Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2008 | 106 | 925–933 doi: 10.1111/j.1471-4159.2008.05437.x

Identification of multiple post-translational modifications in the porcine brain specific p25α

Anne J. Kleinnijenhuis,*,[1] Claus Hedegaard,† Ditte Lundvig,‡ Sabrina Sundbye,‡ Olaf Georg Issinger,* Ole Nørregaard Jensen* and Poul Henning Jensen‡

*Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

†Institute of Genetics and Biotechnology, Faculty of Agricultural Sciences, University of Aarhus, Tjele, Denmark

‡Institute of Medical Biochemistry, University of Aarhus, Aarhus, Denmark

Abstract

P25α is a protein normally expressed in oligodendrocytes and subcellular relocalization of p25α occurs in multiple system atrophy, Parkinson's disease and Lewy body dementia along with ectopic expression in neurons. Moreover, it accumulates in Lewy body inclusions with aggregated α-synuclein and is a potent stimulator of α-synuclein aggregation. P25α is a phosphoprotein and post-translational modifications (PTMs) may play a role in its disease-related abnormalities. To investigate the spectrum of PTMs on p25α we cloned porcine p25α and isolated the protein from porcine brain. Using several complementary tandem mass spectrometry techniques for peptide mass analysis and amino acid sequencing, a comprehensive analysis of the PTMs on porcine p25α was performed. It was found that porcine p25α is heavily modified with a variety of modifications: phosphorylation, di- and trimethylation, citrullination and a HexNAc group. The modifications are localized within p25α's unfolded terminal domains and suggest that their functional states are regulated. This comprehensive mapping of p25α's PTMs will form the basis for future functional studies and investigations of p25α's potential role as a biomarker.

Keywords: citrullination, methylation, *O*-glycosylation, oligodendrocyte, p25α, phosphorylation.

J. Neurochem. (2008) **106**, 925–933.

P25α is a brain-specific protein that normally is expressed in the myelin forming oligodendrocyte (Takahashi *et al.* 1993; Skjoerringe *et al.* 2006). It affects the organization of microtubules (Hlavanda *et al.* 2002; Tirian *et al.* 2003; Lehotzky *et al.* 2004; Orosz *et al.* 2004; Otzen *et al.* 2005) and has accordingly been named tubulin polymerization promoting protein (TPPP) but it binds more proteins like α-synuclein, myelin basic protein (MBP) and glyceraldehyde-6-phosphate dehydrogenase (Lindersson *et al.* 2005; Olah *et al.* 2006; Song *et al.* 2007). In the parkinsonian syndrome multiple system atrophy, subcellular relocalization of p25α occurs in oligodendrocytes from the myelin sheets to the expanding cell bodies and nuclei (Baker *et al.* 2006; Song *et al.* 2007). Changes in its cellular expression are characteristic of the group of neurodegenerative synucleinopathies like Parkinson's disease, Lewy body dementia and multiple system atrophy where neuronal expression occurs with accumulation in the Lewy body inclusion that hallmark the degenerating neurons (Kovacs *et al.* 2004, 2007; Lindersson *et al.* 2005; Baker *et al.* 2006; Jellinger 2006). An active role in the degenerative process has been proposed because the Lewy bodies contain aggregated α-synuclein and p25α is a potent inducer of α-synuclein aggregation *in vitro* (Lindersson *et al.* 2005).

P25α is part of the p25 gene family that is highly conserved among vertebrates and also exists in protozoans (Vincze *et al.* 2006). In humans, 3 members of the gene

Received March 10, 2008; revised manuscript received April 10, 2008; accepted April 16, 2008.

Address correspondence and reprint requests to Prof. Poul Henning Jensen, Institute of Medical Biochemistry, University of Aarhus, Ole Worms Alle 1170, DK-8000 Aarhus C, Denmark.
E-mail phj@biokemi.au.dk

[1]The Present address of Anne J. Kleinnijenhuis is TNO Quality of Life, Zeist, The Netherlands.

Abbreviations used: CDK5, cyclin-dependent kinase 5; CID, collision-induced dissociation; ECD, electron capture dissociation; ERK2, extracellular-regulated kinase 2; ETD, electron transfer dissociation; LC, liquid chromatography; MAP, microtubule-associated protein; MBP, myelin basic protein, MS, mass spectrometry; PKA, protein kinase A; PTM, post-translational modification; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TPPP, tubulin polymerization promoting protein.

family exist: p25α, -β and -γ corresponding to proteins of 219, 170, and 176 amino acids with an α-form specific approximately 43 amino acid residue N-terminal domain being the major distinguishing feature. Besides p25α, only the γ form has been demonstrated at the protein level (Vincze *et al.* 2006). The p25 proteins display structural features resembling unfolded proteins like heat stability (Hlavanda *et al.* 2002) but folding has been demonstrated biophysically (Otzen *et al.* 2005) and NMR studies of the mouse and human recombinant p25γ homologues and the *Caenorhabditis elegans* WR33 p25 homologue demonstrate a central folded core, consisting of five α-helices and one β-turn, which is flanked by unfolded N- and C-terminal domains (Monleon *et al.* 2004; Kobayashi *et al.* 2005; Aramini *et al.* 2007).

Bovine p25α is a phosphoprotein with phosphorylations of Thr14, Ser18, and Ser32 in its N-terminal domain (Takahashi *et al.* 1991; Hlavanda *et al.*2007). Moreover, purified p25α is a substrate for a range of kinases, e.g., tau protein kinase II, phosphatidic acid-stimulated brain kinases, LIM-kinase, cyclin-dependent kinase 5 (Cdk5), extracellular-regulated kinase 2 (ERK2) and protein kinase A (PKA) (Takahashi *et al.* 1991; Yokozeki *et al.* 1998; Martin *et al.* 2002; Acevedo *et al.* 2007; Hlavanda *et al.* 2007) and *in vitro* phosphorylation has demonstrated the phosphorylation on Ser160 in the C-terminal domain of recombinant human p25α (Hlavanda *et al.* 2007). Murine p25α has additionally been demonstrated to be O-glycosylated (Vosseller *et al.* 2006).

