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The SUMO-E3 Ligase PIAS3 Targets Pyruvate Kinase M2

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ABSTRACT

Pyruvate kinase M2 (M2-PK) controls the rate-limiting step at the end of the glycolytic pathway in normal proliferating and tumor cells. Other functions of M2-PK in addition to its role in glycolysis are little understood. The aim of this study was to identify new cellular interaction partners of M2-PK in order to discover novel links between M2-PK and cellular functions. Here we show that the SUMO-E3 ligase protein PIAS3 (inhibitor of activated STAT3) physically interacts with M2-PK and its isoenzyme M1-PK. Moreover, we demonstrate that endogenous SUMO-1-M2-PK conjugates exist in mammalian cells. Furthermore, we show that transient expression of PIAS3 but not the RING domain mutant PIAS3 (C299S, H301A) is consistent with nuclear localization of M2-PK and PIAS3 and M2-PK partially co-localize in the nucleus of these cells. This study suggests a link between PIAS3 and nuclear pyruvate kinase. J. Cell. Biochem. 107: 293–302, 2009. © 2009 Wiley-Liss, Inc.

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Pyruvate kinase (PK, ATP/pyruvate *O*'-phosphotransferase, EC 2.7.1.40) is one of the rate-controlling glycolytic enzymes that catalyze the transfer of a phosphoryl group from phosphoenolpyruvate (PEP) to ADP, to generate pyruvate and ATP [Staal and Rijksen, 1991]. Four distinct pyruvate kinase isoenzymes (L, R, M1, and M2) occur in mammalian cells. M2-PK (alias PKM2) is the embryonic form that replaces the other isoforms in normal proliferating and in tumor cells [Mazurek et al., 2005]. The human M1 and M2 isoenzymes are encoded by one gene; the specific mRNAs are generated by alternative splicing of the M1 specific exon 9 and the M2 specific exon 10, respectively [Takenaka et al., 1991]. M2-PK is subject to allosteric activation by fructose-1,6-bisphosphate (FBP), an intermediate metabolite in the glycolytic pathway. Activation of M2-PK involves a shift from a dimeric to a tetrameric conformation. Tetrameric M2-PK is characterized by a high affinity to its substrate PEP and high activity at physiological PEP concentrations, whereas dimeric M2-PK has a low PEP affinity and is nearly inactive under physiological conditions [Mazurek et al., 2005]. The dimmer–tetramer switch of M2-PK contributes to the regulation of the glycolytic flux in proliferating cells and has been shown to be regulated by direct interaction with viral and cellular oncoproteins [Zwerschke et al., 1999; Mazurek et al., 2001], suggesting that M2-PK activity is tightly controlled in tumor cells. M2-PK is predominantly detected in many if not all human cancers [Mazurek et al., 2005] and lung cancer cells predominantly expressing M2-PK grow to large tumors in a nude mice xenografts model [Christofk et al., 2008]. These data suggest that M2-PK plays an important role for tumor growth. The tetrameric form of M2-PK is associated with other glycolytic enzymes as well as with lactate dehydrogenase, nucleotide diphosphate kinase, and adenylate kinase, within a protein complex, referred to as the glycolytic enzyme complex [Mazurek et al., 1996, 2001]. In contrast, dimeric and also a monomeric form of M2-PK are not associated with the glycloytic complex [Mazurek et al., 1996]. Monomeric M2-PK was

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described as a cytosolic thyroid hormone binding protein [Kato et al., 1989], and, recently nuclear localization and a role in transcriptional regulation was described for M2-PK [Hoshino et al., 2007; Stetak et al., 2007; Lee et al., 2008]; however, non-glycolytic functions of M2-PK are little understood.

