

The ‘PINIT’ motif, of a newly identified conserved domain of the PIAS protein family, is essential for nuclear retention of PIAS3L

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Abstract PIAS proteins, cytokine-dependent STAT-associated repressors, exhibit intrinsic E3-type SUMO ligase activities and form a family of transcriptional modulators. Three conserved domains have been identified so far in this protein family, the SAP box, the MIZ-Zn finger/RING module and the acidic C-terminal domain, which are essential for protein interactions, DNA binding or SUMO ligase activity. We have identified a novel conserved domain of 180 residues in PIAS proteins and shown that its ‘PINIT’ motif as well as other conserved motifs (in the SAP box and in the RING domain) are independently involved in nuclear retention of PIAS3L, the long form of PIAS3, that we have characterized in mouse embryonic stem cells.

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1. Introduction

The PIAS (protein inhibitor of activated signal transducer and activator of transcription (STAT)) protein family contains five members (PIAS1, PIAS3, PIASx_a, PIASx_b, PIASy) essentially identified through their ability to bind to specific proteins (transcription factors, K⁺ channel, RNA helicase II) [1–3]. PIAS3 was the first member discovered as an interleukin-6 (IL-6)-dependent repressor of STAT3 [4]. STATs can be selectively repressed by association with specific PIAS molecules (e.g. PIAS3/STAT3; PIAS1 or PIASy/STAT1; PIASx/STAT4) [5,6]. PIAS proteins also coactivate steroid receptor-dependent transcription [7–9] and modulate the transcriptional properties of p53 and interferon regulatory factor-1 (IRF-1) [10–12]. Pro-apoptotic activities of PIAS1 and of a long form of PIAS3 (PIAS3β/KCHAP) have also been reported, but the link with their repressive function on gene transcription has not been established [12–14].

PIAS proteins function as SUMO (small ubiquitin-related modifier)-E3 ligases upon various transcription factors like STATs, c-jun, IRF-1, p53 and its Mdm2 regulator, and themselves [11,15–17]. Sumoylation of transcription factors can either increase or decrease their activities [18]. However, the connection between sumoylation and PIAS-dependent repressive function remains elusive. For example, the mutant STAT1 K703R protein is an active transcription factor that is repressed by PIAS1 as efficiently as the wild type STAT1 protein, despite its failure to be sumoylated [19].

PIAS proteins contain several conserved domains: the N-terminal SAP (Saf-A/B, Acinus and Pias) box with the LXXLL signature, which is required for the trans-repression of STAT1 activity by PIASy [5,20]; the MIZ-Zn finger/RING domain [21], which is essential for SUMO ligase activity; a C-terminal acidic domain, which is involved in binding of PIAS3 to the nuclear coactivator TIF2 [8]. The direct interaction of PIAS proteins with their sumoylated partners results in the nuclear translocation of the complex. Sumoylation occurs during the transport from cytosol to nucleus and is sometimes enhanced in the nucleus by other PIAS molecules [17,22,23].

PIAS translocation from cytosol to cell nucleus might be dependent on specific domains not yet identified. The aim of the present study was to delineate new conserved domains in this protein family and to establish their role in protein trafficking.

2. Materials and methods

2.1. Cell culture, transfection and lysates

The mouse embryonic stem (ES) S1 cell line [24] was grown in STEM medium (high glucose Dulbecco's modified Eagle's medium-Glutamax with sodium pyruvate; 10% fetal calf serum; 0.1 mM β-mercaptoethanol), supplemented with 500 pM leukemia inhibitory factor (LIF); cells were passaged or medium was changed every other day. COS-1 cells were grown in STEM medium. Transfections were performed on 10 cm cell Petri dishes with 1–5 μg of DNA, using Exgen 500 (Euromedex). ES cell-derived neurons were obtained as described [25].

Whole cell lysates, a mixture of cytosolic and nuclear lysates at a 2:1 ratio, [26], were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electro-transferred onto nitrocellulose membranes in the presence of 0.07% sodium dodecyl sulfate.

