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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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Key words: Embryonic stem cell; E3-type SUMO ligase; Nuclear localization; PIAS; STAT repressor

1. Introduction

The PIAS (protein inhibitor of activated signal transducer and activator of transcription (STAT)) protein family contains five members (PIAS1, PIAS3, PIASxa, PIASxb, 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].

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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

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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                       1        10        20        30        40        50        60        70        80        90        100       110       120       130
                       |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PIAS3_Mmu_AF034080              MVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTLGPSDLSLLS-----LPPGTSP--------------------------------------
PIAS3L_Mmu_BC023128             MVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTLGPSDLSLLS-----LPPGTSPV----------GSPGPLAPIPPTL--LTPGTLLGPKRE
PIAS3L_Mmu_BC051252    MAELGELKHMVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTLGPSDLSLLS-----LPPGTSPV----------GSPGPLAPIPPTL--LTPGTLLGPKRE
PIAS3L_Rat_AF032872    MAELGELKHMVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTLGPSDLSLLS-----LPPGTSPV----------GSPSPLASIPPTL--LTPGTLLGPKRE
PIAS3L_Hu_q9y6x2                MVMSFRVSELQVLLGFAGRNKSGRKHELLAKALHLLKSSCAPSVQMKIKELYRRRFPRKTLGPSDLSLLS-----LPPGTSPV----------GSPGPLAPIPPTL--LAPGTLLGPKRE
PIAS1_Mmu_BC051417     MADSAELKQMVMSLRVSELQVLLGYAGRNKHGRKHELLTKALHLLKAGCSPAVQMKIKELYRRRFPQKIMTPADLSIPNVHSSPMPPTLSPS----------TIPQLTYDGHPASSPLLPVSLLGPKHE
PIASXa_Hu_Q96BT5       MADFEELRNMVSSFRVSELQVLLGFAGRNKSGRKHDLLMRALHLLKSGCSPAVQIKIRELYRRRYPRTLEGLSDLSTIKSSVFSLDGGSSPVEPDLAVAGIHSLPSTSVTPHSPSSPVGSVLLQDTKPT
PIASXb_Hu_AF077954     MADFEELRNMVSSFRVSELQVLLGFAGRNKSGRKHDLLMRALHLLKSGCSPAVQIKIRELYRRRYPRTLEGLSDLSTIKSSVFSLDGGSSPVEPDLAVAGIHSLPSTSVTPHSPSSPVGSVLLQDTKPT
PIASY_Hu_Q8N2W9        MAAELVEAKNMVMSFRVSDLQMLLGFVGRSKSGLKHELVTRALQLVQFDCSPELFKKIKELYETRYAKKNSEPAPQPHRPLDPLTMHSTYDRAG---------AVPRTPLAGPNIDYPVLYGKYLNGLGR
Consensus              .ma...elknMVmSfRVS#LQvLLG%aGRnKsGrKH#Ll.rALhLlks.CsP.vq.KIkELYrrR%prk..gpsdls........$..g.sp.............P.t.........pv....ll..k..

                       131  140       150       160       170       180       190       200       210       220       230       240       250       260
                       |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PIAS3_Mmu_AF034080     -----------PVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTPQQLQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLPPTKNGAEPKRPSR
PIAS3L_Mmu_BC023128    VDMH--PPLPQPVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTPQQLQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLPPTKNGAEPKRPSR
PIAS3L_Mmu_BC051252    VDMH--PPLPQPVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTPQQLQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLPPTKNGAEPKRPSR
PIAS3L_Rat_AF032872    VDMH--PPLPQPVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTPQQLQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLPPTKNGAEPKRPSR
PIAS3L_Hu_q9y6x2       VDMH--PPLPQPVHPDVTMKPLPFYEVYGELIRPTTLASTSSQRFEEAHFTFALTPQQVQQILTSREVLPGAKCDYTIQVQLRFCLCETSCPQEDYFPPNLFVKVNGKLCPLPGYLPPTKNGAEPKRPSR
PIAS1_Mmu_BC051417     LELPHLTSALHPVHPDIKLQKLPFYDLLDELIKPTSLASDNSQRFRETCFAFALTPQQVQQISSSMDI-SGTKCDFTVQVQLRFCLSETSCPQEDHFPPNLCVKVNTKPCSLPGYLPPTKNGVEPKRPSR
PIASXa_Hu_Q96BT5       FEMQQPSPPIPPVHPDVQLKNLPFYDVLDVLIKPTSLVQSSIQRFQEKFFIFALTPQQVREICISRDFLPGGRRDYTVQVQLRLCLAETSCPQEDNYPNSLCIKVNGKLFPLPGYAPPPKNGIEQKRPGR
PIASXb_Hu_AF077954     FEMQQPSPPIPPVHPDVQLKNLPFYDVLDVLIKPTSLVQSSIQRFQEKFFIFALTPQQVREICISRDFLPGGRRDYTVQVQLRLCLAETSCPQEDNYPNSLCIKVNGKLFPLPGYAPPPKNGIEQKRPGR
PIASY_Hu_Q8N2W9        L-------PAKTLKPEVRLVKLPFFNMLDELLKPTELVPQNNEKLQESPCIFALTPRQVELIRNSRELQPGVK---AVQVVLRICYSDTSCPQEDQYPPNIAVKVNHSYCSVPGYYPSNKPGVEPKRPCR
Consensus              ........p..pvhP#!.$k.LPF%#vldeLikPT.Lv..s.#rfqE..fiFALTPqQv..I..Sr#.lpG.k.dyt!QVqLR.Cl.#TSCPQED.%Ppnl.!KVNgklcplPGY.Pp.KnG.EpKRP.R