SUMO, a small ubiquitin-like modifier protein that is highly conserved from yeast to human, is conjugated to a large number of cellular target proteins [Uzunova et al., 2007]. Similar to enzymatic ubiquitination, the conjugation of specific SUMO proteins (SUMO-1–3) to target proteins requires an E1-activating enzyme (Aos1/Uba2) as well as an E2-type SUMO-1-conjugating enzyme [Ubc9; Johnson, 2004]. More recently, proteins that increase the efficiency of SUMO conjugation have been discovered. The members of the mammalian protein inhibitor of activated STAT (PIAS) protein family, originally identified as cytokine-induced inhibitors of the STAT family of transcription factors [Chung et al., 1997], belong to this protein class, referred to as SUMO-E3 ligases. The PIAS family consists of seven structurally related proteins (PIAS1, PIAS3, PIAS3β, PIASxα, PIASxβ, PIASy, and PIASyE6) [Shuai and Liu, 2005]. Similar to many ubiquitin E3 ligases, these proteins contain a predicted RING finger-like structure which has been shown to be essential for their SUMO-E3 ligase activities toward various target proteins. In contrast to the well-known function of polyubiquitin conjugation in proteasome-mediated protein degradation, sumoylation seems to be rarely involved in protein degradation processes. There is evidence that SUMO-2/3 conjugates in human cells are controlled via degradation by the proteasome while SUMO-1 conjugates show little changes upon inhibition of the proteasome [Uzunova et al., 2007]. Sumoylation is a highly dynamic process and its outcomes are highly diverse, ranging from changes in subcellular localization, transcriptional regulation to altered activity and stability of the modified protein [Johnson, 2004]. PIAS proteins do not operate merely as SUMO-E3s, since their co-regulator effects are often independent of their RING finger but dependent on their SIM (SUMO-interacting motif) or SAP (scaffold attachment factor-A/B/acinus/PIAS) domain [Palvimo, 2007]. The modulator activity of the PIAS/SUMO system has so far mainly been shown to involve altered subnuclear targeting and/or assembly of transcription complexes.

The aim of this study was to discover novel cellular M2-PK-binding proteins. We identified the SUMO-E3 ligase PIAS3 [Chung et al., 1997] as a novel M2-PK-interacting protein and analyzed functional consequences of this interaction.

MATERIALS AND METHODS

INTERACTION ANALYSIS IN YEAST

LexA-M2-PK was used as bait in the yeast two-hybrid screen to identify cDNAs for M2-PK-binding proteins from a human WI38 fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) [Zwerschke et al., 1999]. The pB42-WI38 cDNA::TRP1 plasmids express the B42 transactivation domain-prey fusions proteins from the inducible GAL1 promoter. Yeast strain EGY48/pSH1834 (MATa, his3, ura3, trp1, leu2::lexAo6-pLEU2/LexAo8-GAL1-lacZ::URA3) was used for both the LEU2 and β-galactosidase reporter gene assays. For determination of reporter gene activity, EGY48/pSH1834 was transformed with pEG202::HIS3 plasmids expressing the LexA-M2-PK, LexA-M1-PK or LexA-$APP_{599-695}$ fusion protein, together with the plasmid pB42-HA-$PIAS3_{383-619}$::TRP1, and it was selected for leucine prototrophy as previously described [Zwerschke et al., 1999]. Alternatively, β-galactosidase activity was determined in cellular extracts as previously described [Zwerschke et al., 1996]. The various LexA fusion proteins were expressed to the same level, as confirmed by direct immunoblotting, using a polyclonal antibody against LexA.

PLASMIDS

The complete human M2-PK cDNA was isolated by *Eco*RI/*Xho*I restriction digest from the yeast prey vector pB42-M2-PK::TRP1 [Zwerschke et al., 1999] and inserted in frame to GST in the bacterial expression vector pGEX-4T-1 (Amersham Biosciences, Vienna, Austria) to generate pGEX-4T-1/M2-PK, and fused in frame to LexA in the yeast two-hybrid bait vector pEG202 [Zwerschke et al., 1999] to generate the bait vector pEG202/M2-PK. To construct the bait vector pEG202/$APP_{599-695}$, the human APP cDNA encoding for the amino acids 599–695 was amplified by PCR and inserted as an *Eco*RI/*Not*I fragment in frame to LexA into pEG202. To generate pGEX-4T-1/M1-PK and pEG202/M1-PK the sequence encoding the M2-PK specific region was exchanged with the M1-PK specific region isolated as *Sph*I/*Xho*I fragment from the vector pX/M1-PK. The yeast expression vector pEG202/M2-PK_{1-348} encoding for the carboxyl-terminal deletion mutant of M2-PK from amino acid residues 1–348 fused to the B42 transactivation domain was constructed by the deletion of a *Msc*I/*Xho*I fragment in pEG202/M2-PK spanning from the 5′*Msc*I side to the stop codon of M2-PK. The human PIAS3 cDNA was amplified from the RZPD clone IDIMAGp958J08198Q2 (Deutsches Ressourcenzentrum für Genomforschung GmbH, Berlin, Germany) by PCR and ligated as *Eco*RI/*Eco*RI fragment into the *Eco*RI site of the pEG202, pGEX-4T-1 and pUHD10-3 to generate pEG202/PIAS3, pGEX-4T-1/PIAS3, and pUHD10-3/PIAS3 respectively. All plasmids were confirmed by sequencing. The plasmids Flag-mPIAS3 wild-type and Flag-mPIAS3 (C299S, H301A) are gifts of Fang Liu [see, Long et al., 2004].