A protein's post-translational modifications (PTM) represent a finger print of the often reversible signaling pathways impinging on its structure, e.g., kinases and phosphatases, glycosyltransferases and deglycosidases, methyltransferases and demethylases, and may regulate its function as demonstrated by the inhibition of the microtubule assembling activity of p25α by the kinases ERK2, Cdk5 and LIM-kinase (Acevedo *et al.* 2007; Hlavanda *et al.* 2007). Moreover, modifications can represent specific biomarkers for pathogenic processes, e.g., the Ser129 phosphorylation of α-synuclein that highlights cytoplasmic inclusions in Parkinson's disease (Fujiwara *et al.* 2002; Anderson *et al.* 2006).

In the present study, we cloned the porcine p25α to deduce its complete amino acid sequence. Next, we used mass spectrometry to verify the amino acid sequence of isolated porcine brain p25 and characterized the PTMs of the protein with liquid chromatography - tandem mass spectrometry (LC-MS/MS) using collision-induced dissociation (CID) or electron transfer dissociation (ETD) for peptide fragmentation. Porcine p25α was found to be extensively modified by phosphorylations, citrullinations, methylations, and an O-linked HexNAc modification. The modifications both alter the peptides charge and may affect its accessibility for various ligands and thus affect the functional state of p25α.

Material and methods

Cloning of the porcine p25α coding sequence

Using total RNA from porcine cerebellum as template, cDNA was synthesized with the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). Aligning of the human and bovine p25α coding sequences (GenBank acc. no. NM 007030 and NM 173976, respectively) identified homologous sequences both 5′ and 3′ to the coding region. From these sequences, degenerated primers (FWdeg-35 to-19 5′-GGAGGCGCYKCCGCTGC-3′; and RVdeg714 to 693 5′-AGYACAGGAATGTAAYGAAGTG-3′) as well as solely bovine primers (FWbov-35 to-19 5′-GGAGGCGCTGCCGCTGC-3′; and RVbov714 to 693 5′-AGCACAGGAATGTAACGAAGTG-3′) for PCR amplification of the full-length coding sequence were derived (DNA Technology, Aarhus, Denmark). In addition, the bovine p25α coding sequence was compared to porcine genomic data by BLAST recognizing a sequence putatively exon 3 from which the primers (FWpor319 to 342 5′-GGGAAGTCGTGCAGGACCATCACG-3′; and RVpor471 to 448 5′-CGTCACCCCCGAGATGATGGGCGC-3′) were designed (DNA Technology). PCRs were carried out with the enzyme Phusion (Finnzymes, Espoo, Finland) and the standard buffer under the conditions: 98°C for 30 s; 35 cycles of 98°C for 10 s, 55/60°C for 30 s, 72°C for 30 s; 72°C for 10 min; 4°C. Ethidium bromide stained amplicons of interest identified by 1% agarose gel electrophoresis were excised and recovered with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). Since Phusion is a proof-reading enzyme creating blunt ends, 3′-A-overhangs were subsequently generated by DyNAzyme EXT (Finnzymes) in a 10 min reaction at 72°C. Prior to Sanger sequencing, the isolated amplicons were cloned using the TA TOPO Cloning Kit (Invitrogen).

MS analysis of porcine p25α

Porcine brain cytosolic p25α was affinity purified using a column carrying immobilized rabbit anti-human p25α-1 IgG and a buffer containing 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM vanadate as phosphatase inhibitors and PEFA Bloc SC (Pentapharm, Bassel, Switzerland) and 2 mM EDTA as protease inhibitors, whereafter the eluate was resolved by reducing sodium dodecyl sulphate-polyacrylmide gel electrophoresis (SDS-PAGE) as previously described (Song *et al.* 2007). The porcine p25α was in gel digested with trypsin and the peptides were extracted with 5% formic acid solution. Samples were desalted using POROS Oligo R3 (PerSeptive Biosystems, Framingham, MA, USA) microcolumns as described by Gobom *et al.* (1999). Peptides were eluted with 1 μL of 20 mg/mL 2,5-dihydroxybenzoic acid in 70% acetonitrile and 1% phosphoric acid directly onto a MALDI plate (Stensballe and Jensen 2004). MALDI MS/MS spectra were recorded on a MALDI-QTOF (Ultima HT, Waters/Micromass, Manchester, UK) equipped with a nitrogen laser (337 nm) (Stensballe and Jensen 2004).

For LC-MS/MS analysis, peptides were eluted from microcolumns using 70% acetonitrile/0.1% formic acid and then dried in a vacuum centrifuge. Samples were redisolved in approximately 10 μL 0.1% formic acid.and analyzed with LC-MS/MS. Analysis of tryptic digests of p25a was performed using two different LC-MS/MS systems. An Ultimate 3000 LC system (Dionex, Sunnyvale, CA, USA) coupled to a QTOF Micro (Waters/Micromass) tandem

mass spectrometer was used for CID MS/MS experiments. Trap/precolumn and analytical columns were packed with Reprosil C18 (Dr Maisch, Germany) material. An HPLC-ChipCube interfaced to an ion trap – MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with a Protein ID chip #1 (Agilent Technologies), containing a 40 nL enrichment column and a 43 × 75 μm analytical column packed with ZORBAX 300SB C18, was used for ETD and CID experiments. The reaction time for ETD was 100 ms and reagent accumulation time was 30 ms. For both LC-MS/MS systems, LC solvents were 0.5% acetic acid or 0.1% formic acid (solvent A) and peptides were eluted from the analytical column using an acetonitrile gradient (from 0–3 to 80% solvent B (0.1% FA, 90% acetonitrile) in 9 min for the HPLC-ChipCube system and in 30 min for the Ultimate 3000 LC system).