                       261  270       280       290       300       310       320       330       340       350       360       370       380       390
                       |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PIAS3_Mmu_AF034080     PINITPLARLSATVPNTIVINWSSEFGRNYSLSVYLVRQLTAGTLLQKLRAKGIRNPDHSRALIKEKLTADPDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCD
PIAS3L_Mmu_BC023128    PINITPLARLSATVPNTIVVNWSSEFGRNYSLSVYLVRQLTAGTLLQKLRAKGIRNPDHSRALIKEKLTADPDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCD
PIAS3L_Mmu_BC051252    PINITPLARLSATVPNTIVVNWSSEFGRNYSLSVYLVRQLTAGTLLQKLRAKGIRNPDHSRALIKEKLTADPDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCD
PIAS3L_Rat_AF032872    PINITPLARLSATVPNTIVVNWSSEFGRNYSLSVYLVRQLTAGTLLQKLRAKGIRNPDHSRALIKEKLTADPDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCD
PIAS3L_Hu_q9y6x2       PINITPLARLSATVPNTIVVNWSSEFGRNYSLSVYLVRQLTAGTLLQKLRAKGIRNPDHSRALIKEKLTADPDSEVATTSLRVSLMCPLGKMRLTVPCRALTCAHLQSFDAALYLQMNEKKPTWTCPVCD
PIAS1_Mmu_BC051417     PINITSLVRLSTTVPNTIVVSWTAEIGRTYSMAVYLVKQLSSTVLLQRLRAKGIRNPDHSRALIKEKLTADPDSEIATTSLRVSLLCPLGKMRLTIPCRALTCSHLQCFDATLYIQMNEKKPTWVCPVCD
PIASXa_Hu_Q96BT5       PLNITSLVRLSSAVPNQISISWASEIGKNYSMSVYLVRQLTSAMLLQRLKMKGIRNPDHSRALIKEKLTADPDSEIATTSLRVSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNEKKPTWICPVCD
PIASXb_Hu_AF077954     PLNITSLVRLSSAVPNQISISWASEIGKNYSMSVYLVRQLTSAMLLQRLKMKGIRNPDHSRALIKEKLTADPDSEIATTSLRVSLMCPLGKMRLTIPCRAVTCTHLQCFDAALYLQMNEKKPTWICPVCD
PIASY_Hu_Q8N2W9        PINLTHLMYLSSAT-NRITVTW-GNYGKSYSVALYLVRQLTSSELLQRLKTIGVKHPELCKALVKEKLRLDPDSEIATTGVRVSLICPLVKMRLSVPCRAETCAHLQCFDAVFYLQMNEKKPTWMCPVCD
Consensus              PiNiT.L.rLSsavpN.I.!.W.s#.GknYS.svYLVrQLts..LLQrLk.kG!rnP#hsrAL!KEKLtaDPDSE!ATTslRVSLmCPLgKMRLt!PCRA.TCaHLQcFDAalYlQMNEKKPTW.CPVCD

                       391  400       410       420       430       440       450       460       470       480       490       500       510       520
                       |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PIAS3_Mmu_AF034080     KKAPYESLIIDGLFMEILNSCSDCDEIQFMEDGSWCPMKPKKEASEVCPPPGYGLDG-----LQYSAVQEGIQPESKKRVEVIDLTIESSSDEEDL-PPTKKHCPVTSAAIPALPGSKGALTSGHQPSSV
PIAS3L_Mmu_BC023128    KKAPYESLIIDGLFMEILNSCSDCDEIQFMEDGSWCPMKPKKEASEVCPPPGYGLDG-----LQYSAVQEGIQPESKKRVEVIDLTIESSSDEEDL-PPTKKHCPVTSAAIPALPGSKGALTSGHQPSSV
PIAS3L_Mmu_BC051252    KKAPYESLIIDGLFMEILNSCSDCDEIQFMEDGSWCPMKPKKEASEVCPPPGYGLDG-----LQYSAVQEGIQPESKKRVEVIDLTIESSSDEEDL-PPTKKHCPVTSAAIPALPGSKGALTSGHQPSSV
PIAS3L_Rat_AF032872    KKAPYESLIIDGLFMEILNSCSDCDEIQFMEDGSWCPMKPKKEASEVCPPPGYGLDG-----LQYSPVQEGNQSENKKRVEVIDLTIESSSDEEDL-PPTKKHCPVTSAAIPALPGSKGALTSGHQPSSV
PIAS3L_Hu_q9y6x2       KKAPYESLIIDGLFMEILSSCSDCDEIQFMEDGSWCPMKPKKEASEVCPPPGYGLDG-----LQYSPVQGGDPSENKKKVEVIDLTIESSSDEEDL-PPTKKHCSVTSAAIPALPGSKGVLTSGHQPSSV
PIAS1_Mmu_BC051417     KKAPYEHLIIDGLFMEILKYCTDCDEIQFKEDGSWAPMRSKKEVQEVTASYN-GVDGCLSSTLEHQVASHNQSSNKNKKVEVIDLTIDSSSDEEEEEPPAKRTCPSLSPTSPL--SNKGILSLPHQASPV
PIASXa_Hu_Q96BT5       KKAAYESLILDGLFMEILNDCSDVDEIKFQEDGSWCPMRPKKEAMKVSSQPCTKIES--SSVLSKPCSVTVASEASKKKVDVIDLTIESSSDEEED-PPAKRKCIFMSETQSS--PTKGVLM--YQPSSV
PIASXb_Hu_AF077954     KKAAYESLILDGLFMEILNDCSDVDEIKFQEDGSWCPMRPKKEAMKVSSQPCTKIES--SSVLSKPCSVTVASEASKKKVDVIDLTIESSSDEEED-PPAKRKCIFMSETQSS--PTKGVLM--YQPSSV
PIASY_Hu_Q8N2W9        KPAPYDQLIIDGLLSKILSECEDADEIEYLVDGSWCPIRAEKERSCSPQGAILVLGPSDANGLLPAPSVNGSGALGSTGGGGPVGSMENGKPGADVVDLTLDSSSSSEDEEEEEEEEDEDEEGPRPKR-
Consensus              KkApY#sLIiDGLfmeILn.CsD.DEI.%.eDGSWcPmrpkKEas.v...p...l.......L....sv.g.....kkkv.vidlti#sssdee#..pptk..c...s.........kg.l..g.qpssv