2.2. Characterization and cloning of full length mouse piAS3L cDNA

Total RNA (1 μg), prepared from ES cells (Trizol reagent kit; Life Technologies) [27], was reverse-transcribed (RT) with random hexameric primers and MLV reverse transcriptase (Sigma). A quarter

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Abbreviations: STAT, signal transducer and activator of transcription; PIAS, protein inhibitor of activated STATs; ES, embryonic stem; LIF, leukemia inhibitory factor; SUMO, small ubiquitin-related modifier; SAP, Saf-A/B, Acinus and Pias

Fig. 1. Alignment of PIAS family members. PIAS proteins, with their indicated GenBank accession numbers (Mmu = *Mus musculus*; Rat = *Rattus norvegicus*; Hu = Human), have been aligned with the 'multAlin' software. Highly conserved residues are in red, low consensus residues in blue and neutral residues in black. In the consensus line, # indicates E or D, % indicates F or Y, \$ indicates L or M and ! indicates I or V.

of the RT was used for each polymerase chain reaction (PCR) reaction with the following oligonucleotides corresponding to sequences of the mouse *pias3* cDNA (set 1) [4] (accession number: AF034080) and of the complete sequence of rat cDNA (set 2) [14] (accession number: AF032872).

Set 1: 5' primer: 5'-GCCCTGCACCTCCTCAAGTG-3', 3' primer: 5'-GGGCTCAGCTCCATTCTTGG-3'.

Set 2: 5' primer: 5'-GGAATTCATATGGCGAGCTGGGC-GAATTAAAGC-3', 3' primer: 5'-CCCAAGCTTTCAGTCCAAG-GAAATGACGTC-3'.

2.3. PCR amplification on different tissues

PCR reactions were performed on first strand cDNAs prepared from 20 mouse tissues (Mouse Rapid-Scan[®] Gene Expression Panels, OriGene Technologies) as recommended by the manufacturer. Thirty-five cycles of amplification were performed with actin-specific primers (control primers included in the kit) or with primers recognizing both forms of *pias3* (accession number: BC023128):

5' primer: 5'-TTGCCCCCTGGCACCTCTCC-3', 3' primer: 5'-GGGCTCAGCTCCATTCTTGG-3'.

Expected sizes of PCR products were: *pias3*, 362 bp; *pias3L*, 467 bp; β -actin, 570 bp. The identity of each band was confirmed by sequencing.

2.4. Construction of expression vectors

The following Flag-tagged expression vectors, encoding the wild type PIAS3 (PIAS3-WT) or PIAS3L (PIAS3L-WT) and mutated forms of PIAS3L proteins, were constructed as follows:

P513-Flag-*pias3L*-WT: PCR-amplified fragments, corresponding to the full length rat *pias3L* (primer set 2), were cloned, in frame with the Flag tag, into the *NdeI/HindIII* sites of the p513-Flag expression vector, a SV40 promoter-based vector [27].

P513-Flag-*pias3*-WT: This vector was constructed by looping out the 105 bp sequence specific to *pias3L* by site-directed mutagenesis with the following primers:

5' primer: 5'-CCTTGCTTTCTTGGCCCCCTGGCACCTCTCCT-CCTGTGCACCCCGATGTCACCATGAAGCC-3', 3' primer: 5'-GGCTTCATGGTGACATCGGGGTGCACAGGAGGAGAGGTG-CCAGGGGGCAAAGAAAGC-3'.

P513-Flag-*pias3L*-mutants: Point or deletion (Δ) mutations (bold-faced lettering) were introduced into the full length p513-Flag-*pias3L* by site-directed mutagenesis with the following primers. The coordinate of the first residue of the mutated sequence is indicated, based on the rat sequence [14].

L₁₉QVLL/AQVAA: 5' primer: 5'-CCGAGTGTCTGAGGCC-CAGGTGGCCGCCGCTTCGCTGGC-3', 3' primer: 5'-GCCAG-CGAAGCCGGCGGCCACCTGGGCTCAGACACTCGG-3'.

