Mutation of Tyr307 and Leu309 in the Protein Phosphatase 2A Catalytic Subunit Favors Association with the α4 Subunit Which Promotes Dephosphorylation of Elongation Factor-2†

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ABSTRACT: The cellular location and substrate specificity of the catalytic subunit (C) of protein phosphatase 2A (PP2A) depend on its interaction with A and B subunits. The distribution of epitope-tagged wild-type or mutated C subunits was studied by transient expression in COS-7 cells. Wild-type tagged C expressed at low levels formed ABC trimer and AC dimer like the endogenous C. Single mutations of C at the site of phosphorylation (Y307F) or carboxymethylation (L309Q) resulted in recovery of only AC dimer. Double mutation of both residues resulted in association of C with α4 protein (α4), a novel subunit of PP2A, instead of with A and B subunits. Thus, the distribution of C between ABC trimer, AC dimer, and α4C complexes can be affected by modifications of the C-terminal residues. The α4 protein is a homologue of the yeast Tap42 protein that functions downstream of the TOR protein to regulate protein synthesis. Transient overexpression of FLAG-α4 resulted in increased dephosphorylation of elongation factor 2, but had no effect on phosphorylation of either p70S6 kinase or PHAS-I (eIF4E-BP). Signals that affect phosphorylation or methylation of the C subunit of PP2A may promote subunit exchange and direct phosphatase activity to specific intracellular substrates.

Protein phosphatase 2A (PP2A)[1] is an abundant serine/threonine phosphatase that has been implicated in the modulation of various cellular events, including metabolism, cell cycle progression, DNA replication, transcription, and protein synthesis (see refs *1*, *2*). Within cells PP2A exists as a variety of trimers consisting of A, B, and C subunits. A core dimer of A subunit composed of helical HEAT repeats (*3*) and C (catalytic) subunit can bind to a variety of structurally distinct regulatory B subunits. The different B subunits are believed to modify PP2A activity and subcellular localization. Biochemical experiments have shown that different trimers have different substrate specificity (*4*, *5*). Genetic studies in budding yeast have shown that mutation of two different B subunit genes, *CDC55* and *RTS1*, results in different phenotypes. In addition, these two genes do not fully complement one another (*6*).

The C subunit of PP2A has been reported to undergo at least three different posttranslational modifications: carboxymethylation of L309 at the C-terminus by a methyltransferase (*7*−*9*); phosphorylation of Y307 in the C-terminal segment (*10*−*12*); and phosphorylation of an unidentified threonine residue by autophosphorylation-activated protein kinase (*13*). Each of these modifications has been shown to occur stoichiometrically in vitro. Methylation does not cause a large change in activity as measured in biochemical assays (*7*). In contrast, phosphorylation of either tyrosine or threonine residues results in inactivation of the phosphatase, and auto-dephosphorylation has been suggested as a mechanism for reactivation (*10*, *13*).

In *S. cerevisiae*, Tap42 was isolated as a high-copy suppressor of defects in the protein phosphatase Sit4 (*14*), a C subunit corresponding to mammalian PP6 that is related in sequence to PP2A. Tap42 was found in a complex with the C subunit of Sit4 as well as with Pph21/22, the yeast PP2A C subunits. Tap42 is related in sequence (24% identity) to the murine α4 protein which was originally discovered as an Ig-α binding protein in the B cell receptor complex (*15*, *16*). In coprecipitation assays, recombinant GST-α4 bound to the C subunit of PP2A and displaced the A and B subunits from either the AC dimer or the ABC trimer (*17*). In COS-7, Jurkat, or Tag-Jurkat cells expressing tagged α4