                       521  530       540       550       560       570       580       590       600       610       620       630       640       650
                       |--------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
PIAS3_Mmu_AF034080     LRSPAMGTLGSDFLSSLPVHEYPPAF---P---LGADIQGLDLFSFLQTESQQYGPSVIISL-----DEQDTLGHFFQYRGTPSHFLGPLAPTLGSCHGSSTPAPPPGRVSSIVAPGSSLREGHGGPLPS
PIAS3L_Mmu_BC023128    LRSPAMGTLGSDFLSSLPLHEYPPAF---P---LGADIQGLDLFSFLQTESQHYGPSVITSL-----DEQDTLGHFFQYRGTPSHFLGPLAPTLGSSHRSSTPAPPPGRVSSIVAPGSSLREGHGGPLPS
PIAS3L_Mmu_BC051252    LRSPAMGTLGSDFLSSLPLHEYPPAF---P---LGADIQGLDLFSFLQTESQHYGPSVITSL-----DEQDTLGHFFQYRGTPSHFLGPLAPTLGSSHRSSTPAPPPGRVSSIVAPGSSLREGHGGPLPS
PIAS3L_Rat_AF032872    LRSPAMGTLGSDFLSSLPLHEYPPAF---P---LGADIQGLDLFSFLQTESQHYSPSVITSL-----DEQDTLGHFFQFRGTPPHFLGPLAPTLGSSHRSATPAPAPGRVSSIVAPGSSLREGHGGPLPS
PIAS3L_Hu_q9y6x2       LRSPAMGTLGGDFLSSLPLHEYPPAF---P---LGADIQGLDLFSFLQTESQHYGPSVITSL-----DEQDALGHFFQYRGTPSHFLGPLAPTLGSSHCSATPAPPPGRVSSIVAPGGALREGHGGPLPS
PIAS1_Mmu_BC051417     SRTPSLPAVDTSYINTSLIQDYRHPFHMTP---MPYDLQGLDFFPFLSGDNQHYNTSLLAAAAAAVSDDQDLLHSSRFFPYTSSQMFLDQLSAGGSTSLPATNGSSSGSNSSLVS-SNSLRESHGHGVAS
PIASXa_Hu_Q96BT5       -RVPSVTSVDPAAIPPS-LTDYSVPFHHTPISSMSSDLPGEQRRN--DINNELKLGTSSDTV------QQ
PIASXb_Hu_AF077954     -RVPSVTSVDPAAIPPS-LTDYSVPFHHTPISSMSSDLPGLDFLSLIPVDPQYCPPMFLDSL------TSPLTASSTSVTTTSSHESSTHVSSSSSRSETGVITSSGSNIPEIISLD
PIASY_Hu_Q8N2W9        -RCPFQKGLVPAC
Consensus              .R.P....l.pa.........y...f...p......d..gld.........q.................q...........t.s...........s.............................

                       651  660       670
                       |--------+---------|
PIAS3_Mmu_AF034080     GPS-----LTGCRSDVISLD
PIAS3L_Mmu_BC023128    GPS-----LTGCRSDVISLD
PIAS3L_Mmu_BC051252    GPS-----LTGCRSDVISLD
PIAS3L_Rat_AF032872    GPS-----LTGCRSDVISLD
PIAS3L_Hu_q9y6x2       GPS-----LTGCRSDIISLD
PIAS1_Mmu_BC051417     RSSADTASIFGIIPDIISLD
PIASXa_Hu_Q96BT5
PIASXb_Hu_AF077954
PIASY_Hu_Q8N2W9
Consensus              ....................
```

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′-GGAATTCCATATGGCGGAGCTGGGC-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 *Nde*I/*Hin*dIII 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′-CCTTGCTTTCTTTGCCCCCTGGCACCTCTCCT-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].