Δ P₁₁₇PLP: 5' primer: 5'-CCTAAGCGTGAGGTGGCAATGCA-TCAGCCTGTGCACCCCGATGTCACC-3', 3' primer: 5'-GGTG-

ACATCGGGGTGCACAGGCTGATGCATGTCCACCTCACGCT-TAGG-3'.

PI₂₄₂NIT/PSDST: 5' primer: 5'-CCAAGAGGCCAGCCGTCC-GAGCGACAGCACACCCTTGGCTCG-3', 3' primer: 5'-CGAGC-CAAGGGTGTGCTGCTCGCTCGGACGGCTGGGCCTCTTGG-3'.

C₃₄₃AHLQS/GADLQG: 5' primer: 5'-CCGTGTCGTGCCCTCA-CCGGTGGCGATCTGCAGGGTTTCGATGCTGCC-3', 3' primer: 5'-GGCAGCATCGAAACCCTGCAGATCGGCACCGGTGAGG-GCACGACACGG-3'.

Δ C₃₆₆PVC: 5' primer: 5'-CAGATGAATGAGAAGAAGCCGA-CATGGACGGACAAGAAGGCTCCCTATGAGTCG-3', 3' primer: 5'-CGACTCATAGGGAGCCTTCTTGTCCTCCATGTCGGCTT-CTTCTCATTCATCTG-3'.

Each construct was verified by sequencing.

2.5. PIAS3L-specific antibody and immunodetection

A PIAS3L-specific rabbit polyclonal antibody (Ab 1395) was raised by repeated injection of an ovalbumin-coupled peptide (TPGTLLGPKREVDMMHPLPQ), present only in the large variant of PIAS3 (PIAS3L).

Antibody synthesis: 300 μ g of peptide (in a 1:1 phosphate-buffered saline (PBS)/complete Freund adjuvant emulsion) was injected in anesthetized New Zealand female rabbit as 70 intradermal induration points, with a glass syringe. One month later, 40 ml of blood was drawn every week four times and the serum was tested by Western blot on COS-1 cells transfected with p513-Flag-*pias3L*. The rabbit was then boosted with 150 μ g of peptide (in a 1:1 PBS/incomplete Freund adjuvant emulsion) and killed 12 days later, under anesthesia. Before injection of the specific peptide, 5 ml of blood, drawn from the ear artery, was used to derive the pre-immune serum from the same rabbit.

Immunoprecipitation: Total cell lysates (200 μ g protein in 1 ml of Tris-buffered saline (TBS)) were precleared with 40 μ l of a 50% (v/v) protein A Sepharose slurry for 1 h at 4°C. The supernatant was incubated with 10 μ l of Ab 1395 or pre-immune sera for 1 h and reacted with 40 μ l of protein A Sepharose for another 1 h. The recovered beads were washed three times with ice-cold TBS, 0.1% NP40. Flag-tagged proteins were immunoprecipitated following the same procedure with 30 μ l of anti-Flag M2 affinity gel (Sigma).

2.6. Immunocytochemistry

COS-1 cells were transfected with 2 μ g of p513-Flag-*pias3* or *pias3L* constructs. Cells were fixed with 4% formaldehyde in PBS for 10 min at room temperature and permeabilized with 0.1% Triton X-100 for 20 min. Fixed cells were rinsed with PBS and incubated with blocking buffer (PBS, 3% bovine serum albumin (BSA), 0.1% Triton X-100) for 1 h, followed by incubation with the primary antibodies at room temperature for 1 h in PBS with 5% BSA and 0.1% Triton X-100. After washing with PBS, cells were incubated for 1 h with species-