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[1] Abbreviations: PP2A, protein phosphatase 2A; A, 60 kDa regulatory subunit of PP2A; B, family of regulatory subunits of PP2A; α4, phosphoprotein that binds to the Ig-α chain of B cell receptor; eEF2, eukaryotic elongation factor-2; PHAS-I or eIF4E-BP, phosphoprotein that binds initiation factor 4E; TOR, target of rapamycin protein; Tap42, yeast protein related to α4 that functions as an alternative phosphatase subunit; FLAG, epitope tag of sequence DYKDDDDK; HA, epitope tag of hemagglutinin A with sequence YPYDVPDYA; $(HA)_3$-C_{WT}, triple HA tagged, wild-type C subunit; $(HA)_3$-C_{Y307F}, triple HA tagged C subunit singly mutated at residue 307; $(HA)_3$-C_{L309Q}, triple HA tagged C subunit singly mutated at residue 309; $(HA)_3$-C_{DM}, triple HA tagged C double mutated at Y307F, L309Q.

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protein, PP2A C subunit was recovered bound to α4 by immunoprecipitation (*17*−*19*). Thus, the α4 protein represents a novel PP2A subunit that forms complexes with the C subunit without the A subunit.

The mechanisms involved in the regulation of the interaction of the C subunit of PP2A with the A, B, and α4 subunits are not known. One hypothesis is that modification of the C subunit by methylation or phosphorylation influences subunit association. In this study, we have investigated whether mutations of Y307 or L309 residues in the PP2A C subunit alter the distribution of the C subunit between dimer (AC), trimer (ABC), or α4-containing complexes. The results obtained indicate that wild-type C subunit was found in complexes with ABC and AC forms of PP2A in COS-7 cells. C subunits containing the single mutations, Y307F or L309Q, formed mostly AC dimers, whereas C subunit containing the double mutation, Y307F/L309Q, was bound to the α4 protein instead of the A subunit.

In yeast, the Tap42 protein associated with PP2A C subunits functions in a positive role downstream of TOR and promotes protein synthesis (*14*). This may result from the ability of α4−C complexes to dephosphorylate these regulatory proteins whose net dephosphorylation would stimulate protein synthesis. Possible candidates are the α subunit of eukaryotic initiation factor-2 (eIF-2α) or eukaryotic elongation factor-2 (eEF2). There is good evidence that eIF-2α is dephosphorylated by PP1 (*20*−*22*), while eEF2 is dephosphorylated by PP2A (*23*−*28*). In the present study, we found that overexpression of FLAG-α4 resulted in a selective decrease in phospho-eEF2. Together, these results indicate that modification of the COOH-terminus of the C subunit influences its interaction with the A, B, and α4 subunits to alter the substrate specificity of intracellular pools of PP2A.

EXPERIMENTAL PROCEDURES

Plasmid Constructs and Mutagenesis. Mutations of the PP2A C subunit were produced by polymerase chain reaction with the HL-14 clone (*29*) using sense primer starting from the second codon and the degenerate antisense primer 5′-AC GGA TCC AAA ATT TCA TTA C(A/T)G GAA G(A/T)A GTC TGG GGT ACG-3′ (41-mer). The resulting DNA was subcloned into mammalian expression vector p*K*H3 (*30*) to generate N-terminal triple hemaglutinin (HA)-tagged C subunit [$(HA)_3$-C]. Mutants were selected by automated sequencing of the 3′ ends. Murine α4 epitope tagged with FLAG on the N-terminus (FLAG-α4) was described previously (*17*). PHAS-I expression vector pCMV-PHAS-I was kindly provided by Dr. J. C. Lawrence, Jr. (University of Virginia, Charlottesville, VA).

Cell Culture and DNA Transfection. COS-7 cells were grown at 37 °C with a humidified atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine and 10% (v/v) newborn calf serum (GIBCO). Cells at 50% confluency were transiently transfected with FuGene 6 (Boehringer Mannheim). Transfection efficiency was estimated by measuring β-galactosidase expression in cells transfected in parallel with a vector encoding β-galactosidase.