L$_{19}$QVLL/AQVAA: 5′ primer: 5′-CCGAGTGTCTGAGGCC-CAGGTGGCCGCCGGCTTCGCTGGC-3′, 3′ primer: 5′-GCCAG-CGAAGCCGGCGGCCACCTGGGCCTCAGACACTCGG-3′.

ΔP$_{117}$PLP: 5′ primer: 5′-CCTAAGCGTGAGGTGGCAATGCA-TCAGCCTGTGCACCCCGATGTCACC-3′, 3′ primer: 5′-GGTG-ACATCGGGGTGCACAGGCTGATGCATGTCCACCTCACGCT-TAGG-3′.

PI$_{242}$NIT/PSDST: 5′ primer: 5′-CCAAGAGGCCCAGCCGTCC-GAGCGACAGCACACCCTTGGCTCG-3′, 3′ primer: 5′-CGAGC-CAAGGGTGTGCTGTCGCTCGGACGGCTGGGCCTCTTGG-3′.

C$_{343}$AHLQS/GADLQG: 5′ primer: 5′-CCGTGTCGTGCCCTCA-CCGGTGCCGATCTGCAGGGTTTCGATGCTGCC-3′, 3′ primer: 5′ - GGCAGCATCGAAACCCTGCAGATCGGCACCGGTGAGG-GCACGACACGG-3′.

ΔC$_{366}$PVC: 5′ primer: 5′-CAGATGAATGAGAAGAAGCCGA-CATGGACGGACAAGAAGGCTCCCTATGAGTCG-3′, 3′primer: 5′-CGACTCATAGGGAGCCTTCTTGTCCGTCCATGTCGGCTT-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 (TPGTLLGPKREVDMHPPLPQ), present only in the large variant of PIAS3 (PIAS3L).

Antibody synthesis: 300 μg 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 indulation 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 μg 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 μg protein in 1 ml of Tris-buffered saline (TBS)) were precleared with 40 μl of a 50% (v/v) protein A Sepharose slurry for 1 h at 4°C. The supernatant was incubated with 10 μl of Ab 1395 or pre-immune sera for 1 h and reacted with 40 μl 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 μl of anti-Flag M2 affinity gel (Sigma).

2.6. Immunocytochemistry

COS-1 cells were transfected with 2 μg 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-

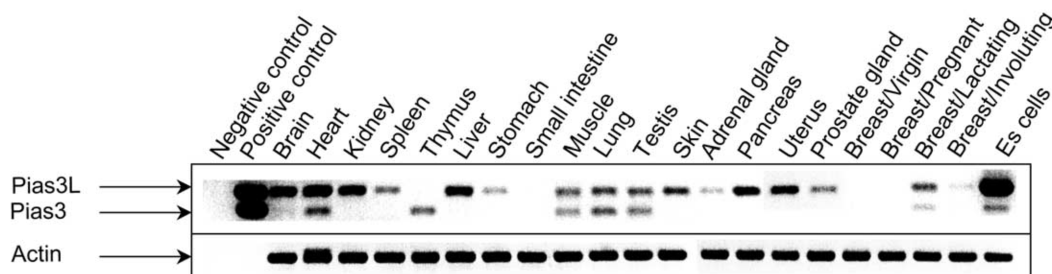

Fig. 2. Differential expression profiles of *pias3* and *pias3L*. PCR experiments, with primers recognizing both *pias3* and *pias3L* cDNAs and with control *actin* primers, were performed on a commercially available subset of tissues with the 'Rapid Scan' kit. One quarter of each PCR reaction was run 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 pg 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.

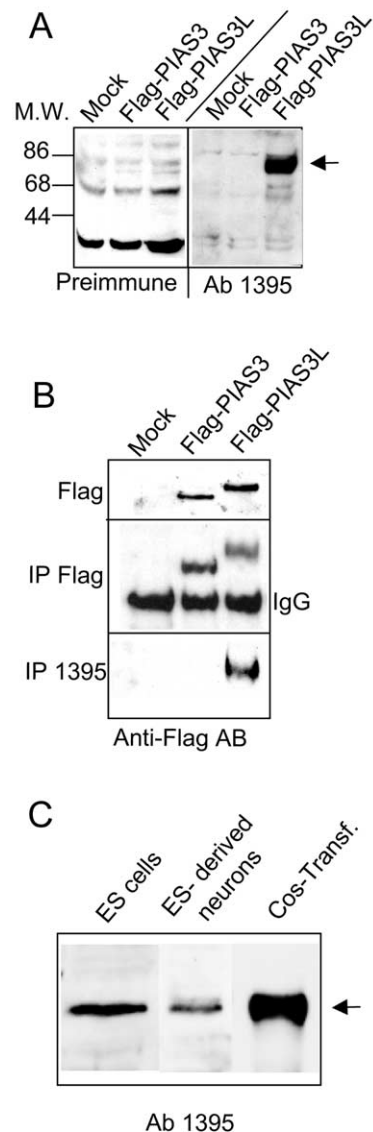

Fig. 3. Characterization of PIAS3L protein with a PIAS3L-specific polyclonal antibody. A: Western blot analysis was performed on 50 µg of total cell lysates from COS-1 cells (mock or transfected with p513-Flag-pias3 or pias3L encoding vectors) with the pre-immune or Ab 1395 polyclonal antibodies, as 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