IN VITRO INTERACTION ANALYSIS

GST pull down assays were performed essentially as described [Mannhardt et al., 2000], using 5 μg purified GST, GST-M2-PK or GST-PIAS3 proteins and U-2 OS cell lysates.

CO-IMMUNOPRECIPITATION EXPERIMENTS

Co-immunoprecipitation experiments were performed essentially as described [Mannhardt et al., 2000].

WESTERN BLOTTING EXPERIMENTS

Immunoblot analysis was performed essentially as described [Mannhardt et al., 2000] using antibodies against LexA (sc-7544, IgG1, mouse monoclonal Santa Cruz Biotechnology, Vienna, Austria), M2-PK (clone DF4, mouse monoclonal, ScheBo Biotech, Wettenberg, Germany), SUMO-1 (33–2,400, mouse monoclonal, Zymed, Vienna, Austria, or A-712, rabbit polyclonal, BostonBiochem, USA), M1/2-PK (200–1,178, goat polyclonal, Rockland,

Vienna, Austria), HA1 (sc-805, rabbit polyclonal, Santa Cruz Biotechnology) and PIAS3 (AP,1245a, rabbit polyclonal, Abgent). Further antibodies used: anti-rabbit, W401B, IgG, HRP conjugate; anti-mouse W4021, IgG, HRP conjugate; anti-goat, W4011, IgG, HRP conjugate (Promega, Vienna, Austria). Anti-goat IgG (FITC-conjugate Jackson, Vienna, Austria). Anti-rabbit IgG, TRITC-conjugate (DAKO, Vienna, Austria).

IN VIVO SUMOYLATION ASSAY

To prevent desumoylation of the target proteins, U-2 OS cells were resuspended in SUMO immunoprecipitation buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2% SDS) and incubated at 95°C for 10 min to inactivate desumoylating enzymes [Buschmann et al., 2001]. The lysate was diluted 1:10 in dilution buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100) and centrifuged at 20,000*g* and 4°C. After preclearing with A/G agarose beads (50 μg/1 mg lysate) M2-PK was precipitated over night with goat polyclonal antibodies against pyruvate kinase (Rockland Immunocemicals, Gilbertsville, PA) and A/G agarose beads. The precipitates were washed with dilution buffer, separated by SDS–PAGE and blotted onto a PVDF membrane. The PVDF membrane was probed with monoclonal anti-M2-PK or monoclonal anti-SUMO-1 antibodies.

CELL CULTURE AND TRANSFECTION

The human osteosarcoma cell line U-2 OS was cultured in DMEM plus 10% fetal calf serum as described [Mannhardt et al., 2000] and the mouse NIH3T3 cell line was cultured in DMEM plus 10% calf serum as described [Zwerschke et al., 1999]. Transient transfection of the expression vector pUHD-3/PIAS3 was performed using Effectene (Qiagen, Hilden, Germany).

INDIRECT IMMUNOFLUORESCENCE ANALYSIS

Indirect immunofluorescence analysis was performed essentially as described previously [Mannhardt et al., 2000]. Briefly, cells were grown to about 80% confluence on glass cover slips and transiently transfected (Effectene, Qiagen). At 30 h post-transfection, cells were prepared for indirect immunofluorescence according to standard protocols, including fixation with 4% PFA/0.2% Triton X-100 and incubation with primary antibodies (α-PIAS3 antibody, Abgent, clone AP,1245a, Vienna; α-M2-PK antibody, Rockland, Vienna) and TRITC- or FITC-conjugated secondary antibodies. Nuclear staining was conducted with TO-PRO-3 (Molecular Probes, Göttingen, Germany). Samples were viewed by indirect immunofluorescence microscopy using the confocal scanning system MicroRadiance (Bio-Rad, Hempstead, UK) in combination with a Zeiss Axiophot microscope. The following filters were used for FITC-derived and TRITC-derived fluorescence: excitation for FITC 488 nm, TRITC 543 nm and emission for FITC 515–530 nm and TRITC >570 nm.

