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Phosphorylation of more than one site is required for tight interaction of human tau protein with 14-3-3ζ

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ABSTRACT

Serine residues phosphorylated by protein kinase A (PKA) in the shortest isoform of human tau protein (τ3) were sequentially replaced by alanine and interaction of phosphorylated τ3 and its mutants with 14-3-3 was investigated. Mutation S156A slightly decreased interaction of phosphorylated τ3 with 14-3-3. Double mutations S156A/S267A and especially S156A/S235A, strongly inhibited interaction of phosphorylated τ3 with 14-3-3. Thus, two sites located in the Pro-rich region and in the pseudo repeats of τ3 are involved in phosphorylation-dependent interaction of τ3 with 14-3-3. The state of τ3 phosphorylation affects the mode of 14-3-3 binding and by this means might modify tau filament formation.

Structured summary:
MINT-7233358, MINT-7233372, MINT-7233384: *14-3-3 zeta* (uniprotkb:P63104) and *Tau 3* (uniprotkb:P10636-3) *bind* (MI:0407) by *molecular sieving* (MI:0071)
MINT-7233323, MINT-7233334, MINT-7233346: *Tau 3* (uniprotkb:P10636-3) and *14-3-3 zeta* (uniprotkb:P63104) *bind* (MI:0407) by *crosslinking studies* (MI:0030)
MINT-7233285, MINT-7233297, MINT-7233310: *14-3-3 zeta* (uniprotkb:P63104) and *Tau 3* (uniprotkb:P10636-3) *bind* (MI:0407) by *comigration in non-denaturing gel electrophoresis* (MI:0404)

1. Introduction

The microtubule-associated tau protein is abundantly expressed in the axons of neurons and plays a crucial role in axonal transport [1]. Six major isoforms of tau detected in the human brain are generated by alternative splicing and contain from 352 (the shortest τ3 isoform) to 441 (the largest τ4 isoform) residues [1,2]. These isoforms differ by the presence of either three (τ3) or four (τ4) imperfect repeats forming microtubule-binding region and by the presence of variable number of inserts in the N-terminal half [1,2]. Tau belongs to the group of intrinsically disordered proteins [3], however, short stretches located in the region of imperfect repeats tend to form β-strands partially masked by mobile N- and C-terminal tails [4]. Certain stimuli induce conformational changes unmasking these β-strands [5] and leading to formation of filaments and neurofibrillar tangles. These aggregates are hallmarks of Alzheimer's disease and related taupathies [1,2]. Different factors affect transition of soluble tau monomers to insoluble filaments and among them limited proteolysis, formation of disulfide bonds and phosphorylation [1,2,5]. Tau contains more than 20 sites phosphorylated by a number of different protein kinases [6]. Phosphorylation of certain sites induces dissociation of tau from microtubules, provokes phosphorylation of other sites and/or partial proteolysis thus increasing the probability of aggregation [2]. Therefore the fate of phosphorylated tau is of special interest.

The recently published data indicate that tau interacts with 14-3-3 [6]. The family of 14-3-3 proteins is presented by seven isoforms with especially high content in the brain [7]. These proteins form homo- or heterodimers and predominantly interact with proteins containing phosphorylated Ser (or Thr) in canonical motifs [8]. Earlier published data indicate that 14-3-3 interacts with the microtubule-binding site of τ4 and this interaction is independent of tau phosphorylation by cdc2-like protein kinase [6]. It is supposed that serving as an adapter 14-3-3, stimulates tau phosphorylation by a number of different protein kinases [6,9–11]. 14-3-3 promotes aggregation of unphosphorylated tau [11–13] and is presented in neurofibrillar tangles in Alzheimer's

Abbreviations: PKA, protein kinase A; PKB, protein kinase B; τ3, the shortest isoform of human tau protein; pτ3, τ3 phosphorylated by protein kinase A; PMSF, phenylmethane sulfonylfluoride; ME, β-mercaptoethanol

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disease [14,15]. The recent data indicate that phosphorylation by protein kinase A (PKA) or protein kinase B (PKB) increases the interaction of tau with 14-3-3 and modifies the effect of 14-3-3 on tau aggregation [12,13,16].

Until recently the role of different phosphorylation sites of tau on its interaction with 14-3-3 was not analyzed in detail. This paper deals with analysis of participation of certain sites phosphorylated by PKA in the interaction of the shortest isoform of human tau protein (τ3) with 14-3-3.