Mono Q Chromatography Analyses of PP2A. For Mono Q chromatography, COS-7 cells were sonicated in buffer consisting of 25 mM Tris-HCl, pH 7.4, 30 mM 2-mercaptoethanol, 1 mM sodium *o*-vanadate, and 1× mini-complete protease inhibitor (Boehringer Mannheim). After centrifugation at 100000*g* for 30 min at 4 °C, 1 mg total protein of the supernatant was loaded onto a Mono Q HR5/5 column (Pharmacia) preequilibrated in buffer A (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 30 mM 2-mercaptoethanol). Fractions were collected every minute for 50 min (flow rate 0.5 mL/min). The column was washed with buffer A for 16 min, followed by a linear gradient to buffer B (25 mM Tris-HCl, pH 7.4, 500 mM NaCl, and 30 mM 2-mercaptoethanol) over 24 min, and then buffer B for the final 10 min.

For PP2A activity, aliquots of the fractions were immediately assayed using *p*-nitrophenyl phosphate (pNPP) as substrate: 0.1 mL of the fraction was incubated in a 0.3 mL reaction with 20 mM pNPP, 50 mM MOPS, pH 7.4, 20 mM $MgCl_2$, 2 mM $MnCl_2$, and 1 mM DTT (final concentrations) at 30 °C in the presence or absence of 5 nM okadaic acid (OA). Reactions were stopped with 0.17 mL of 2.5 M Na_2CO_3 (final concentration 900 mM). The optical density at 405 nm ($OD_{405\ nm}$) was determined with 0.2 mL of the reaction mix using a 96-well plate reader (Molecular Devices). PP2A activity was defined as the difference between the absence and presence of 5 nM OA (1 unit = $OD_{405\ nm}$/min × 1000). For Western analysis, 0.1 mL of the fractions from Mono Q chromatography was precipitated with 20% trichloroacetic acid and 0.1% deoxycholate. The precipitate was redissolved in 1× SDS sample buffer, resolved on an 8.5% SDS−polyacrylamide gel, and immunoblotted. Blotting was performed with anti-peptide antibodies against PP2A C subunit (*11*), PP2A B55 subunit (Upstate Biotechnology), PP2A A subunit (Upstate Biotechnology), HA epitope (12CA5; Berkeley Antibody Co.), or FLAG epitope (M2; Kodak). Immunoblots were developed using the Renaissance chemiluminescence reagent (NEN Life Science Products). For immunoprecipitation, Mono Q chromatography was carried out using 1 mL of 2.5 mg/mL extract, and 0.2 mL of each fraction was diluted to 0.5 mL with buffer A prior to immunoprecipitation.

Immunoblot and Immunoprecipitation of PP2A in Soluble Cell Extracts. Cells transiently transfected for 20 h were lysed in buffer consisting of 25 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.5% IGEPAL CA-630 (replacement for NP-40), 30 mM 2-mercaptoethanol, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 1 mM sodium *o*-vanadate, and 1 μM microcystin-LR. After centrifugation at 20000*g* for 10 min at 4 °C, the supernatant was collected as the soluble extract. Protein concentration was determined using the Bio-Rad protein assay with bovine serum albumin as standard. For Western analyses, 10−25 μg of soluble extract protein was used per lane. Antibodies for total and phospho-specific eEF2 were used as described previously (*31*). Antibody against p70 S6 kinase was from Santa Cruz, and antibody for PHAS-I was a generous gift from Dr. J. C. Lawrence, Jr. (University of Virginia, Charlottesville, VA). For immunoprecipitation, 0.5 mg of soluble extract protein was diluted to 1 mL in the same buffer used for cell lysis, and mixed with 1 μg of 12CA5 antibody bound to protein A−Sepharose (Pharmacia) or 2 μL of FLAG M2 resin (Kodak) for 90 min at 4 °C. One-quarter of the final immunoprecipitate was subjected to immunoblotting with anti-epitope antibodies.