```
                            1                                            50                                                                             100
Hs_pias3_aah30556      109 PLPQPVHPDV TMKPLPFYEV YGELIRPTTL ASTSS...QR FEEAHFTFAL TPQQVQQILT SREVLP..GA KCDYTIQVQL RFC.LCETS. CPQEDYFP.P NLFVKVNG
Mm_pias3_q8r598        109 PLPQPVHPDV TMKPLPFYEV YGELIRPTTL ASTSS...QR FEEAHFTFAL TPQQLQQILT SREVLP..GA KCDYTIQVQL RFC.LCETS. CPQEDYFP.P NLFVKVNG
Rn_pias3_o70260        118 PLPQPVHPDV TMKPLPFYEV YGELIRPTTL ASTSS...QR FEEAHFTFAL TPQQLQQILT SREVLP..GA KCDYTIQVQL RFC.LCETS. CPQEDYFP.P NLFVKVNG
Hs_pias1_human         127 SALHPVHPDI KLQKLPFYDL LDELIKPTSL ASDNS...QR FRETCFAFAL TPQQVQQISS SMDIS...GT KCDFTVQVQL RFC.LSETS. CPQEDHFP.P NLCVKVNT
Mm_pias1_mouse         127 SALHPVHPDI KLQKLPFYDL LDELIKPTSL ASDNS...QR FRETCFAFAL TPQQVQQISS SMDIS...GT KCDFTIQVQL RFC.LSETS. CPQEDHFP.P NLCVKVNT
Mm_piasy_q8r165        115 LPTKTLKPEV RLVKLPFFNM LDELLKPTEL VPQSA...EK LQESPCIFAL TPRQVEMIRN SRELQP..GV KA...VQVVL RIC.YSDTS. CPQEDQYP.P NLAVKVNH
Hs_piasy_q8n2w9        122 LPAKTLKPEV RLVKLPFFNM LDELLKPTEL VPQNN...EK LQESPCIFAL TPRQVELIRN SRELQP..GV KA...VQVVL RIC.YSDTS. CPQEDQYP.P NLAVKVNH
Ci_ak115495              1 VNPPQATPIV QFKPSPFYKQ ISEVLQPSCI TNSKS...KM AHSKNFTFLL HHQREVLCKD RVQVQSMIRQ SPKNHTQIQL RFC.KLETS. CEQPDALP.P HLTVKVNN
Am_pias frag             1                                                                       VAS SRDCRA..GS KMDYXVQIQM RFC.LQETS. CEQEDCFP.P SIAVKVNG
Bm frag                  1                                                                                   SN KLDYIIQAQL RFC.LLETS. CEQEDHFP.P SVNVKVNN
Dm_q9xym4              116 NSQLPIHPDV RLKKLAFYDV LGTLIKPTTL VPRNT...QR VQEVPFYFTL TPQQATEIAS NRDIRN..SS KVEHAIQVQL RFC.LVETS. CDQEDCFP.P NVNVKVNN
Ag_eaa09481 frag        158 YGDYPIHPDV QLKKLAFFDV MATLLKPATL VPSNTA..QR VQEGSYFFHL TPQQANEIAL NRDISN..SA KIEHNVQVQL RFC.LLETT. CEQDDHFP.P NIVLKVNN
Mm_piasx_beta_q99jx5   128 PPIPPVHPDV QLKNLPFYDV LDVLIKPTSL VQSSI...QR FQEKFFIFAL TPQQVREICI SRDFLP..GG RRDYTVQVQL RIC.LAETS. CPQEDNYP.N SLCIKVNG
Hs_piasx_beta_o75928   137 PPIPPVHPDV QLKNLPFYDV LDVLIKPTSL VQSSI...QR FQEKFFIFAL TPQQVREICI SRDFLP..GG RRDYTVQVQL RIC.LAETS. CPQEDNYP.N SLCIKVNG
Hs_piasx_alpha_q96bt5  137 PPIPPVHPDV QLKNLPFYDV LDVLIKPTSL VQSSI...QR FQEKFFIFAL TPQQVREICI SRDFLP..GG RRDYTVQVQL RIC.LAETS. CPQEDNYP.N SLCIKVNG
Mm_piasx_alpha_q9d5w7   99 PPIPPVHPDV QLKNLPFYDV LDVLIKPTSL VQSSI...QR FQEKFFIFAL TPQQVREICI SRDFLP..GG RRDYTVQVQL RIC.LAETS. CPQEDNYP.N SLCIKVNG
Dv_q8t9s2               12 KRMHTVYPRE HFKRLPFYEV LAVLLQPTRL KTDGR...GS THNTSLPFQF LPHHVNDILK SLTRNTA.SM WTEFGAEVHL RFC.LFDRH. EQQDDSYP.Y DLRLEVNN
Ce_q9u326              159 TPKKMYADNF EPLPLPFYDV ISVLLKPVEL HSSDSPTLKQ TKQLQFPFLL TAEHISKISY RADV...... TPLPRYELQL RFFNLTEPVQ GPQKDDFP.L NCYARVDD
At_q9ffs2              157 PGIKSPGSTF SQIMERFYPF VKLGHVLVSF EVK.....AG YTMLAHDFYI SKNMPHSL.. .........Q EKIRLFVAQT DN...IDTS. ..ACISNP.P EVRFLLNG
Vc_o04238              145 NGPSSSHSAV LTIMERFYPR MKLGPIIVTI EAQ.....PG YGASAVDPHI TKNNVHS... .........D KKIWLLVAQT DN...IETS. ..ACLISP.Q EVNFLLNG
Sc_siz2_nfi1_yeast     142 PPYSQQQALA YSFTSPFYKP IVQIPDANKK LKQS....AG RGCTKMKPKV SKSNHDLLKS NK.......S YKLYLFSGFS IPF.IYETV. GHEAIDFP.Y PCELVFNG
Sc_siz1_q04195         166 SSSSFAVPTI HFKESPFYKI QRLIPELVMN VEVT....GG RGMCSAKFKL SKADYNLLSN PNS......K HRLYLFSGMI NP...LGSR. GNEPIQFP.F PNELRCNN
Nc_eaa33693            116 DWYNQQRHDF QFKPSVFYEI QYRIGEVKDC EPMQ....QH RQTVSIKLRV ADHPQLSNVG NDP......S LRIMMYCAAG KN........ GVQDIAFP.Y QSELKVNG
Sp_o94451              111 PFAPVVHSRI RFRKSPFYDI LEQFNAPFVV PACV....GT RNTISFSFHV TPPALSKLL. NDPK.....Q YRVYLFSTPS ET...IGF.. GNCLMEFPTP QMELRINN
Dd_q8mmx3              765 KKLQLNDSSF EEKQSPFFQE LDKLVITSFV STVTV....S FSLDLETWKI IEAAQQKLLN PSLPPPPL.. KDDYSIQIRL Y....NKQS. GSD.IHYG.S DIYLSVNG
At_q9fkk4              157 PESFYCEICR LTRADPFWVT VAHPLSPVRL TATTIP.... .NDGASTMQS VERTFQITRA DKDLLA.... KPEYDVQAWC ML..LNDKV. .LFRMQWP.Q YADLQVNG
Os_aao38837            168 PPHFYCDMCR ITRADPFWVT VNHPVLPVSI TPCKVA.... .SDGSYAVQY FEKTFPLSRA NWEMLQ.... KDEYDLQVWC IL..FNDSV. .PFRMQWP.L HSDIQING
Ce_q21971              137 AQQYAVTAHP NPVPQPYYHP EVRQLYETTA GYIVD..... RELLAEG.HV TPGSNGVEAM LT........ HDASDFLKVS P.....QTD. .VLFSIWP.V TKSNDPNI
Consensus 85%                    hps h ph   shhp  h  h  shph    ph            p   h h h  pp   h  p ph        p    hph     h hhphh  h    hs  ph hph
PHD sec. structure                                aa aaaaa   b b       aa aa   bbbbbb bbbbbbbbbb b                   bbbbbbb bb                    bbbbb