Cloning, purification and *in vitro* phosphorylation of recombinant human p25α proteins

Human p25α(Δ156–219) was constructed by PCR using cDNA coding for human p25α protein and the following primers (DNA Technology): 5′-CACCCATGGCTGACAAGGCC-3′ and 5′-CACGGATCCCTACGTCACCCCTGA-3′. The PCR fragment was inserted into a pET-11d vector (Novagen, VWR Int., Rodovre, Denmark) using *Nco*I and *Bam*HI restriction enzymes (New England Biolabs). Correct insertion was verified by DNA sequencing (Eurofins-MWG, Martinsried, Germany). Expression and purification of recombinant human p25α, p25α(Δ3–43) and p25α(Δ156–219) was performed as previously described for p25α (Otzen *et al.* 2005).

Recombinant human p25α, p25α(Δ3–43), and p25α(Δ156–219)(50μg/mL) were incubated with human PKA (1μg/mL), protein kinase C (PKC) (2μg/mL), p38α (5 μg/mL) (all from KinaseDetect ApS, Odense, Denmark) in 25 mM Tris, pH 7.5, 5 mM NaCl, 3.75 mM $MgCl_2$, 5 mM dithiotreitol, 0.025% Brij35, 125 μM ATP, 1 μCi/μL γP^{32}-ATP for 30 min. To 10 μL of each sample (corresponding to 0.5 μg of p25) 10 μL of sample buffer was added and subsequently analyzed by SDS-PAGE, followed by Coomassie Blue staining and autoradiography.

Results

Porcine brain was chosen as a source to study brain-specific PTMs of p25α in non-pathological brain tissue because it is possible to obtain this tissue fresh and in amounts amenable for protein analyses. First, the sequence of the porcine p25α was established to facilitate the characterization of its PTMs.

Cloning of the porcine p25α coding sequence

All nine combinations of the three sets of primers were used for PCR. A distinct band of 396 bp was raised with FWpor319 to 342 and RVdeg714 to 693. Cloning and sequencing showed it to be the 3′-part of the p25α coding sequence. The primers FWbov-35 to -19 and RVbov714 to 693 raised several faint bands in the area of 700–800 bp. The DNA fragments were eluted from the gel *en bloc* and cloned but a clone holding p25α coding sequence was not identified. The eluate was hence used as template in a semi-nested PCR with the primers FWbov-35 to -19 and RVpor471 to 448, which resulted in a single, strong 506 bp band that proved to be the 5′-part of the p25α encoding sequence. The partial coding sequences obtained were superimposed resulting in a 710 bp contig covering completely the coding region of porcine p25α (Supplementary material Fig. S1; GenBank accession EU522721). Alignment of the porcine and human p25α nucleotide sequences demonstrates 90% identity. Translation of the nucleotides to the amino acid sequence shows that porcine p25α is a 221 amino acid protein as compared to the human of 219 amino acids (Fig. 1). Alignment demonstrates 93% sequence identity and the presence of two single amino acid insertions within the N-terminal part in the porcine sequence corresponding to Ser4 and Gly47 in the porcine sequence. Six of the 14 changed amino acids are conservative changes between basic Arg and Lys residues.

MS analysis of porcine p25α

Brain p25α from porcine cytosol was isolated by using an affinity column with immobilized rabbit p25α-1 IgG and the porcine p25α protein was resolved from its copurifying proteins by SDS-PAGE. The purified protein displayed an electrophoretic migration identical to the recombinant human p25α standard (Fig. 2). The porcine p25α band was excised and subjected to tryptic digestion prior to mass spectrometry analysis.

Approximately, 65% of the porcine p25α protein sequence was identified using LC-MS/MS and MALDI-MS/MS (Fig. 1a). LC-MS/MS was performed using both CID and ETD for fragmentation of peptide ions. Several PTMs were identified in porcine p25α, as summarized in Table 1 and Fig. 1.

Two phosphorylation sites were identified in the N-terminal part of the p25 protein, namely Thr15 and one site in a peptide containing the potential sites Ser33, Ser36, Ser40 or Ser51. Additionally three dehydroalanine residues corresponding to S161, S162, and S201 were revealed by MS/MS assignment. Phosphorylated or *O*-glycosylated residues can undergo facile loss of the phosphate or glycan moieties, respectively, leaving a dehydroalanine residue in the peptide. Interestingly, a peptide with a mass corresponding to doubly phosphorylated AISSPTVSR (containing the S161 and S162 residues) was observed with MALDI-QToF MS after enriching for phosphopeptides using an optimized immobilized metal affinity chromatography protocol (Stensballe and Jensen 2004), but because of the low signal no MS/MS spectra could be obtained to confirm this. Alternatively, the observed dehydroalanine residues could represent natural PTMs. For example lantibiotics, which have been analyzed with tandem MS extensively (Kleinnijenhuis *et al.* 2003), contain many naturally occurring dehydroalanine residues.

An interesting group of modifications that was found in porcine p25α has a charge reducing effect in proteins, namely citrullination, dimethylation, and trimethylation. In total,

```
P     1 MADSRPKPAKPANRTPPKSPGDPAKDKAAKRLSLESEGASEGAAAGGPELSALEEAFRRF
H     1 MAD-KAKPAKAANRTPPKSPGDPSKDRAAKRLSLESEGAGEGAAAS-PELSALEEAFRRF
        ***   **** ************ ** ************ *****  **************

P    61 AVHGDTRATGKEMHGKNWSKLCRDCQVIDGRNVTVTDVDIVFSKIKGKSCRTITFEQFKE
H    59 AVHGDARATGREMHGKNWSKLCKDCQVIDGRNVTVTDVDIVFSKIKGKSCRTITFEQFQE
        ***** **** *********** ************************************ *

P   121 ALEELAKKRFKDKSSEEAVREVHRLIEGKAPIISGVTKAISSPTVSRLTDTTKFTGSHKE
H   119 ALEELAKKRFKDKSSEEAVREVHRLIEGKAPIISGVTKAISSPTVSRLTDTTKFTGSHKE
        ************************************************************

P   181 RFDPSGRGKGRAGRVDLVDESGYVPGYKHAGTYDQKVQGGK
H   179 RFDPSGKGKGKAGRVDLVDESGYVSGYKHAGTYDQKVQGGK
        ****** *** ************* ****************
```

Fig. 1 Porcine and human p25α. The porcine p25α amino acid sequence (P) was deduced from the cloned nucleotide sequence and aligned with the human (H) sequence. Identical amino acid residues are marked by an asterisk below the human sequence. The two insertions in the porcine sequence are indicated by a line in the human sequence. The positions of the 5 α-helices identified in the NMR structures of human, murine p25γ and the *C. elegans* p25 homologue are marked by lines below the human sequence. Tryptic peptides sequenced by LC-MS/MS are indicated above the porcine sequence by dotted arrow lines. The post-translationally modified residues in the porcine sequence, which are all conserved in the human sequence, are marked by colored boxes (phosphorylation blue, a peptide containing a phosphorylation of unknown position is marked gray; citrullination green; methylations red; HexNAc yellow; dehydroalanine orange). The dehydroalanine at Ser161, 162 has likely been phosphorylated as a peptide corresponding to a double phosphorylated AISSPTVSRLTDTTK was identified by MALDI-QTof.