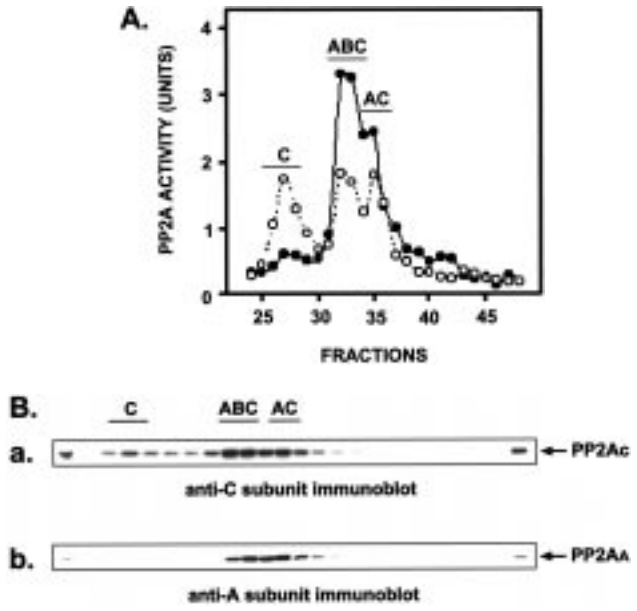

FIGURE 1: Analysis of PP2A in cell extracts by Mono Q chromatography. (A) Extracts of COS-7 cells were fractionated by Mono Q chromatography, and PP2A activity was assayed as described under Experimental Procedures. Fresh extracts (solid line) were compared to freeze−thaw extracts (dashed line). (B) Every fraction from 24 to 48 from Mono Q was immunoblotted (one fraction per lane) with antibody against PP2A C subunit (panel a) or A subunit (panel b). The location of monomer (C), trimer (ABC), and dimer (AC) forms of PP2A was deduced from immunoblotting for the separate subunits (data not shown for anti-B immunoblot). Total cell extract (10 μg of protein) was used as positive control in the first and last lanes.

RESULTS

Analysis of PP2A in Cell Extracts by Mono Q Chromatography. For more than 20 years PP2A has been purified from tissues as dimeric and trimeric complexes, but little is known about the distribution of the catalytic subunit (C) in tissue culture cells. To analyze PP2A in the cytosol of COS-7 cells, fresh extracts were separated by Mono Q chromatography, followed by phosphatase assay using *p*-nitrophenyl phosphate (pNPP) as substrate (Figure 1A, solid line). The activity profile showed a minor peak centered at fraction 27, a major peak that was partially resolved into two components, centered at fractions 32 and 35, followed by a minor peak centered at fraction 41. Immunoblot analyses of every fraction (24−48) showed the PP2A C subunit (36 kDa) was present in the first three peaks (Figure 1B, frame a) and the phosphatase activity was proportional to the intensity of C subunit detected by immunoblotting. Immunoblotting of the upper half of the filters indicated that the A subunit (60 kDa) was present in the two major peaks, but not in the first or last minor peaks (Figure 1B, frame b). Immunoblotting also showed that the B55 subunit (55 kDa) was present only in the first major peak centered at fraction 32 (not shown). Therefore, the first three peaks corresponded to the C monomer, ABC trimer, and AC dimer forms of PP2A, respectively. When fresh extracts were subjected to a single cycle of freezing and thawing before Mono Q chromatography, the yield of the ABC trimer was much lower, and increased amounts of the C monomer and AC dimer of PP2A were recovered (Figure 1A, dashed line). Apparently, dissociation of the ABC trimer into AC dimer and C monomer occurred as a result of the freezing and thawing. Only freshly prepared cell extracts were used in the following studies.