2. Materials and methods

2.1. Proteins

The cDNA corresponding to the human shortest tau isoform containing 352 residues and denoted as τ3 (Swiss-Prot ID P10636-3) was PCR amplified and cloned as described earlier [16]. Tau mutant S156A was obtained by the megaprimer method using *Pfu* DNA polymerase (Sileks) and the following primer TCCCGCACCCCGGCACTTCCAACCCCA. The double mutants S156A/S235A and S156A/S267A were obtained using TCCAAGTGTGGCGCATTAGGCAAC (for S235A) and TCGAAGATTGGGGCACTGGACAAT (for S267A) primers (mutagenic codons are underlined) and cDNA encoding S156A tau mutant as a matrix. Resulting constructs were verified by DNA sequencing and used for protein expression and purification [16]. Recombinant human 14-3-3ζ and the catalytic subunit of mouse PKA were expressed and purified as described earlier [16,17]. Protein concentration was determined spectrophotometrically using $\varepsilon_{0.1\%}$ equal to 0.987 and 0.203 for 14-3-3ζ and tau, respectively.

2.2. Protein phosphorylation

Tau or its mutants (5–15 μM) were phosphorylated at 37 °C in a buffer containing 20 mM Tris–HCl pH 7.5, 10 mM glycerol-1-phosphate, 1 mM $MgCl_2$, 0.1 mM phenylmethane sulfonylfluoride (PMSF), 15 mM β-mercaptoethanol (ME), 150–250 μM ATP and trace amounts of [γ-^{32}P]ATP in the presence of catalytic subunit of PKA. Kinetics and stoichiometry of phosphorylation were determined by incorporation of radioactive phosphate using a phosphocellulose-paper assay [16].

2.3. Native gel electrophoresis

Fixed amounts of 14-3-3 were mixed with increasing quantities of tau or its mutants in buffer BB containing 20 mM Tris–HCl pH 7.6, 150 mM NaCl, 5% glycerol, 0.1 mM PMSF and 15 mM ME. The mixture was incubated for 40 min at 37 °C and subjected to native gel electrophoresis [18] with subsequent quantitative densitometry. Under conditions used, the square under the peak of 14-3-3 was proportional to the quantity of protein loaded on the gel.

2.4. Chemical crosslinking

Isolated phosphorylated τ3 or the mixture of 14-3-3 with phosphorylated tau (or its mutants) were incubated in buffer BB for 40 min at 37 °C and glutaraldehyde was added up to the final concentration 0.045%. After 15 min incubation at 30 °C the reaction was stopped by addition of SDS-sample buffer. The protein composition of the samples was determined by SDS-electrophoresis on a 5–20% gradient polyacrylamide gel [19]. The gels were stained by Coomassie R250 and subjected to autoradiography.

2.5. Size-exclusion chromatography

The samples (each 150 μl) of isolated 14-3-3, phosphorylated tau or its mutants or mixtures containing 14-3-3 and phosphorylated tau species were preincubated for 30 min at 37 °C and loaded on a Superdex 200 (HR 10/30) column equilibrated with 20 mM Tris–acetate pH 7.6, 150 mM NaCl, 5 mM $MgCl_2$, 15 mM ME.

3. Results

PKA transfers about 2 moles of phosphate per mole of τ3 and phosphorylates Ser156, 235, 267, 320 and 327 (corresponding to Ser214, 324, 356, 409 and 416 of the longest τ4 isoform) [20]. The rate and level of phosphorylation of these sites decreases in the order S156 > S235 > S327 ⩾ S320 > S267 for τ3 isoform [21]. The primary structure at Ser156 (RTPpSLP) is close to the consensus sequence (RXXpSXP) recognized by 14-3-3 [8] and it was therefore postulated that this site might be responsible for τ3-14-3-3 interaction [12,16]. In order to check this suggestion we replaced Ser156, i.e. the main site of PKA phosphorylation, by Ala and analyzed the effect of two other phosphorylation sites (Ser235 and Ser267) on the interaction of phosphorylated τ3 with 14-3-3. The wild type τ3 and its Ala mutants were subjected to phosphorylation up to 1.7–2.0 moles P/mole protein. Fixed amounts of 14-3-3 were mixed with increasing quantities of unphosphorylated (τ3), phosphorylated τ3 (pτ3) or its phosphorylated mutants and subjected to native gel electrophoresis. Unphosphorylated τ3 only weakly interacts with 14-3-3 (Fig. 1) [16], whereas addition of even small quantities of phosphorylated τ3 was accompanied by disappearance of the band corresponding to the isolated 14-3-3 and accumulation of a new band having lower electrophoretic mobility, which presumably corresponds to the complex 14-3-3-pτ3 (Fig. 1A). Indeed, when this band was cut out, subjected to the SDS-gel electrophoresis and silver-stained we detected two protein bands corresponding to 14-3-3 and pτ3 (Fig. 1B). Phosphorylated S156A mutant was less effective in 14-3-3 binding than the wild type τ3 and even at high concentrations of S156A significant portion of 14-3-3 remained free (Fig. 1). Thus, although phosphorylated Ser156 contributes to the tight binding of τ3 to 14-3-3, it is not unique for this interaction.