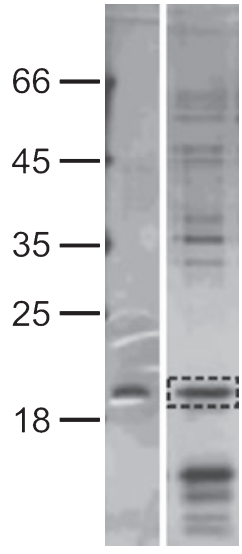

Fig. 2 Purification of porcine p25α for MS analysis. Brain cytosol was subjected to affinity purification using immobilized anti-p25α IgG, the eluate was resolved by reducing SDS-PAGE and visualized by silver staining where after the p25α band was excised for MS analysis. Lane 1, recombinant human p25α (0.5 μg), Lane 2, eluate from the affinity column. The p25α band excised for further analysis is marked by hatched box. Molecular size markers in kDa is shown to the left.

three citrullinated arginine residues were identified and localized, namely Arg83, Arg167, and Arg194 (Figs 1 and 3; Table 1). These residues were always identified in peptides containing a missed trypsin cleavage site, because trypsin does not cleave after a citrullinated Arg residue. The CID tandem MS spectra of the peptide AGRVDLVDES GYVPGYK and the corresponding citrullinated peptide (on Arg3) can be seen in Fig. 3. The signal obtained for the citrullinated peptide was about 10% of the signal obtained for the non-citrullinated peptide. The better ionization efficiency of non-citrullinated peptides compared to citrullinated peptides (because of a charge reduction in citrullinated peptides), however, has an unknown effect on the observed stoichiometry with MS. Furthermore, trypsin should cleave non-citrullinated AGRVDLVDESGYVPGYK after Arg3, but the peptide AGR was not detected. Therefore the observed stoichiometry with MS cannot be used to quantify citrullination *in vivo*. The effect of citrullination on the charge distribution in a peptide is nicely demonstrated by the tandem MS spectra shown in Fig. 3. The CID spectrum of the non-citrullinated peptide, with charge on Arg3, contains mainly b ions, whereas the y ions are much more prominent in the spectrum of the citrullinated peptide. The inset of Fig. 3 clearly shows the 1 Da increase in mass in the b_3 ions, which is another indication for citrullination. Preliminary studies on human brain p25α have demonstrated the citrullination of

Table 1 Identification of multiple post-translational modifications in porcine brain p25α. All modification sites were determined using LC-MS/MS, except the modification marked with an asterisk. They were determined using MALDI-QToF MS/MS

Detected peptide	Modification	Modification site	Position in p25α
TPPKSPGDPAK	Phosphorylation	T1	T15
RLSLESEGASEGAAAGGPELSALEEAFR/ LSLESEGASEGAAAGGPELSALEEAFRR	Unmodified	–	–
RLSLESEGASEGAAAGGPELSALEEAFR/ LSLESEGASEGAAAGGPELSALEEAFRR	Phosphorylation*	Not determined	Not determined
LSLESEGASEGAAAGGPELSALEEAFR	Unmodified	–	–
FAVHGDTR	Unmodified	–	–
LCRDCQVIDGR	Unmodified	–	–
LCRDCQVIDGR	Citrullination	R3	R83
NVTVTDVDIVFSK	Unmodified	–	–
TITFEQFK	Unmodified	–	–
TITFEQFKEALEELAK	Unmodified	–	–
EALEELAK	Unmodified	–	–
EALEELAKK	Unmodified	–	–
LIEGKAPIISGVTK	Unmodified	–	–
LIEGKAPIISGVTK	Dimethylation	K5	K149
LIEGKAPIISGVTK	Trimethylation	K5	K149
APIISGVTK	Unmodified	–	–
APIISGVTK	HexNAc	T8	T157
APIISGVTKAISSPTVSR	Unmodified	–	–
APIISGVTKAISSPTVSR	Dimethylation	K9	K158
APIISGVTKAISSPTVSR	Trimethylation	K9	K158
AISSPTVSR	Unmodified	–	–
AISSPTVSR	Dehydroalanine	S3, S4	S161, S162
AISSPTVSRLTDTTK	Unmodified	–	–
AISSPTVSRLTDTTK	Citrullination	R9	R167
AGRVDLVDESGYVPGYK	Unmodifieda	–	–
AGRVDLVDESGYVPGYK	Citrullination	R3	R194
VDLVDESGYVPGYK	Unmodified	–	–
VDLVDESGYVPGYK	Dehydroalanine	S7	S201
VDLVDESGYVPGYKHAGTYDQK	Unmodified	–	–
VDLVDESGYVPGYKHAGTYDQK	Trimethylation	K14	K208