Expression and Distribution of $(HA)_3$-Tagged PP2A Catalytic Subunits. To study the effect of modifications of the C subunit of PP2A on its behavior in cells, we produced four different N-terminal triple (HA)-tagged constructs: (1) wild-type catalytic subunit [$(HA)_3$-C_{WT}] that served as control; (2) tyrosine 307 mutated to phenylalanine [$(HA)_3$-C_{Y307F}] to eliminate a known site of phosphorylation; (3) leucine 309 mutated to an uncharged but polar residue, glutamine [$(HA)_3$-C_{L309Q}], to interfere with the recognition of the C-terminus by methyltransferase; and (4) double mutation of both Y307F and L309Q [$(HA)_3$-C_{DM}] to interfere with both phosphorylation and methylation. Transient transfection of COS-7 cells gave low levels of ectopic expression of these epitope-tagged catalytic subunits that were detected at 39 kDa by immunoblotting cell lysates with anti-HA antibodies (not shown). Immunoblotting with anti-C subunit antibodies showed that the 39 kDa protein was present only in trace amounts relative to the endogenous 36 kDa C subunit. Even when taking into account transfection efficiency (30−50%), we estimated that the amount of $(HA)_3$-C in cells was only ca. 5% of the endogenous PP2A. Consistent with these estimates based on immunoblotting, no significant increase in PP2A activity could be detected in the cell extracts or in the activity profiles after Mono Q chromatography for cells transfected with tagged C, compared to controls transfected with empty vector. Therefore, the tagged PP2A was expressed at the level of a tracer in these studies and did not alter the distribution of the endogenous C.

The distributions of epitope-tagged wild-type and mutated versions of PP2A C subunit were analyzed by Mono Q chromatography. The $(HA)_3$-C_{WT} was separated into two major peaks, corresponding to the ABC trimer and the AC dimer (Figure 2A, frame a). The (HA)-tagged C subunit eluted slightly later in the gradient than the endogenous C subunit (Figure 2A, frame a, dotted bars vs solid bars) probably due to the six acidic residues introduced by the triple (HA)-tag. The results showed that $(HA)_3$-C_{WT} associated with endogenous A and B55 subunits to form ABC trimer. In contrast to the behavior of wild-type C, single point mutations of either Y307F or L309Q yielded a single major peak after Mono Q chromatography, corresponding to AC dimer in COS-7 cells, with essentially no ABC trimer detected (Figure 2A, frames b, c, dotted bars). These results suggest that the single mutations in the C subunit did not interfere with formation of the AC dimer, but did affect the interaction between B subunits and the AC dimers. Double mutation of the C subunit at residues 307 and 309 resulted in a broad peak that eluted from Mono Q chromatography much later in the gradient (Figure 2A, frame d). This peak of $(HA)_3$-C_{DM} did not coincide with either the ABC trimer or the AC dimer based on immunoblot analyses for the A and B subunits, indicating that this mutant C subunit was not associated with the A subunit.

The α4 protein is capable of forming a complex with C subunit without participation of the A subunit (*17*, *18*), and could account for the form of PP2A that eluted from the Mono Q column in the later peak. Expression of epitope-tagged α4 (FLAG-α4) alone in COS-7 cells resulted in elution from the Mono Q column in a broad peak, in the same fractions in which $(HA)_3$-C_{DM} eluted (not shown).