The double mutation S156A/S267A, further decreased interaction of phosphorylated τ3 with 14-3-3 (Fig. 1). The phosphorylated double mutant S156A/S235A was very ineffective in 14-3-3 binding and its interaction with 14-3-3 was comparable with that of unphosphorylated τ3 (Fig. 1C). This indicates that phosphorylation of Ser267 located in the fourth repeat and especially Ser235 located in the third repeat significantly contributes to the binding of τ3 to 14-3-3.

Chemical crosslinking was utilized to confirm these results. Two bands were detected after crosslinking of isolated phosphorylated τ3 by glutaraldehyde, namely a strong radioactive band with M_r 46 kDa, corresponding to τ3 monomer and a weak band with M_r 94 kDa corresponding to τ3 dimer (Fig. 2A and B). Three main bands were detected after crosslinking the wild type τ3 with 14-3-3. The sharp band with M_r 28 kDa corresponds to the 14-3-3 monomer. The band with M_r 46 kDa corresponds to τ3 monomer and the diffuse band with $\sim M_r$ 120–150 kDa corresponds to the 14-3-3-τ3 complex. The bands corresponding to the 14-3-3-τ3 complex had a higher M_r and were much more intense than the band corresponding to τ3 dimer (Fig. 2C and D). The data obtained with the S156A mutant were qualitatively similar to that obtained with the wild type τ3. After crosslinking of phosphorylated S156A/S235A or S156A/S267A mutants with 14-3-3 we detected four bands. In addition to the three above mentioned bands we found a band with M_r 58 kDa corresponding