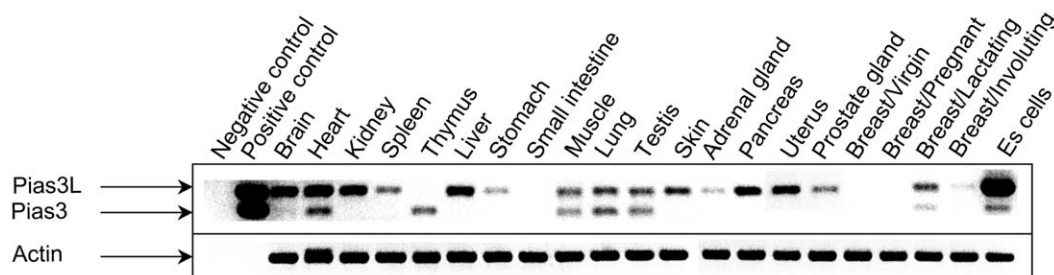


Fig. 2. Differential expression profiles of *pias3* and *pias3L*. PCR experiments, with primers recognizing both *pias3* and *pias3L* cDNAs and with control *actin* primers, were performed on a commercially available subset of tissues with the 'Rapid Scan' kit. One quarter of each PCR reaction was run on a 2% agarose gel and the identity of each band verified by DNA sequencing. Arrows points to the specific PCR products. 'Negative control' was performed in the absence of DNA. 'Positive control' corresponds to a PCR reaction performed with a mixture of 1 μ g of p513-Flag-*pias3* and *pias3L* plasmid DNAs.

specific fluorescence-labeled secondary antibodies. Samples were washed twice with PBS, counterstained with Hoechst 33258 for 10 s and mounted in 75% glycerol, 20% PBS and 5% propylgallate. Antibodies were diluted as followed: rabbit Ab 1395 (1/200), mouse monoclonal anti-Flag M2 (1/500), anti-mouse or anti-rabbit IgG-Cy3 (1/300, Jackson Immunoresearch).

2.7. Computer analysis

Protein alignments were performed with the multAlin (Infobiogen site: <http://www.infobiogen.fr/services/deambulum> [28]) and the PipeAlign (IGBMC site: <http://www-igbmc.u-strasbg.fr> [29]) software.

The different protein sequences were retrieved from GenBank and pasted into the 'multAlin' windows (for Fig. 1). Sequence alignments were achieved using PipeAlign and refined manually to maximize sequence conservations and the superposition of the predicted secondary structures, using the PHD software [30] for each individual sequence (for Fig. 4).

3. Results and discussion

3.1. Cloning of a novel variant of *pias3* cDNA (*pias3L*) from mouse ES cells

To characterize additional STAT3 repressors from mouse ES cells, we performed RT-PCR analyses with total RNA and primers corresponding to the *pias3* gene (set 1, Section 2). A major amplification product was obtained, which revealed an insertion of 105 bp, in comparison with the already reported *pias3*, corresponding to a 35 amino acid in-frame extension of the PIAS3 protein. Data base search performed with the cDNA fragment revealed that it was identical to the rat *Kchap* cDNA which encodes a K⁺ channel-associated protein that had previously been cloned by double-hybrid screen from a rat brain cDNA library [31]. A mouse cDNA corresponding to an extended form of *pias3* (named *pias3L*) has recently been reported [12]. The nucleotide and protein sequences of this cDNA are identical to our sequence between amino acids 10 and 628, but it lacks the first nine residues present in our mouse ES cDNA clone, as well as in the *Kchap* rat homologue and in a recently released mouse cDNA sequence (accession number BC051252). Comparison of protein sequences of various PIAS proteins allowed us to identify the initiation codon, 10 residues upstream of the previously defined start codon of PIAS3, and a conserved insertion element, absent from the originally cloned *pias3* cDNA (Fig. 1). This element, which corresponds to a retained intron ([14] and our unpublished results) and encodes a 35 (in PIAS3L) or 49 (in PIASXa/b) residue sequence, is poorly conserved among the different PIAS proteins but is surrounded by two highly conserved blocks, GXSP and PVHPDV (Fig. 1).

3.2. Differential expression pattern of *pias3* and *pias3L* in mouse tissues

The fact that only *pias3L*, but not *pias3*, was originally detected in mouse ES cells prompted us to characterize the expression profile of *pias3* and *pias3L* in different tissues with primers recognizing both forms (see Section 2). Except for muscle, lung and testis, where both forms were about equally present, differential expression was observed in all other tissues assayed (Fig. 2). We also confirmed that the major form present in ES cells is *pias3L*. We also found that *pias3L* is present in lactating breast but not in virgin, pregnant or involuting breast (Fig. 2). The significance of regulated co-expression or exclusive expression of these different *pias3* forms is presently unknown.