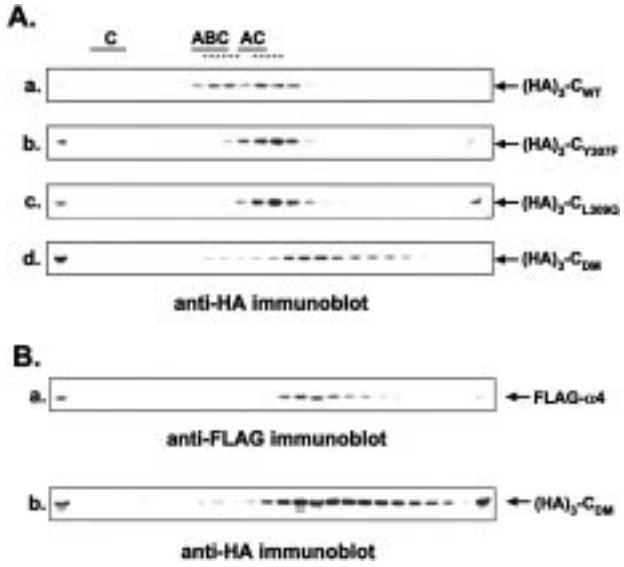

FIGURE 2: Distribution of $(HA)_3$-tagged PP2A catalytic subunits expressed in COS-7 cells. Using conditions as in Figure 1, fresh cell extracts of COS-7 cells were analyzed for the distribution of PP2A by Mono Q chromatography. Solid lines indicate endogenous and dashed lines HA-tagged C subunit. Immunoblotting of fractions with anti-HA antibody was used to reveal the distribution of the following HA epitope-tagged PP2A catalytic subunits: (a) wild type (WT); (b) Y307F; (c) L309Q; (d) double mutant (DM)Y307F/L309Q. (B) Extracts of COS-7 cells transfected with both FLAG-α4 and $(HA)_3$-$PP2A_{DM}$ were fractionated by Mono Q chromatography, and individual fractions were immunoblotted with antibodies against (a) FLAG epitope (M2 antibody) and (b) HA epitope (12CA5).

Coexpression of FLAG-α4 with $(HA)_3$-C_{DM} indicated that the two proteins coeluted in a broad peak (Figure 2B). Coexpression of the two proteins did not affect the elution profile of the endogenous C subunit determined by Western blotting or the PP2A activity profile (not shown).

Coimmunoprecipitation of $(HA)_3$-C_{DM} and FLAG-α4. To test whether $(HA)_3$-C_{DM} and FLAG-α4 were bound together, rather than simply coeluting in the same fractions, coimmunoprecipitation studies were carried out. In anti-FLAG immunoprecipitates, $(HA)_3$-C_{DM} coimmunoprecipitated with FLAG-α4, and in anti-HA immunoprecipitates, FLAG-α4 coimmunoprecipitated with $(HA)_3$-C_{DM} from cell lysates (Figure 3A). The $(HA)_3$-C_{DM} and FLAG-α4 proteins could also be coimmunoprecipitated from individual fractions following Mono Q chromatography (Figure 3B). Immunoblot analyses of immunoprecipitates showed that $(HA)_3$-C_{DM} was complexed with FLAG-α4 across the entire peak. These results show that $(HA)_3$-C_{DM} and FLAG-α4 interact in a stable complex, although other protein(s) may be part of the complex.

Effect of FLAG-α4 Expression on Protein Dephosphorylation. The Tap42 protein has a positive function downstream of TOR in yeast, and promotes protein synthesis (*14*). Therefore, we examined the effect of FLAG-α4 on the dephosphorylation of factors involved in regulation of protein translation, including eEF2, a protein known to be a substrate for PP2A in mammalian cells (*32*−*34*). Expression of FLAG-α4 plus $(HA)_3$-C_{DM} (not shown) or FLAG-α4 alone (Figure 4A, panel a) selectively reduced phosphorylation of eEF2 (Figure 4A, panel b), as detected with a phospho-specific antibody, without effect on the total eEF2 protein (Figure 4A, panel c). The phosphorylation level of eEF2 recovered

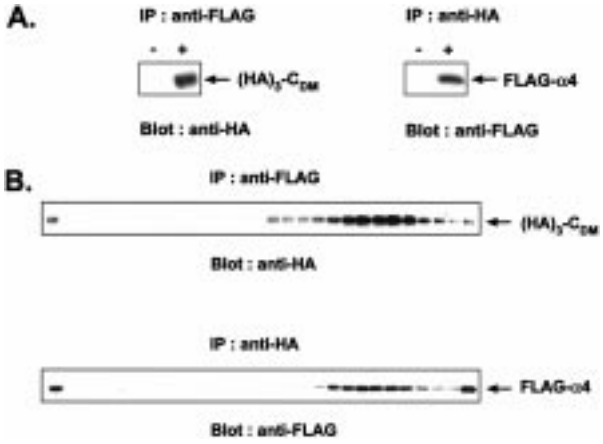

FIGURE 3: Coimmunoprecipitation of $(HA)_3$-$PP2A_{DM}$ and FLAG-α4 expressed in COS-7 cells. A cytosolic fraction was prepared from COS-7 cells transfected with vectors for both FLAG-α4 and $(HA)_3$-$PP2A_{DM}$. Samples were immunoprecipitated (IP) with anti-FLAG or anti-HA antibodies and blotted with anti-HA or anti-FLAG antibodies, respectively. (A) Whole cytosolic fraction processed with (+) and without (−) added antibody; (B) fractions after Mono Q chromatography.