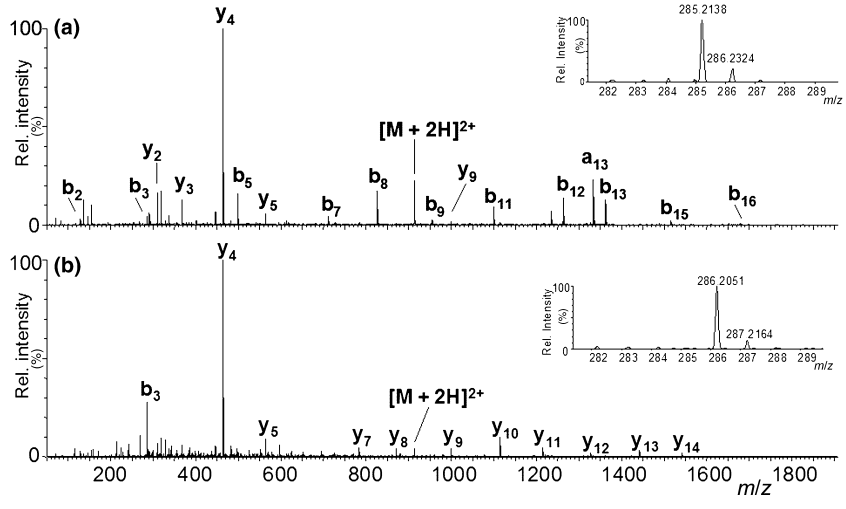

Fig. 3 Identification of citrullination of Argenine-194. CID spectra of (a) non-citrullinated and (b) citrullinated AGRVDLV DESGYVPGYK (R3). The insets show the b_3 ions of the non-citrullinated (top) and citrullinated (bottom) peptide.

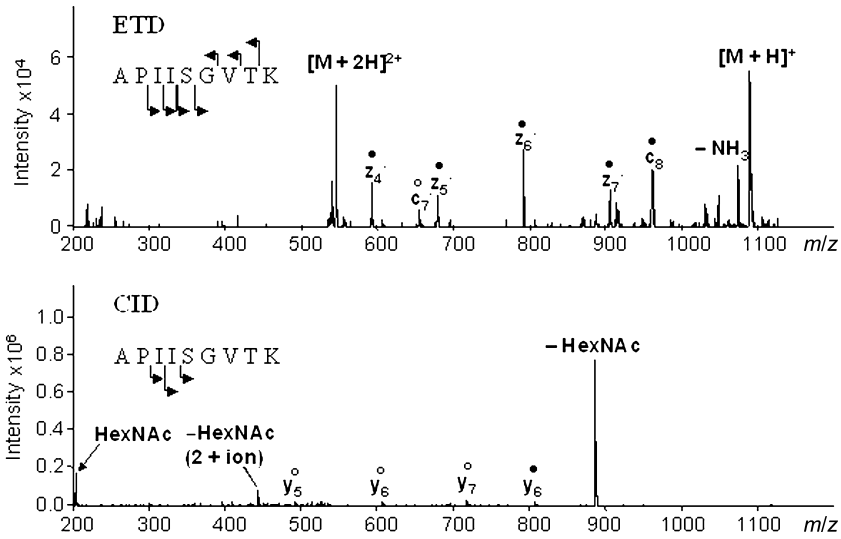

Fig. 4 Identification of an O-linked glycosylation at Threonine-157. ETD (top) and CID (bottom) spectra of peptide APIISGVTK containing a HexNac modification on Thr8. Fragment ions marked with a dot retained the modification; fragment ions marked with a circle did not contain the modification. The fragmentation patterns are shown in the spectra. Small arrows represent fragment ions that were only observed non-modified. Big arrows represent fragment ions that retained the modification.

Arg9 of AISSPTVSRLTDTTK corresponding to Arg167 that we demonstrate to be present in the porcine sequence.

Additionally, several methylated lysine residues were identified in porcine p25α. The methylation sites included two dimethylation sites (Lys149 and Lys158) and three trimethylation sites (Lys149, Lys158 and Lys208) (Fig. 1; Table 1). Similar to citrullination, methylation has a charge reducing effect on peptides and results in missed tryptic cleavage sites.

Finally, a HexNAc group was detected in porcine p25α (Fig. 1; Table 1). The detection of GlcNAc in mouse p25α has been described before (Vosseller *et al.* 2006). The modified amino acid, Thr157, is different in porcine p25α, compared to the Ser151 residue in mouse p25α. With CID as fragmentation technique the HexNAc site could not be determined, because the modification was lost from the precursor ion. This is illustrated by the intense 203 neutral loss and the HexNAc peak at m/z 204 in Fig. 4. With ETD the HexNAc group could be localized as the fragment ions retained the labile modification. The results that are described in this paper clearly illustrate that several MS approaches are needed to obtain a comprehensive view on the PTM pattern of proteins.

In vitro phosphorylation

The *in vivo* phosphorylation sites of brain p25α is located in the unfolded termini and their significance was further evaluated by comparing the *in vitro* phosphorylation of wild-type p25α to deletion mutants lacking the N- and C-termini p25α(Δ3–43) and p25α(Δ156–219) (Fig. 5). The PhosphoMotif finder in the Human reference protein database was used to identify potential candidate kinases that could phosphorylate C-terminal Ser161 and PKA and PKC exhibited a high score whereas the p38 was less likely. The *in vitro* phosphorylation demonstrates that p25α is a substrate for

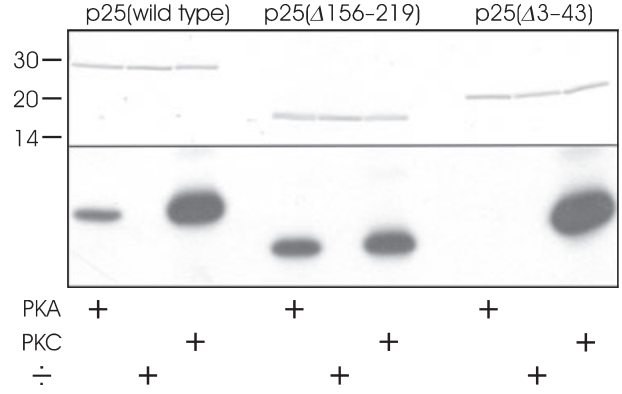

Fig. 5 *In vitro* phosphorylation of human p25α(wt) and p25α(Δ3–43) and p25α(Δ156–219). Purified human recombinant p25α(wt), p25α(Δ3–43) and p25α(Δ156–219) were incubated with PKA, PKC or no kinase as negative control and γP^{32}-ATP whereafter 0.5 μg were subjected to SDS-PAGE, Coomassie Blue staining and autoradiography. Top panel, Coomassie blue staining. Molecular size markers in kDa shown to the left. Lower panel, autoradiography of the gel.