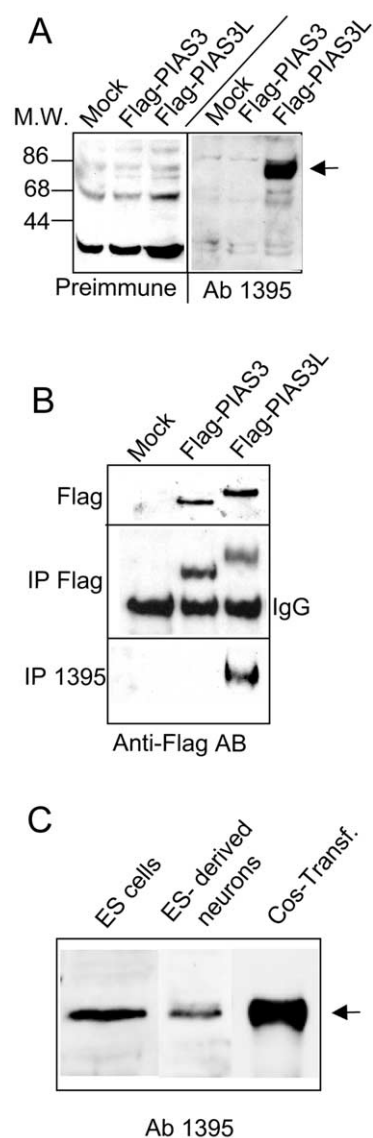


Fig. 3. Characterization of PIAS3L protein with a PIAS3L-specific polyclonal antibody. A: Western blot analysis was performed on 50 µg of total cell lysates from COS-1 cells (mock or transfected with p513-Flag-*pias3* or *pias3L* encoding vectors) with the pre-immune or Ab 1395 polyclonal antibodies, as indicated. The arrow points to the position of the PIAS3L protein. B: The same lysates as above were either probed directly with the anti-Flag antibody (Flag) or after immunoprecipitation (200 µg) with 5 µg/ml of mouse anti-Flag (IP Flag) or with 10 µl of the rabbit polyclonal Ab 1395 (IP 1395) antibodies. C: Total cell lysates derived from mouse ES cells (50 µg; ES), ES-derived neurons (50 µg) and COS-1 cells overexpressing transfected FLAG-PIAS3L (10 µg; Cos-Transf.) were probed with the 1395 polyclonal antibody (Ab 1395).

3.3. Characterization of the PIAS3L protein

To verify that the protein corresponding to the *pias3L* transcript was actually synthesized, Western blot experiments were performed, using protein extracts from cells transfected with p513-Flag vectors encoding the PIAS3 or PIAS3L proteins. While the pre-immune serum recognized non-specific bands in the different cell lysates, a polyclonal antibody (Ab 1395) directed against a peptide sequence unique to PIAS3L (Section 2) specifically reacted with the FLAG-PIAS3L but not with the FLAG-PIAS3 proteins (Fig. 3A). When the same lysates were reacted with the anti-Flag antibody, the presence