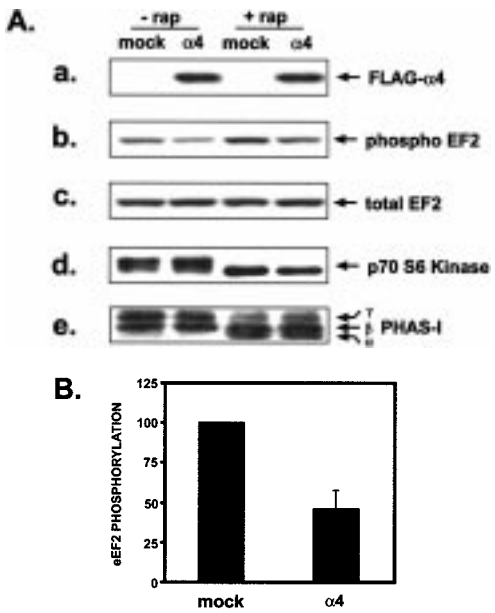

FIGURE 4: Effect of transient FLAG-α4 expression on phosphorylation of proteins involved in translation. (A) Effects of transient transfection of COS-7 cells with empty vector (mock) or FLAG-α4 (α4) were analyzed by preparation of extracts and immunoblotting with antibodies against (a) FLAG (M2), (b) phospho-eEF2, (c) total eEF2, (d) p70 S6 kinase, or (e) PHAS-I. Cells cotransfected with vectors for FLAG-α4 and PHAS-1 were processed and proteins immunoblotted with anti-PHAS-I. Cells were treated with vehicle (−rap) or 100 nM rapamycin (+rap) for 30 min prior to lysis. (B) The dephosphorylation of eEF2 illustrated in panel b was quantitated by densitometry in four independent experiments to show there was a reproducible and significant difference, 46% ± 11, $p < 0.01$, by Student's t test.

from all the cells was decreased by a significant amount (46% ± 11, $p < 0.01$), even though fewer than half of the cells were transfected (Figure 4B). This implies a much more extensive dephosphorylation of eEF2 in those cells in the population that were expressing FLAG-α4. Rapamycin treatment enhanced phosphorylation of eEF2 by 50−60%, and transient expression of FLAG-α4 still caused about 50% dephosphorylation (Figure 4A, panel b). In contrast, transient

expression of FLAG-α4 had no effect on the phosphorylation of other proteins involved in regulation of translation, namely, p70 S6 kinase (Figure 4A, panel d) or PHAS-I (Figure 4A, panel e), based on their migration in SDS−PAGE. Rapamycin treatment of cells did cause dramatic dephosphorylation of these proteins. The drug affected all the cells, and there was a complete change in electrophoretic mobility of the proteins. This response was unaffected by expression of FLAG-α4. The results show that overexpression of FLAG-α4 caused dephosphorylation of eEF2, without changing phosphorylation of PHAS-I or p70 S6 kinase.

DISCUSSION

PP2A has been thought to exist in cells predominantly as a trimer (ABC). The dimer form (AC) recovered by purification from tissues has been considered an artifact due to partial proteolysis or dissociation of the B subunit during purification (*35*). However, PP2A is also present in cells as the AC dimer. Kremmer et al. (*36*) suggested that the AC dimer represented about one-third of the total PP2A, based on selective immunoprecipitation of trimers and dimers. Our analysis by Mono Q chromatography, using rapid cell lysis and sample processing with a cocktail of protease inhibitors, showed an appreciable fraction of PP2A was recovered as dimer, and even a small fraction as C monomer. Disruption of trimer into dimer and monomer was observed upon freezing and thawing as previously reported (*37*), and this interconversion supported our assignment of the different PP2A forms separated by Mono Q chromatography.