PKC and confirms that it is a substrate for PKA (Fig. 5) whereas p38 is unable to phosphorylate it (data not shown). However, the PKA phosphorylation sites are exclusively located in the N-terminus because deletion of this segment in p25α(Δ3–43) completely inhibits the phosphorylation (Fig. 5) in agreement with (Hlavanda *et al.* 2007). Deletion of the C-terminal segment diminished the phosphorylation by PKC considerably as compared to the PKA phosphorylation although some phosphorylation of p25α(Δ156–219) occurs (Fig. 5). The exclusive PKA phosphorylation of the N-terminal segment, which is identical in the wild-type and the Δ156–219 proteins, allows the phosphorylation by PKC to be normalized to PKA and gives the ratio (PKC/PKA)Δ156–219/ (PKC/PKA)wild-type = 0.419 ± 0,083 (n = 3). This

suggests that approximately half the PKC phosphorylation sites are present within the C-terminal domain. However, the data does not allow for the comparison of the PKC phosphorylation in the N- and C-terminal domains because we were unable to normalize the higher phosporylation by PKC of the p25α(Δ3–43) protein to the total protein. Accordingly, p25α possesses exclusively phosphorylation sites for PKA in its N-terminal extension. By contrast PKC phosphorylation sites are present both in its C-terminal extension and within the rest of the molecule.

Discussion

The expression of oligodendroglial p25α is developmentally regulated with its onset corresponding with the initiation of myelin production (Skjoerringe *et al.* 2006). Its subcellular localization changes in parallel from initially being localized to the microtubule organizing centre followed by a more diffuse cytoplasmic localization during *in vitro* differentiation of cultured rat oligodrocytes (Goldbaum *et al.* 2008) to finally also encompassing the distant myelin structures recently demonstrated in human brain (Song *et al.* 2007). Changes in this localization occur during the course of neurodegenerative diseases where p25α in the parkinsonian disorder multiple system atrophy disappears from the myelin and builds up in the oligodendroglial cell bodies that expand dramatically. During this accumulation, additional cytopathology occurs with the accumulation of misfolded α-synuclein in the oligodendroglial cytoplasm along with p25α and ubiquitin. Similar inclusions containing misfolded α-synuclein develop in degenerating neurons in multiple system atrophy, Parkinson's disease and Lewy body dementia where they also co-localize with p25α that appears to be abnormally expressed in neurons during neurodegenerative disorders (Kovacs *et al.* 2004, 2007; Lindersson *et al.* 2005; Baker *et al.* 2006; Jellinger 2006). Accordingly, p25α displays significant changes in its cellular expression and subcellular localization during normal development and disease processes. However, it is important to note that these changes equally well may represent disease facilitating factors, be disease-associated epiphenomenons or even a protective response. A need for caution in making early strong conclusions is highlighted by the Ser129 phosphorylation, which initially was characterized as an inducer of α-synuclein-mediated cytopathology but where a recent report describes a protective effect of the phosphorylation (Gorbatyuk *et al.* 2008).

The cloning of porcine p25α confirms the high degree of sequence conservation with 93% amino acid sequence identity to the human protein and demonstrates that the sequence variation including the two single amino acid insertions preferentially is localized to the unfolded α-form specific N-terminal domain. Our comprehensive mass spectrometric analyses of tryptic peptides revealed a series of phosphorylations, citrullinations, methylations and a single O-glycosylation, among which citrullinations and methylations are novel PTMs in p25α. The PTM sites are for the most highly evolutionary conserved and between the human α-, β- and γ-forms (Supplementary material Fig. S2). The PTMs show a preference for the highly conserved C-terminus, which contains a hot spot with 6 PTM sites within about 20 residues from the C-terminal α-helix, and this suggests that functions relying on the C-terminal could be subject to a high degree of enzymatic regulation.

The functional role of p25α is still obscure although studies indicate a role in the regulation of microtubular dynamics, myelin homeostasis and in pathophysiological processes. It was originally purified as a phosphoprotein co-purifying with a tau kinase fraction from bovine brain and later demonstrated to be a substrate for a range of kinases, e.g., glycogen synthase kinase 3, PKA, PKC, phosphatidic acid-stimulated kinase, tau kinases, ERK2, Cdk5, LIM-kinase (Takahashi *et al.* 1991; Yokozeki *et al.* 1998; Martin *et al.* 2002; Acevedo *et al.* 2007; Hlavanda *et al.* 2007). A functional relation to microtubule dynamics has also been suggested because p25α was identified as a factor that bundles microtubules in bovine brain extracts (Takahashi *et al.* 1991; Hlavanda *et al.* 2002), thus giving rise to the name TPPP for bovine p25α (Hlavanda *et al.* 2002). The association to microtubules may be regulated by phosphorylation as ERK2 and Cdk5 phosphorylation of recombinant human p25α abrogates its MT assembling activity (Hlavanda *et al.* 2007) and thus demonstrates some analogy to the inhibition of tau binding to MT by phosphorylation (Stoothoff and Johnson 2005).

Conclusively, brain p25α phosphorylation has been identified at residues corresponding to human Thr14, Ser18, and Ser32 (Hlavanda *et al.* 2007). We find additionally evidence for a phosphorylation of porcine Ser161, Ser162, corresponding to human Ser159, 160, based on a peptide with a mass corresponding to a doubly phosphorylated AIS-SPTVSR peptide combined with the demonstration of dehydroalanine residues at these positions in another peptide. This is further corroborated by the *in vitro* phosphorylation of human recombinant p25α at Ser159 and Ser160 (Hlavanda *et al.* 2007).