                       110                                     150                                                            200          211
Hs_pias3_aah30556      KLCPLPGYLPPT KNGAE.PKRP SRPINITPLA RLSATVP.NT IVVNWSSEFG RNYSLSVYLV R.QLTAGTLL QKLRAKGIRN P..DHSRALI KEKLTADPDS E 306 (313)
Mm_pias3_q8r598        KLCPLPGYLPPT KNGAE.PKRP SRPINITPLA RLSATVP.NT IVVNWSSEFG RNYSLSVYLV R.QLTAGTLL QKLRAKGIRN P..DHSRALI KEKLTADPDS E 306 (313)
Rn_pias3_o70260        KLCPLPGYLPPT KNGAE.PKRP SRPINITPLA RLSATVP.NT IVVNWSSEFG RNYSLSVYLV R.QLTAGTLL QKLRAKGIRN P..DHSRALI KEKLTADPDS E 315 (313)
Hs_pias1_human         KPCSLPGYLPPT KNGVE.PKRP SRPINITSLV RLSTTVP.NT IVVSWTAEIG RNYSMAVYLV K.QLSSTVLL QRLRAKGIRN P..DHSRALI KEKLTADPDS E 323 (328)
Mm_pias1_mouse         KPCSLPGYLPPT KNGVE.PKRP SRPINITSLV RLSTTVP.NT IVVSWTAEIG RTYSMAVYLV K.QLSSTVLL QRLRAKGIRN P..DHSRALI KEKLTADSDS E 323 (328)
Mm_piasy_q8r165        SYCSVPGYYPSN KPGVE.PKRP CRPINLTHLM YLSSAT..NR ITVTW.GNYG KSYSVALYLV R.QLTSSDLL QRLKTIGVKH P..ELCKALV KEKLRLDPDS E 307 (200)
Hs_piasy_q8n2w9        SYCSVPGYYPSN KPGVE.PKRP CRPINLTHLM YLSSAT..NR ITVTW.GNYG KSYSVALYLV R.QLTSSELL QRLKTIGVKH P..ELCKALV KEKLRLDPDS E 314 (196)
Ci_ak115495            VSVVLPNFIPPT KAGME.PRRP NRPLNITNLC RHSSSGQ.NT LEVRYSSSDH EEYCVTVNLV K.QLFAEDLL QKLKSQPVLS A..ATTRYRI KEKLKRDLDS D 200 (0)
Am_pias frag           KLCPLPNPIPTN KPGVE.PKRP PRPVNISPLV KLSPTVA.NQ IHVTWSADYG RRYAIAIYLV R.KLSSAELL SRLKSRDVRH S..DYTRGLI KEKLNEDADS E 144 (25)
Bm frag                KMCPLPNPIPTN KPTPE.PKRP PRPVNTSSLV KLSPTVS.NT IQVTWAADFT RAYVLSVFMV K.KLTSAELL QRLKNKGTKN P..DYTRSLI KEKLSEDYDS E 135 (74)
Dm_q9xym4              KLCQLPNVIPTN RPNVE.PKRP PRPVNVTSNV KLSPTVT.NT ITVQWCPDYT RSYCLAVYLV K.KLTSTQLL QRMKTKGVKP A..DYTRGLI KEKLTEDADC E 313 (241)
Ag_eaa09481 frag      KLCPLPNPIPTN KPGVE.PKRP PRPVNITAQV KLSPTVA.NH ISVSWCTEYN RG                                                        310 (0)
Mm_piasx_beta_q99jx5   KLFPLPGYAPPP KNGIE.QKRP GRPLNITSLV RLSSAVP.NQ ISISWASEIG KNYSMSVYLV R.QLTSAMLL QRLKMKGIRN P..DHSRALI KEKLTADPDS E 325 (287)
Hs_piasx_beta_o75928   KLFPLPGYAPPP KNGIE.QKRP GRPLNITSLV RLSSAVP.NQ ISISWASEIG KNYSMSVYLV R.QLTSAMLL QRLKMKGIRN P..DHSRALI KEKLTADPDS E 334 (287)
Hs_piasx_alpha_q96bt5  KLFPLPGYAPPP KNGIE.QKRP GRPLNITSLV RLSSAVP.NQ ISISWASEIG KNYSMSVYLV R.QLTSAMLL QRLKMKGIRN P..DHSRALI KEKLTADPDS E 334 (238)
Mm_piasx_alpha_q9d5w7  KLFPLPGYAPPP KNGIE.QKRP GRPLNITSLV RLSSAVP.NQ ISISWASEIG KNYSMSVYLV R.QLTSAMLL QRLKMKGIRN P..DHSRALI KEKLTADPDS E 296 (238)
Dv_q8t9s2              QPLALPESIPVH SSGGV.SGRI RLPINILPSC FLDASVK.NK VSVSWRPELD REYVAGLFLV R.KKSVATIL RELRQRRMQS A..TLTKALV KKKAQRQASC D 210 (232)
Ce_q9u326              SVVQLPNVIPTN KTNAE.PKRP SRPVNITSNM NRYKKE..HT VAVEWLAD.K RVWAAGVYFV H.RVNSDILF KRLNQNVSRH RSLEVLKQEV IKKLS.GGED D 356 (331)
At_q9ffs2              KNVE.KRVNIAM DTG......P QLPTNVTAQL KYGT....NL LQVM..GNFK GNYIIIIAFT GLVVPSEKPV LK........ ...DYLQSGV IE...ASPDS D 317 (459)
Vc_o04238              KGID.TRTNFRM DPG......P QMPTNVTSVL KFGT....NL LQAV..GQFN GHYIILVAYM SVASLSEHPV LPP....... ...DYVQPAV TS...VDSDS D 305 (523)
Sc_siz2_nfi1_yeast     TKLEDNVKGLKK QNG......T GNPANLTPYL KVPTEM..NH LDLHY.LNID KEYSISCFIV E.VFSPEALL GKILKRPKII K..QATTAYI KRTLNEQDDD D 326 (400)
Sc_siz1_q04195         VQIKDNIRGFKS KPG......T AKPADLTPHL KPYTQQ..NN VELIY.AFTT KEYKLFGYIV E.MITPEQLL EKVLQHPKII K..QATLLYL KKTLREDEEM G 349 (555)
Nc_eaa33693            GEVKANLRGLKN KPG......S TRPVDITDSL RLRPNTYQNN IDFTY.ALTR EELVSRIT.. K.KIRAESVV TEIANKANDP D......... .......... . 277 (202)
Sp_o94451              QVAHANYRRLKG KPG......T TNPADITDLV SKYAGPPGNN VVIYY.MNST KSYSVVVCFV K.VYTIENLV DQIKSRKA.. ...ESKEKII ERIKNDNQDA D 293 (434)
Dd_q8mmx3              KSINNEQFKKIR TKSFSNPCVI QKPIDITHLV KRSD....NR VNLQI.QNRT SGIMVVQLL. K.NQTLQQVV EEIKMKSEM. ...EESQSAD KRQKKNEDD. . 949 (369)
At_q9fkk4              VPVRAINRPGGQ LLGVN..GRD DGPI.ITSCI RDGV....NR ISLS..GGDV RIFCFGVRLV K.RRTLQQVL NLIPEEGKGE T.FEDALARV RRCIGGGGGD D 342 (531)
Os_aao38837            IPIRVVNRQPTQ QLGVN..GRD DGPV.LTAYV REGS....NK IVLS..RSDS RTFCLGVRIA K.RRSVEQVL SLVPKEQDGE N.FDNALARV RRCVGGGTEA D 353 (362)
Ce_q21971              QYEVYINQKSVM N.....SHMQ VKSIFIKDFL ISGM....NS IHIVY.NGPQ QMYKYACEFV T.RKSVIELR KILIQKRVIE G..QQLATQQ KQQYMQMANK . 311 (323)
Consensus 85%            h h          p hh     c  shphp   h p hs s   pp h h hh p   p hhhhhhhh p    ph   hh pph  p        p hph   ppphp   p  c
PHD sec. structure                                      b                bbbbb       bbbbbbbbb b    aaaaa aaaaaa              bbbbbbb bbbbbbb
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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