Fig. 4. Characterization of the 'PINIT' domain in PIAS proteins. Multiple alignment of the 'PINIT' domain sequences in PIAS proteins was done with PipeAlign and subsequently corrected manually based on secondary structure elements. The designation of each protein includes the species abbreviation followed by the gene name or the accession number (or the SwissProt database name when available), 'frag' indicates that the sequence corresponds to a protein fragment. Expressed sequence tags (ESTs) are designated by their GenBank accession number. The numbers at the beginning and at the end of each sequence indicate the positions of the first and last of the aligned residues in the respective protein sequence; the number in parentheses at the end of each sequence indicates the number of residues remaining after the last shown position. The shading of conserved residues is according to the consensus and includes residues conserved in at least 85% of the aligned sequences. In the consensus line, h indicates hydrophobic residues (A,C,F,I,L,M,V,W,Y,T,S,G; yellow background); s indicates small residues (A,C,S,T,D,V,G,P; green background, white letters); c indicates charged residues (D,E,H,K,R; pink background); p indicates polar residues (D,E,H,K,N,Q,R,S,T; blue letters) and strictly conserved residues are shown by inverse shading (white against a black background). The predicted secondary structure elements were positioned using the PHD program on the multiple alignment and are indicated below the alignment (a indicates α -helix; b indicates β -strand). Abbreviations: Ag, *Anopheles gambiae*; Am, *Apis mellifera*; At, *Arabidopsis thaliana*; Bm, *Bombyx mori*; Ce, *Caenorhabditis elegans*; Ci, *Ciona intestinalis*; Dd, *Dictyostelium discoideum*; Dm, *Drosophila melanogaster*; Dv, *Dermacentor variabilis*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Nc, *Neurospora crassa*; Os, *Oryza sativa*; Rn, *Rattus norvegicus*; Sc, *Saccharomyces cerevisiae*; Sp, *Schizosaccharomyces pombe*; Vc, *Vicia faba*.

of PIAS3 or PIAS3L proteins was revealed in the corresponding extracts, as expected (Fig. 3B, top). Immunoprecipitation of cell lysates with the anti-Flag or Ab 1395 sera also revealed the proteins, further stressing the specificity of the anti-PIAS3L Ab 1395 antibody (Fig. 3B, middle and bottom). The endogenous PIAS3L protein was also detected in mouse ES and in ES cell-derived neuron lysates (Fig. 3C).

PIAS3 has previously been shown to interact with STAT3 proteins in IL-6-stimulated cells [4]. We did not, however, detect any interaction between STAT3 and PIAS3 or PIAS3L in mouse ES cells, whether LIF-induced or not (our unpublished observation). This might be related to the fact that STAT3 does not interact with PIAS3 when this protein is associated with particular proteins in the nucleus, such as the MITF protein [32].

3.4. Identification of a novel protein module, including the conserved 'PINIT' motif, in the PIAS family

The presence of a new domain in the PIAS3L protein, compared to PIAS3, led us to perform a deeper analysis of protein domain conservation between the different PIAS isoforms. Such a study may help to characterize new members of the growing PIAS family and to define essential protein domains involved in PIAS functions. Using the PipeAlign software [29] we defined a well conserved new protein domain of 181 amino acids, which we named the 'PINIT domain'. This domain starts at the PFY motif (position 133 of PIAS3L) and ends at an acidic residue (E or D) at position 315. Among different protein motifs that are conserved within this domain, we have identified the 'PINIT' motif (position 241 of PIAS3L) as a hallmark of this newly defined domain (Fig. 4). This