Lysine methylation has been most extensively studied on histones where they may play a role in the modulation of chromatin structure and where the enzymatic reversion of methylation has been demonstrated by lysine-specific demethylase 1 (Shi *et al.* 2004). However, these lysine methylations have recently been demonstrated on more brain proteins including α-tubulin fragments (Iwabata *et al.* 2005) and found on factors involved in translation (Polevoda and Sherman 2007). Protein citrullination by peptidylarginine deiminases removes the imino moiety from the side chain of a basic Arg residue and thus converts it to a neutral amino

acid (Gyorgy *et al.* 2006). This process has been demonstrated to play a significant role for MBP where citrullination reduces its affinity for the negatively charged phospholipid in the myelin sheet thus making the structure less rigid and also enhances the proteolytic degradation of MBP (Musse and Harauz 2006). The single O-glycosylation on the porcine p25α has previously been demonstrated on murine p25α (Vosseller *et al.* 2006). Interestingly, the mouse O-glycosylation was present on Ser151 whereas Thr157 is modified in porcine p25α and this suggest the structural context C-terminal to the sixth helix is more important for this modification than the specific residue.

Our report has several lines of implication for future experimental work on p25α. First knowledge of the spectrum of PTM on a protein informs about the enzymatic systems working on the peptide. Second, the identified sites of modifications give information about the structure of p25α in brain and where its surface characteristics and functional properties may be dynamically regulated. Third, the identified PTMs and their sites will facilitate comparisons of p25α and its modifications during development and as putative biomarkers in disease tissue, e.g., by means of quantitative techniques like isotope coded affinity tags. Fourth, these insights further allow for the building of novel hypotheses that can be tested in various *in vitro* and *in vivo* models by the removal of specific modifications sites in transgenic p25α proteins.

Acknowledgments

This work was supported by the Lundbeck Foundation (AK, PHJ), The Danish Research Council for Health and Disease 271-05-0166, Aarhus University Research Foundation, ONJ is a Lundbeck Foundation Research Professor.

Supplementary material

The following supplementary material is available for this article:

Fig. S1 Alignment of the nucleotide sequences of porcine (GenBank acc. no. EU522721) and human p25α was found to be 90% identical.

Fig. S2 Amino acid sequence alignment of p25α homologues demonstrating conservation of amino acids carrying post-translational modifications.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1471-4159.2008.05437.x (This link will take you to the article abstract).

Please note: Blackwell Publishing is not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

References

Acevedo K., Li R., Soo P., Suryadinata R., Sarcevic B., Valova V. A., Graham M. E., Robinson P. J. and Bernard O. (2007) The phosphorylation of p25/TPPP by LIM kinase 1 inhibits its ability to assemble microtubules. *Exp. Cell Res.* **313**, 4091–4106.

Anderson J. P., Walker D. E., Goldstein J. M. *et al.* (2006) Phosphorylation of Ser-129 is the dominant pathological modification of alpha-synuclein in familial and sporadic Lewy body disease. *J. Biol. Chem.* **281**, 29739–29752.

Aramini J. M., Rossi P., Shastry R. *et al.* (2007) *Solution NMR structure of tubulin polymerization-promoting protein family member 3 from Homo sapiens*. http://www.pdb.org/pdb/explore/explore.do?structureId=2JRF

Baker K. G., Huang Y., McCann H., Gai W. P., Jensen P. H. and Halliday G. M. (2006) P25alpha immunoreactive but alpha-synuclein immunonegative neuronal inclusions in multiple system atrophy. *Acta Neuropathol.* **111**, 193–195.

Fujiwara H., Hasegawa M., Dohmae N., Kawashima A., Masliah E., Goldberg M. S., Shen J., Takio K. and Iwatsubo T. (2002) Phosphorylated alpha-synuclein is ubiquitinated in alpha-synucleinopathy lesions. *Nat. Cell Biol.* **4**, 160–164.

Gobom J., Nordhoff E., Mirgorodskaya E., Ekman R. and Roepstorff P. (1999) Sample purification and preparation technique based on nano-scale reversed-phase columns for the sensitive analysis of complex peptide mixtures by matrix-assisted laser desorption/ionization mass spectrometry. *J. Mass Spectrom.* **34**, 105–116.

Goldbaum O., Jensen P. H. and Richter-Landsberg C.(2008) The tubulin polymerization promoting protein TPPP/p25α is developmentally regulated in cultured rat brain oligodendrocytes and affected by proteolytic stress. *Glia* (In press).

Gorbatyuk O. S., Li S., Sullivan L. F., Chen W., Kondrikova G., Manfredsson F. P., Mandel R. J. and Muzyczka N. (2008) The phosphorylation state of Ser-129 in human alpha-synuclein determines neurodegeneration in a rat model of Parkinson disease. *Proc. Natl Acad. Sci. USA* **105**, 763–768.

Gyorgy B., Toth E., Tarcsa E., Falus A. and Buzas E. I. (2006) Citrullination: a posttranslational modification in health and disease. *Int. J. Biochem.Cell Biol.* **38**, 1662–1677.

Hlavanda E., Kovacs J., Olah J., Orosz F., Medzihradszky K. F. and Ovadi J. (2002) Brain-specific p25 protein binds to tubulin and microtubules and induces aberrant microtubule assemblies at substoichiometric concentrations. *Biochemistry* **41**, 8657–8664.

Hlavanda E., Klement E., Kokai E., Kovacs J., Vincze O., Tokesi N., Orosz F., Medzihradszky K. F., Dombradi V. and Ovadi J. (2007) Phosphorylation blocks the activity of tubulin polymerization-promoting protein (TPPP): identification of sites targeted by different kinases *J. Biol. Chem.* **282**, 29531–29539.

Iwabata H., Yoshida M. and Komatsu Y. (2005) Proteomic analysis of organ-specific post-translational lysine-acetylation and -methylation in mice by use of anti-acetyllysine and -methyllysine mouse monoclonal antibodies. *Proteomics* **5**, 4653–4664.

Jellinger K. A. (2006) P25alpha immunoreactivity in multiple system atrophy and Parkinson disease. *Acta Neuropathol.* **112**, 112.

Kleinnijenhuis A. J., Duursma M. C., Breukink E., Heeren R. M. A. and Heck A. J. R. (2003) Localization of intramolecular monosulfide bridges in lantibiotics determined with electron capture induced dissociation. *Anal. Chem.* **75**, 3219–3225.