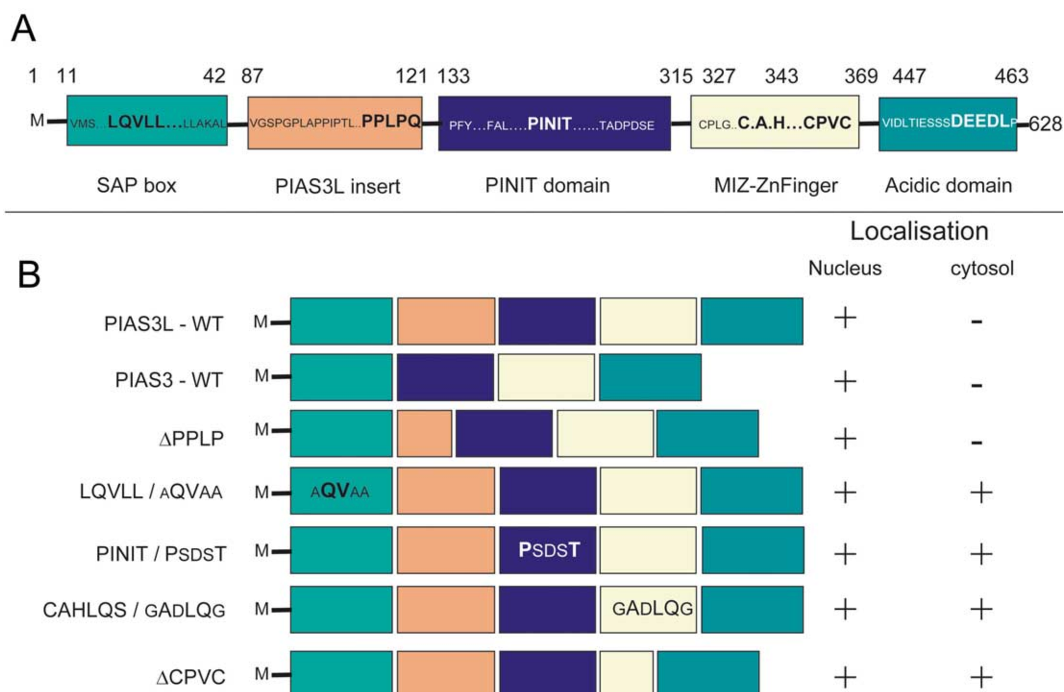

Fig. 5. Independent conserved domains of PIAS3L are involved in its nuclear retention. A: General features of PIAS proteins are represented (not to scale) with coordinates referring to PIAS3L (accession number: BC051252). Within each structured domain (colored boxes), important motifs are boldfaced. 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 ×400.