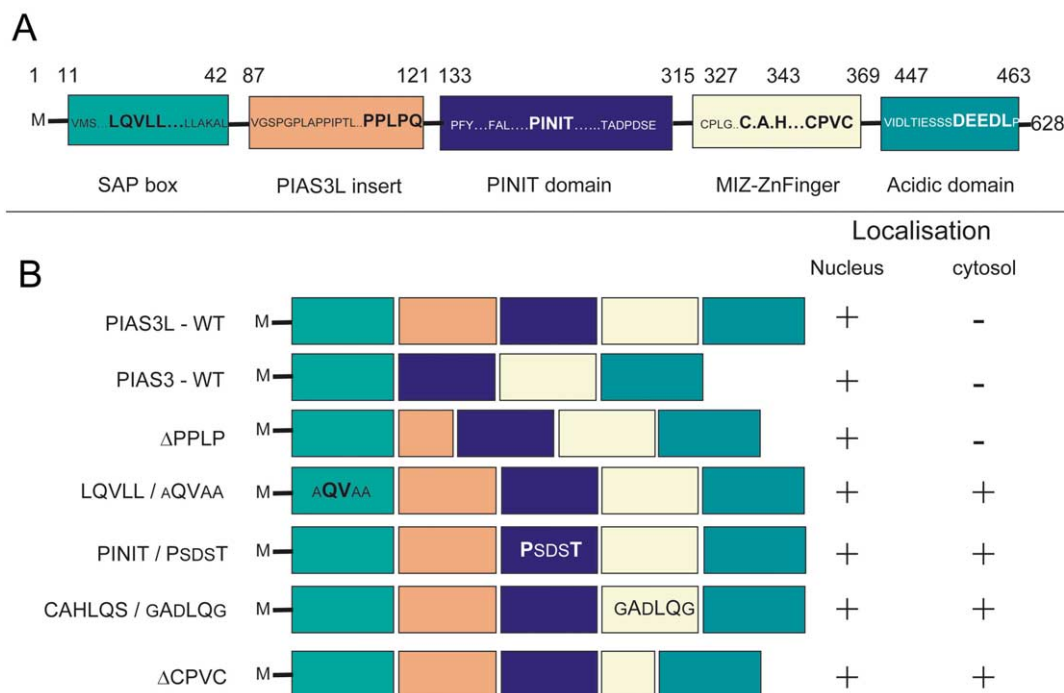


Fig. 5. Independent conserved domains of PIAS3L are involved in its nuclear retention. A: General features of PIAS proteins are represented (not to scale) with coordinates referring to PIAS3L (accession number: BC051252). Within each structured domain (colored boxes), important motifs are boldfaced. B: The structures of the wild type (WT) PIAS3 or WT and mutated PIAS3L proteins are represented with the same color code as in A. The non-conserved sequences are represented by spaces separating the colored boxes. The name of each mutant and the precise nature of the mutations are indicated. Subcellular localizations of these proteins (overexpressed in COS-1 cells) are also indicated. C: Immunostaining of mock or transfected COS-1 cells with the different p513-Flag vectors, with the Cy3 secondary antibodies revealing the primary Ab 1395 (endogenous PIAS3L) or Flag antibodies. Cell nuclei were directly labelled with Hoechst 33258. Visualization of the fluorescence separately or overlaid is presented. Magnification is $\times 400$.