Transient expression of $(HA)_3$-C_{WT} resulted in recovery of the tagged C subunit in ABC trimeric and AC dimeric forms, in the same ratios as observed for the endogenous C subunit. There are transcriptional and translation mechanisms limiting ectopic expression of C to a low level relative to endogenous C (*38, 39*). Therefore, the tagged C was only present in trace amounts. Restrictions that prevent overexpression become an advantage because tagged, mutant forms of C can be studied without significant alteration of the endogenous pool of C. The single mutations of Y307F or L309Q resulted in the C subunit being recovered only in AC dimers.[2] Double mutation of Y307F/L309Q resulted in elution of the C subunit much later in the gradient, where it was associated with the α4 subunit, but not with either the A or the B subunit. Inui et al. (*18*) recently reported augmented binding of α4 to PP2A C subunit by culturing Jurkat cells in low serum (2 or 0.4%) for 72 h. Low serum (0.1%) or no serum are conditions that gave reduced phosphorylation of Y307 in PP2A in 10T1/2 cells (*11*). In addition, carboxymethylation of L309 is low at the G_0/G_1 boundary (*40*), where cells without serum would arrest. Ogris et al. (*41*) reported loss of B55 subunit binding to AC dimers when certain C subunit mutants were stably expressed in clones of NIH3T3 cells. Our results are consistent with the hypothesis that covalent modifications of Y307 and L309 in the C subunit of PP2A affect distribution of the phosphatase between ABC, AC, and α4C. How the covalent modifications of the C subunit might govern PP2A subunit exchange in vivo and how the cell cycle might influence PP2A subunit exchange are still open questions. The discovery that mutations that cause loss of A subunit function are associated with types of human cancer highlights the importance of these molecular interactions (*42*).

The yeast Tap42 protein associates with C subunits of the 2A type, functions in a positive role downstream of TOR, and promotes protein synthesis (*14*). When we found that mutated versions of C preferred to associate with α4, we sought regulatory proteins whose net dephosphorylation would stimulate or activate protein synthesis. Possible candidates are the α subunit of eukaryotic initiation factor 2 (eIF-2α) that is phosphorylated and inhibited by several eIF-2α protein kinases, and eukaryotic elongation factor 2 (eEF2) that is phosphorylated and inhibited by eEF2 kinase (previously called CaM kinase III) (*32*−*34*). There is good evidence that eIF-2α is dephosphorylated by PP1 (*20*−*22*, *26*−*28*), while various studies show that eEF2 is dephosphorylated with considerable specificity by PP2A (*23*−*25*, *32*−*34*). We found here that transient overexpression of FLAG-α4 produced a significant and selective decrease in the level of phospho-eEF2. The α4-bound phosphatase (PP2A or PP6) might dephosphorylate eEF2 directly by being targeted to this substrate. Alternatively, since eEF2 kinase is itself regulated by phosphorylation (*43*, *44*), the effects of α4 on eEF2 could be indirect, by inactivation of the kinase. However, neither eEF2 nor eEF2 kinase was detected in immunoprecipitates of FLAG-α4 (not shown). This suggests that these PP2A substrates and the FLAG-α4 phosphatase do not form stable complexes.

Previously, eEF2 was found to be dephosphorylated in response to insulin in CHO cells overexpressing insulin receptor, and this was associated with stimulation of the rate of peptide chain elongation (*45*). The effect of insulin on eEF2 dephosphorylation was prevented by either rapamycin or wortmannin treatment. In the present study, under basal conditions rapamycin treatment resulted in increased phosphorylation of eEF2 but did not block dephosphorylation of eEF2 in cells expressing FLAG-α4. How rapamycin and TOR affect phosphatase association with α4 remains to be determined,[3] but the finding that α4 can promote dephosphorylation of eEF2 even in the presence of rapamycin represents new evidence toward elucidating this signaling pathway.

[2] A recent report shows that C mutated as L309A is recovered as AC dimer, not ABC trimer (*47*), in agreement with results presented here.

[3] TOR recently was reported to phosphorylate Tap42 (α4) in a rapamycin-sensitive reaction (*46*).

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