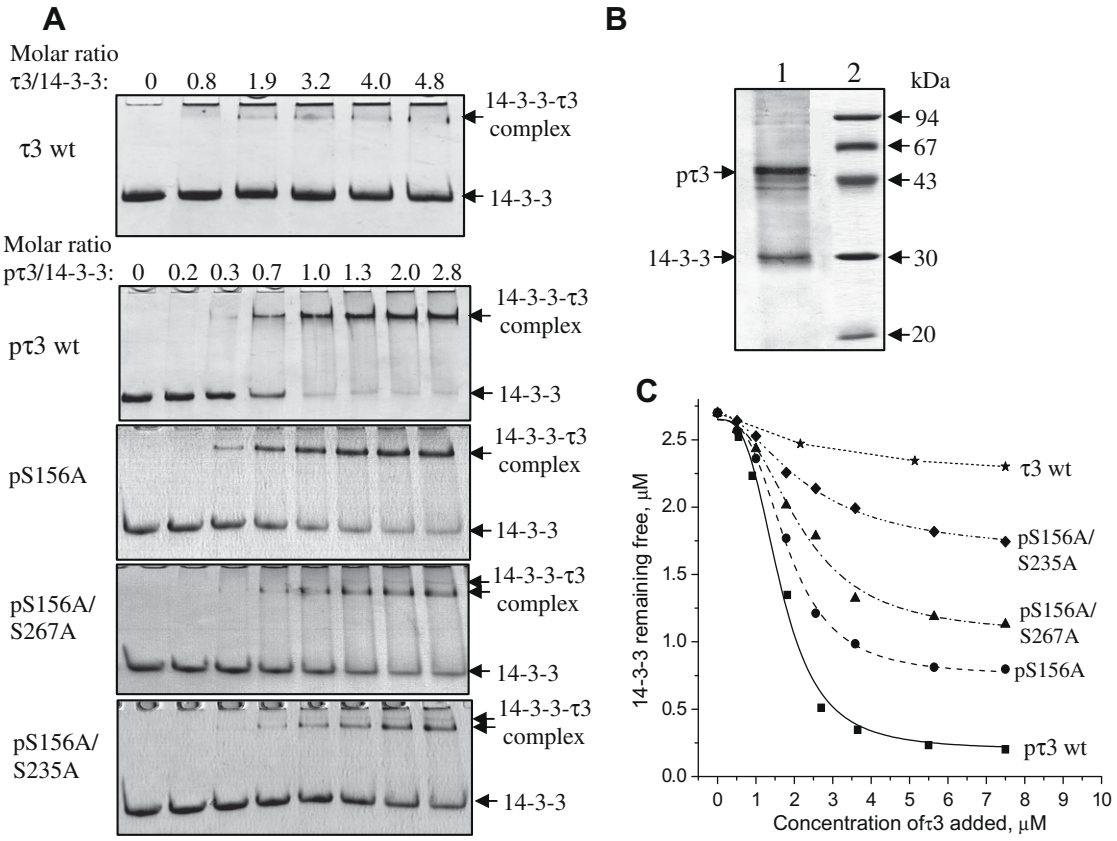

Fig. 1. Interaction of unphosphorylated or phosphorylated wild type the shortest isoform of human tau protein (τ3) and its phosphorylated mutants with 14-3-3 analyzed by native gel electrophoresis. 14-3-3 at fixed concentration 2.7 μM was mixed with increasing quantities of unphosphorylated tau (τ3) or phosphorylated τ3 (pτ3) and phosphorylated mutants (pS156A, pS156A/S235A and pS156A/S267A). Molar ratio τ3/14-3-3 in the sample loaded on the gel is indicated above each lane. (A) Native gel electrophoresis of the mixture of 14-3-3 and different species of τ3. Isolated τ3 and its mutants do not enter the gel. Positions of isolated 14-3-3 and 14-3-3-τ3 complexes are marked by arrows. (B) SDS-gel electrophoresis of the band corresponding to the 14-3-3-τ3 complex (lane 1) and molecular mass markers (lane 2). (C) Dependence of 14-3-3 remaining free on the total concentration of unphosphorylated τ3 (τ3), phosphorylated τ3 (pτ3) or its phosphorylated mutants. The data presented are representative of three independent experiments.

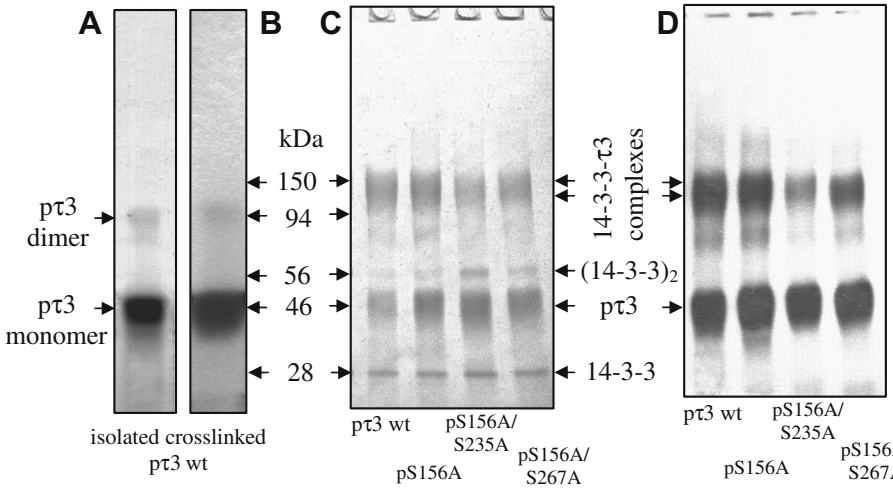

Fig. 2. Chemical crosslinking of phosphorylated τ3 and its mutants with 14-3-3. Isolated phosphorylated wild type τ3 (A and B), or the mixture of phosphorylated wild type protein and its mutants (final concentration 4.9 μM) with 14-3-3 (final concentration 2.7 μM) (C and D) were treated with glutaraldehyde (final concentration 0.045%). The samples were subjected to the SDS-gel electrophoresis (A and C) and autoradiography (B and D). The nature of tau species undergoing crosslinking with 14-3-3 is indicated under each lane, position of isolated 14-3-3, isolated pτ3, dimers of 14-3-3 $(14\text{-}3\text{-}3)_2$ and crosslinked complexes of 14-3-3-τ3 and their M_r are marked by arrows.

to dimer of 14-3-3. This band was more intense in the case of the S156A/S235A mutant indicating that the larger portion of 14-3-3 remained free. Moreover, in the case of the S156A/S235A mutant the intensity and radioactivity of the band corresponding to the 14-3-3-τ3 complex was weaker than the corresponding bands in the case of the wild type protein or two other mutants (Fig. 2). Thus, phosphorylation of at least two sites (S156 and S235 or S156 and S267) is important for tight interaction of 14-3-3 and τ3.