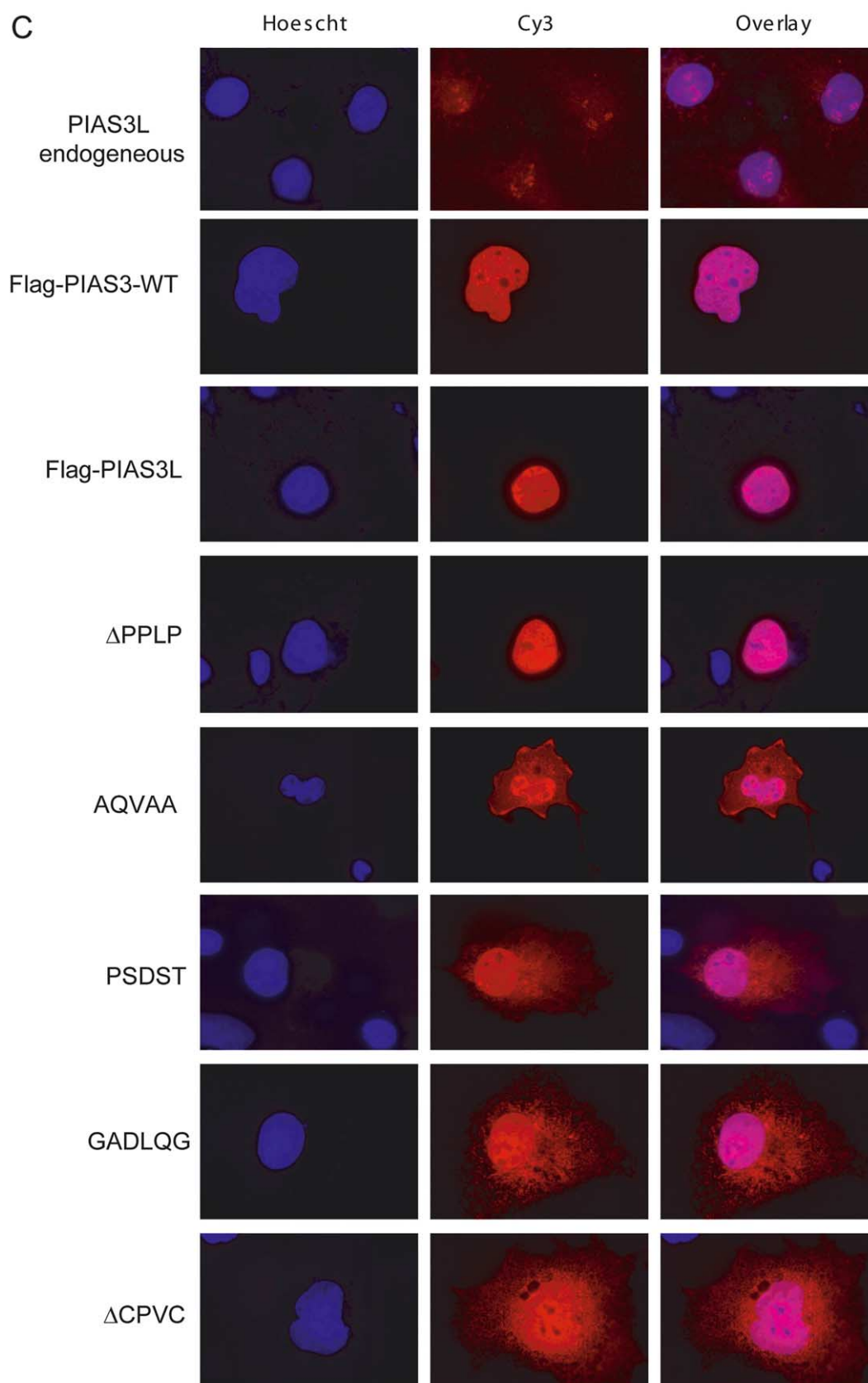


Fig. 5 (Continued).

domain is well conserved among all vertebrate PIAS family members, as well as in PIAS-like proteins characterized in invertebrates up to yeast.

3.5. Conserved domains, including the newly defined 'PINIT' motif, are involved in nuclear localization of the PIAS3L protein

Protein trafficking between nucleus and cytosol is essential for some of the PIAS activities and we have searched for domains involved in differential PIAS3L protein localization. First of all, it was of interest to determine if PIAS3 and PIAS3L have similar cellular localizations. We also investigated the effect of mutations in previously defined PIAS domains (like the SAP box and the RING domain) in the context of the PIAS3L protein. Finally, the importance of the 'PINIT' motif for protein localization was analyzed.

The p513-Flag-pias3L vector and its derivatives were transfected in COS-1 cells. The general features of the PIAS proteins with the positions of each domain given with respect to the PIAS3L sequence and of the PIAS3 variants expressed from the p513-Flag vectors are presented (Fig. 5A,B). A representative selection of immunostained COS-1 cells is shown in Fig. 5C.

The endogenous PIAS3L protein is mainly detected as dots in the cell nucleus, in agreement with previous studies showing a speckled nuclear staining pattern of PIAS1 [23], or PIASy [22]. The overexpressed wild type PIAS3 and PIAS3L, as well as the Δ PLP mutant, were strictly nuclear and mainly located in regions excluding condensed chromatin. By contrast, the localization of PIAS3L mutants, altered either within the N-terminal LQVLL motif, the MZ-Zn finger/RING domain or the newly defined 'PINIT' motif, was disrupted: the overexpressed mutant proteins are no longer restricted to the nucleus. This clearly indicates that separate motifs, present within the SAP box, the MZ-Zn finger/RING or the PINIT domains, are essential for nuclear localization and suggests that PIAS3L may be part of independent subcomplexes formed with different nuclear partners.

In conclusion, besides bringing new insights into the structure of PIAS3 and PIAS3L, this study reveals that both forms may coexist in various tissues, while exclusive expression of one or the other form was observed in some specific organs. In addition, awareness about a new structured domain in PIAS proteins will help to characterize the different PIAS-containing subcomplexes involved in the multiple functions of this protein family.

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