Kobayashi N., Koshiba S., Inoue M., Kigawa T. and Yokoyama S.(2005) *Solution Structure of Mouse CGI-38 Protein* http://www.pdb.org/pdb/explore/explore.do?structureId=1WLM

Kovacs G. G., Laszlo L., Kovacs J. *et al.* (2004) Natively unfolded tubulin polymerization promoting protein TPPP/p25 is a common marker of alpha-synucleinopathies. *Neurobiol. Dis.* **17**, 155–162.

Kovacs G. G., Gelpi E., Lehotzky A., Hoftberger R., Erdei A., Budka H. and Ovadi J. (2007) The brain-specific protein TPPP/p25 in

pathological protein deposits of neurodegenerative diseases. *Acta Neuropathol.* **113**, 153–161.

Lehotzky A., Tirian L., Tokesi N., Lenart P., Szabo B., Kovacs J. and Ovadi J. (2004) Dynamic targeting of microtubules by TPPP/p25 affects cell survival *J. Cell Sci.* **117**, 6249–6259.

Lindersson E., Lundvig D., Petersen C. *et al.* (2005) p25alpha Stimulates alpha-synuclein aggregation and is co-localized with aggregated alpha-synuclein in alpha-synucleinopathies. *J. Biol. Chem.* **280**, 5703–5715.

Martin C. P., Vazquez J., Avila J. and Moreno F. J. (2002) P24, a glycogen synthase kinase 3 (GSK 3) inhibitor. *Biochim. Biophys. Acta* **1586**, 113–122.

Monleon D., Chiang Y., Aramini J. M., Swapna G. V., Macapagal D., Gunsalus K. C., Kim S., Szyperski T. and Montelione G. T. (2004) Backbone 1H, 15N and 13C assignments for the 21 kDa Caenorhabditis elegans homologue of 'brain-specific' protein. *J. Biomol. NMR* **28**, 91–92.

Musse A. A. and Harauz G. (2006) A tale of two citrullines–structural and functional aspects of myelin basic protein deimination in health and disease. *Neurochem. Res.* **32**, 137–158.

Olah J., Tokesi N., Vincze O. *et al.* (2006) Interaction of TPPP/p25 protein with glyceraldehyde-3-phosphate dehydrogenase and their co-localization in Lewy bodies. *FEBS Lett.* **580**, 5807–5814.

Orosz F., Kovacs G. G., Lehotzky A., Olah J., Vincze O. and Ovadi J. (2004) TPPP/p25: from unfolded protein to misfolding disease: prediction and experiments. *Biol. Cell* **96**, 701–711.

Otzen D. E., Lundvig D. M., Wimmer R., Nielsen L. H., Pedersen J. R. and Jensen P. H. (2005) p25alpha is flexible but natively folded and binds tubulin with oligomeric stoichiometry. *Protein Sci.* **14**, 1396–1409.

Polevoda B. and Sherman F. (2007) Methylation of proteins involved in translation. *Mol. Microbiol.* **65**, 590–606.

Shi Y., Lan F., Matson C., Mulligan P., Whetstine J. R., Cole P. A., Casero R. A. and Shi Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* **119**, 941–953.

Skjoerringe T., Lundvig D. M., Jensen P. H. and Moos T. (2006) P25alpha/Tubulin polymerization promoting protein expression by myelinating oligodendrocytes of the developing rat brain. *J.Neurochem.* **99**, 333–342.

Song Y. J., Lundvig D. M., Huang Y., Gai W. P., Blumbergs P. C., Hojrup P., Otzen D., Halliday G. M. and Jensen P. H. (2007) p25alpha relocalizes in oligodendroglia from myelin to cytoplasmic inclusions in multiple system atrophy. *Am. J. Pathol.* **171**, 1291–1303.

Stensballe A. and Jensen O. N. (2004) Phosphoric acid enhances the performance of Fe(III) affinity chromatography and matrix-assisted laser desorption/ionization tandem mass spectrometry for recovery, detection and sequencing of phosphopeptides. *Rapid Commun. Mass Spectrom.* **18**, 1721–1730.

Stoothoff W. H. and Johnson G. V. (2005) Tau phosphorylation: physiological and pathological consequences. *Biochim. Biophys. Acta* **1739**, 280–297.

Takahashi M., Tomizawa K., Ishiguro K., Sato K., Omori A., Sato S., Shiratsuchi A., Uchida T. and Imahori K. (1991) A novel brain-specific 25 kDa protein (p25) is phosphorylated by a Ser/Thr-Pro kinase (TPK II) from tau protein kinase fractions. *FEBS Lett.* **289**, 37–43.

Takahashi M., Tomizawa K., Fujita S. C., Sato K., Uchida T. and Imahori K. (1993) A brain-specific protein p25 is localized and associated with oligodendrocytes, neuropil, and fiber-like structures of the CA3 hippocampal region in the rat brain. *J. Neurochem.* **60**, 228–235.

Tirian L., Hlavanda E., Olah J., Horvath I., Orosz F., Szabo B., Kovacs J., Szabad J. and Ovadi J. (2003) TPPP/p25 promotes tubulin assemblies and blocks mitotic spindle formation. *Proc. Natl Acad. Sci. USA* **100**, 13976–13981.

Vincze O., Tokesi N., Olah J. *et al.* (2006) Tubulin polymerization promoting proteins (TPPPs): members of a new family with distinct structures and functions. *Biochemistry* **45**, 13818–13826.

Vosseller K., Trinidad J. C., Chalkley R. J. *et al.* (2006) O-linked N-acetylglucosamine proteomics of postsynaptic density preparations using lectin weak affinity chromatography and mass spectrometry *Mol. Cell Proteomics* **5**, 923–934.

Yokozeki T., Homma K., Kuroda S., Kikkawa U., Ohno S., Takahashi M., Imahori K. and Kanaho Y. (1998) Phosphatidic acid-dependent phosphorylation of a 29-kDa protein by protein kinase Calpha in bovine brain cytosol. *J.Neurochem.* **71**, 410–417.