Acanthamoeba* actin shows methylation of His-73 and not the N-terminus processing, these two different modifications most likely did not arise in parallel.

The methodology reported here for determination of methylhistidine in actin offers a significant advantage over previous approaches (20, 29, 30). The *o*-phthalal-

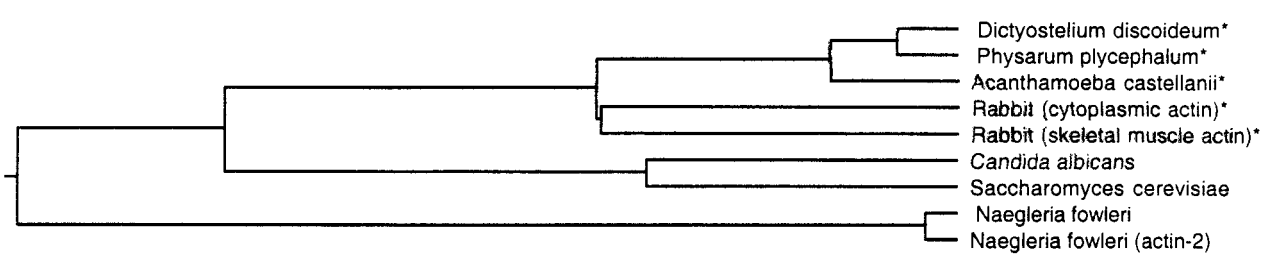

FIG. 6. Phylogenetic tree. Actin sequences were obtained from GenBank. Complete sequences of actin were aligned by the clustal method using the multiple sequence alignment program MegAlign (31). A phylogenetic tree was constructed by applying a neighborhood-joining method to the distance and alignment data (32). The actin sequence of *N. fowleri* was used in place of *N. gruberi*, which was not available. Species labeled with an asterisk contain 3-methylhistidine in their actin.

dehyde derivative of methylhistidine can be clearly resolved chromatographically from that of other amino acids while taking advantage of the sensitivity of the fluorescent labeling. We were able to detect less than 1 mol % 3-methylhistidine in yeast actin, a much lower limit than the 20 mol % reported previously in *N. gruberi* (20). Whereas previous methods required a minimum of 500 μg of actin (30), just 100 μg of actin was sufficient to quantify 3-methylhistidine in this study.

Because the presence of the methyl group on His-73 changes the pK_a of the imidazole, methylation could play an important role quantitatively in ionic processes such as interaction with the phosphate ion released following the hydrolysis of ATP within the cleft of the protein (7, 8). Likewise, methylation might modulate the ionic interaction with one or more of many actin binding proteins that interact with the actin filament between adjacent monomers in the part of the protein where His-73 is located. The extra hydrophobicity and bulk of the methyl group could also play a role in these processes. We simply do not have the answers to these questions yet because we have not had access to the same actin in the methylated vs the nonmethylated state.

The demonstration that yeast does not methylate actin now allows the possibility of testing these theories directly. Aspenstrom and Karlsson (28) have devised an actin expression system in yeast in which chicken nonmuscle β-actin is coexpressed with the endogenous yeast actin, and the two actins are separated chromatographically following cell lysis. This avian actin has a structure identical to β-nonmuscle actin from mammalian sources. Using this system, one could now compare the behavior of the β-actin isolated from yeast with that isolated from either avian or mammalian tissue to address the role of this modification in different *in vitro* assays. The finding that yeast actin is not modified also suggests that it may be useful as a methyl-acceptor substrate to identify the actin methyltransferase in higher organisms. In summary, our demonstration of the lack of actin histidine methylation in yeast may be a crucial step in leading to a better understanding of the role of this modification on actin function.

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