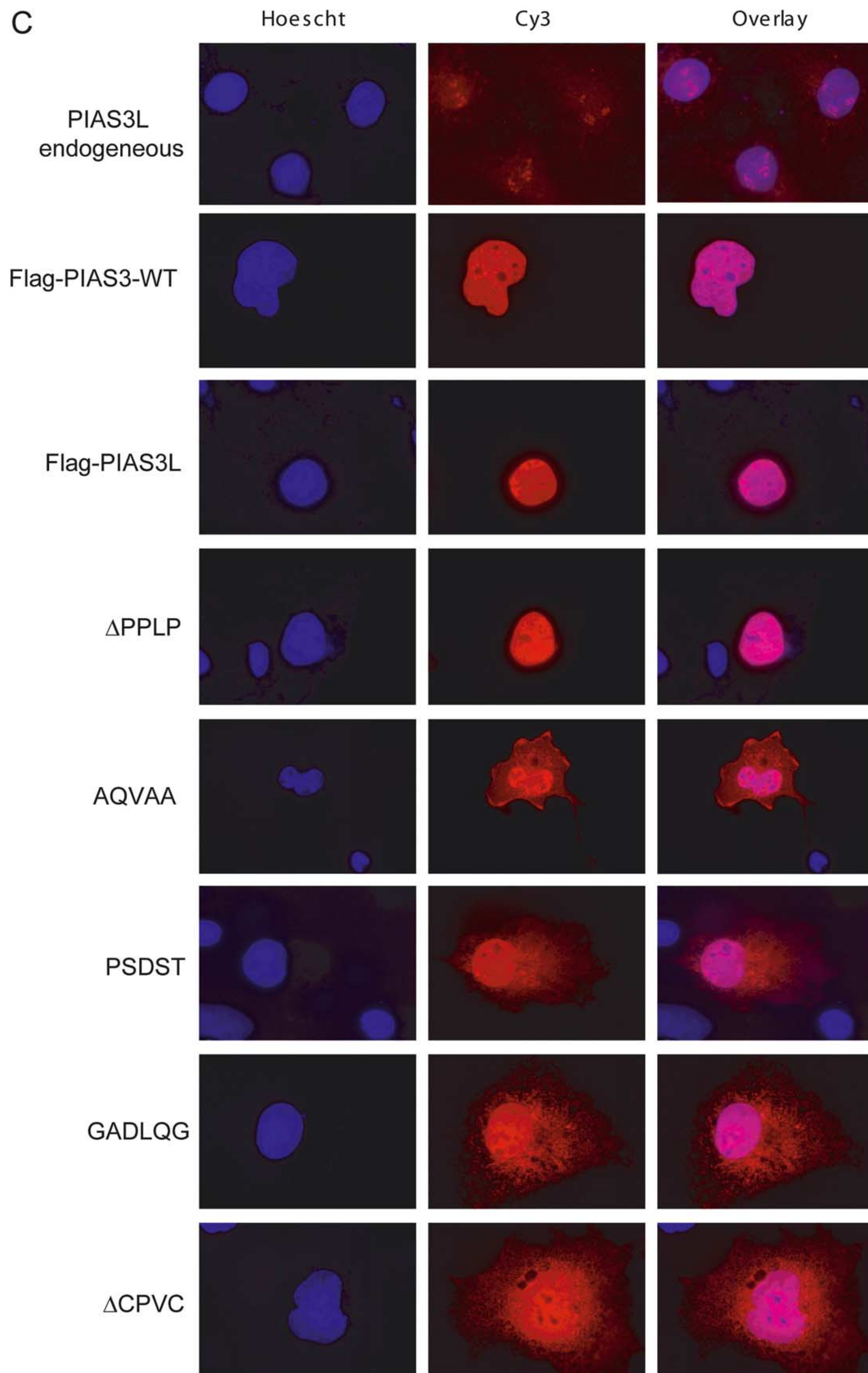

Fig. 5 (*Continued*).

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 ΔPPLP 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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References

[1] Hilton, D.J. (1999) Cell. Mol. Life Sci. 55, 1568–1577.
[2] Janne, O.A., Moilanen, A.M., Poukka, H., Rouleau, N., Karvonen, U., Kotaja, N., Hakli, M. and Palvimo, J.J. (2000) Biochem. Soc. Trans. 28, 401–405.
[3] Heinrich, P.C., Behrmann, I., Haan, S., Hermanns, H.M., Muller-Newen, G. and Schaper, F. (2003) Biochem. J. 374, 1–20.
[4] Chung, C.D., Liao, J., Liu, B., Rao, X., Jay, P., Berta, P. and Shuai, K. (1997) Science 278, 1803–1805.
[5] Liu, B., Gross, M., ten Hoeve, J. and Shuai, K. (2001) Proc. Natl. Acad. Sci. USA 98, 3203–3207.
[6] Arora, T., Liu, B., He, H., Kim, J., Murphy, T.L., Murphy, K.M., Modlin, R.L. and Shuai, K. (2003) J. Biol. Chem. 278, 21327–21330.
[7] Kotaja, N., Aittomaki, S., Silvennoinen, O., Palvimo, J.J. and Janne, O.A. (2000) Mol. Endocrinol. 14, 1986–2000.
[8] Jimenez-Lara, A.M., Heine, M.J. and Gronemeyer, H. (2002) FEBS Lett. 526, 142–146.
[9] Kotaja, N., Vihinen, M., Palvimo, J.J. and Janne, O.A. (2002) J. Biol. Chem. 277, 17781–17788.
[10] Starr, R. and Hilton, D.J. (1999) BioEssays 21, 47–52.
[11] Schmidt, D. and Muller, S. (2002) Proc. Natl. Acad. Sci. USA 99, 2872–2877.
[12] Nakagawa, K. and Yokosawa, H. (2002) FEBS Lett. 530, 204–208.
[13] Liu, B. and Shuai, K. (2001) J. Biol. Chem. 276, 36624–36631.
[14] Wible, B.A., Wang, L., Kuryshev, Y.A., Basu, A., Haldar, S. and Brown, A.M. (2002) J. Biol. Chem. 277, 17852–17862.
[15] Kotaja, N., Karvonen, U., Janne, O.A. and Palvimo, J.J. (2002) Mol. Cell. Biol. 22, 5222–5234.
[16] Nishida, T. and Yasuda, H. (2002) J. Biol. Chem. 277, 41311–41317.
[17] Miyauchi, Y., Yogosawa, S., Honda, R., Nishida, T. and Yasuda, H. (2002) J. Biol. Chem. 277, 50131–50136.
[18] Verger, A., Perdomo, J. and Crossley, M. (2003) EMBO Rep. 4, 137–142.
[19] Rogers, R.S., Horvath, C.M. and Matunis, M.J. (2003) J. Biol. Chem. 278, 30091–30097.
[20] Aravind, L. and Koonin, E.V. (2000) Trends Biochem. Sci. 25, 112–114.
[21] Jackson, P.K. (2001) Genes Dev. 15, 3053–3058.
[22] Lee, P.S., Chang, C., Liu, D. and Derynck, R. (2003) J. Biol. Chem. 278, 27853–27863.
[23] Tan, J.A., Hall, S.H., Hamil, K.G., Grossman, G., Petrusz, P. and French, F.S. (2002) J. Biol. Chem. 277, 16993–17001.
[24] Hogan, B.L., Beddington, R., Costantini, F. and Lacy, E. (1994) Manipulating the Mouse Embryo. A Laboratory manual, 2nd edn., pp. 255–272, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[25] Boeuf, H. et al. (2001) J. Biol. Chem. 276, 46204–46211.
[26] Sadowski, H.B. and Gilman, M.Z. (1993) Nature 362, 79–83.
[27] Duval, D., Reinhardt, B., Kedinger, C. and Boeuf, H. (2000) FASEB J. 14, 1577–1584.
[28] Corpet, F. (1988) Nucleic Acids Res. 16, 10881–10890.
[29] Plewniak, F. et al. (2003) Nucleic Acids Res. 31, 3829–3832.
[30] Rost, B. (1996) Methods Enzymol. 266, 525–539.
[31] Wible, B.A., Yang, Q., Kuryshev, Y.A., Accili, E.A. and Brown, A.M. (1998) J. Biol. Chem. 273, 11745–11751.
[32] Levy, C., Nechushtan, H. and Razin, E. (2002) J. Biol. Chem. 277, 1962–1966.