The method of size-exclusion chromatography was also used for analyzing the interaction of 14-3-3 with phosphorylated τ3 and its mutants. Isolated τ3 (and its mutants) and isolated 14-3-3 are eluted as separate peaks with elution volumes 12.2 and 14.1 mL, respectively (Fig. 3). Formation of the complex 14-3-3-pτ3 is accompanied by an increase in the amplitude and broadening of the peak eluted at 12.3–12.4 mL, which leads to a significant increase of the optical density at elution volume 13 mL (Fig. 3). Moreover, as we have shown earlier [16], the fractions eluted between 12.5 and 13.5 mL contain both phosphorylated τ3 and 14-3-3. Increase of the amplitude at elution volume 13 mL was observed only in the case of complexes formed by 14-3-3 with phosphorylated wild type τ3 and its S156A mutant, but not in the case of double mutants of τ3 (Fig. 3), thus indicating that the double mutants only weakly interact with 14-3-3.

4. Discussion

Recently published data indicate that phosphorylation by PKA enhances interaction of tau with 14-3-3 [12,13,16]. Sadik et al. [12] assumed that this tight interaction is mediated by phosphorylation of Ser214 of τ4 (corresponding to Ser156 of τ3). However mutation S156A only weakly affected interaction of phosphorylated τ3 with 14-3-3 (Figs. 1–3) thus indicating that this site is not unique for 14-3-3 binding. Additional mutation S267A decreased interaction of S156A mutant with 14-3-3, and mutation S235A induced even stronger inhibitory effect on the interaction of phosphorylated τ3 with 14-3-3. The pronounced effect of the S235A mutation is probably due to the fact that the primary structure at Ser235 (KCGpSLG) is similar to the consensus sequence (RXXpSX(P/G)) recognized by 14-3-3 [8]. Thus, phosphorylation of at least two sites located in the Pro-rich domain (Ser156) and in the microtubule-binding regions (Ser235 and/or Ser267) affects interaction of τ3 with 14-3-3. It is worthwhile to mention that the earlier postulated phosphorylation-independent site of 14-3-3 binding is also located within the microtubule-binding region of tau [6].

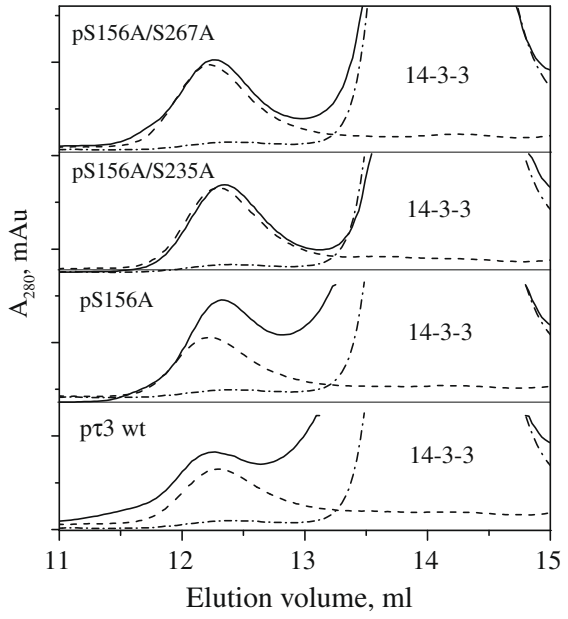

Fig. 3. Size-exclusion chromatography of isolated phosphorylated τ3 and its mutants (12 μM per monomer, dashed lines), of isolated 14-3-3 (24 μM per monomer, dash-dotted lines) or of the mixtures of 14-3-3 and τ3 (or its mutants, solid lines). The amplitude of 14-3-3 peak is much larger than that of τ3 and it is not shown.

We suppose that the mode of 14-3-3 binding depends on the location and quantity of phosphorylated sites in τ3 structure. Weak interaction of 14-3-3 with unphosphorylated (or weakly phosphorylated) tau might promote formation of filaments as reported earlier [12–14]. At the same time the highly specific and tight interaction of 14-3-3 with phosphorylated tau results in formation of stable complexes and might prevent conformational changes leading to filament formation.

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