RESULTS

IDENTIFICATION OF PIAS3 AS M2-PK BINDING PROTEIN

To better understand the function of M2-PK, a yeast two-hybrid screen for cellular M2-PK-binding proteins was conducted. We used full-length M2-PK fused in frame to the LexA-DNA-binding domain as bait [Zwerschke et al., 1999]. LexA-M2-PK can bind to the LexA binding sites of a synthetic LEU2 reporter gene, and thereby allows monitoring of the interaction between M2-PK and a second hybrid protein containing the B42 transactivation domain fused in-frame to a heterologous cDNA. When a galactose inducible human fibroblast cDNA expression library (pB42-WI38 cDNA::TRP1) was co-expressed with LexA-M2-PK in the yeast strain EGY48/pSH1834 and screened for transformants that grew on leucine-deficient medium, cDNAs encoding M2-PK and fragments thereof were repeatedly isolated (G. Spoden and W. Zwerschke, unpublished findings), reflecting the tendency for M2-PK to form tetramers and dimers [Zwerschke et al., 1999]. Intriguingly, the M2-PK bait identified a cDNA encoding for the intact carboxyl-terminal substrate binding domain (amino acids 383–619) of protein inhibitor of activated STAT3 (PIAS3) [Chung et al., 1997], which was more recently identified as a SUMO-E3 ligase involved in SUMO-1 modification and transcriptional repression of interferon regulatory factor-1 [Nakagawa and Yokosawa, 2002] and micro-phthalmia transcription factor [Miller et al., 2005]. Co-expression of the PIAS3$_{383–619}$-HA1-B42 fusion protein with LexA-M2-PK (Fig. 1A) enabled the yeast strain EGY48/pSH1834 to grow on galactose but not glucose minimal plates, indicating that leucine prototrophy depends on expression of the M2-PK-B42 fusion protein (Fig. 1B). PIAS3 also bound to the M1-PK isoenzyme in this assay (Fig. 1B), suggesting that the M2-PK specific region is not necessary for the pyruvate kinase/PIAS3 interaction. The inability of the unrelated fusion protein LexA-APP$_{599–695}$ to bind to B42-HA-PIAS3$_{383–619}$ indicates that the M-type pyruvate kinase sequences are essential for binding to PIAS3. These findings were corroborated using lacZ as a second reporter gene. High β-galactosidase activity (blue color) was observed when B42-HA-PIAS3$_{383–619}$ was co-expressed with either LexA-M2-PK or LexA-M1-PK, but not with the unrelated fusion protein LexA-APP$_{599–695}$ (Fig. 1B, bottom panel).

To analyze whether M2-PK also interacts with the PIAS3 full-length protein, purified GST-M2-PK was incubated with extracts from human U-2 OS cells overexpressing wild-type PIAS3. PIAS3 specifically bound to GST-M2-PK in this assay, whereas no binding was observed for GST (Fig. 2A, left). Moreover, the incubation of GST-PIAS3 full-length protein with lysates from human U-2 OS cells demonstrates that human M2-PK binds specifically to purified GST-PIAS3, but not to GST alone (Fig. 2A, right). These data suggest that M2-PK interacts with PIAS3 in vitro. To map the PIAS3-interacting region on M2-PK, we incubated GST-PIAS3 either with the M2-PK full-length protein (aa 1–531) or the carboxyl-terminal deletion mutant M2-PK$_{1–348}$ lacking the M2-PK specific region (Fig. 2B, left). Both proteins were expressed in yeast cells and the lysates incubated with purified GST-PIAS3 and GST. We found that M2-PK$_{1–348}$ bound as strongly as the M2-PK full-length protein to GST-PIAS3 (Fig. 2B, left). This underlines the specificity of the PIAS3/M2-PK protein–protein interaction and suggests that M2-PK$_{1–348}$ is sufficient for PIAS3 binding. This is in keeping with the finding that the B42-HA-PIAS3$_{383–619}$ protein specifically interacts with both M-type pyruvate kinase isoenzymes (Fig. 1), whose amino acid sequences are identical in this region. The interaction of M2-PK with the intact carboxyl-terminal substrate binding domain of the PIAS3 protein found in living yeast cells (Fig. 1) was corroborated in

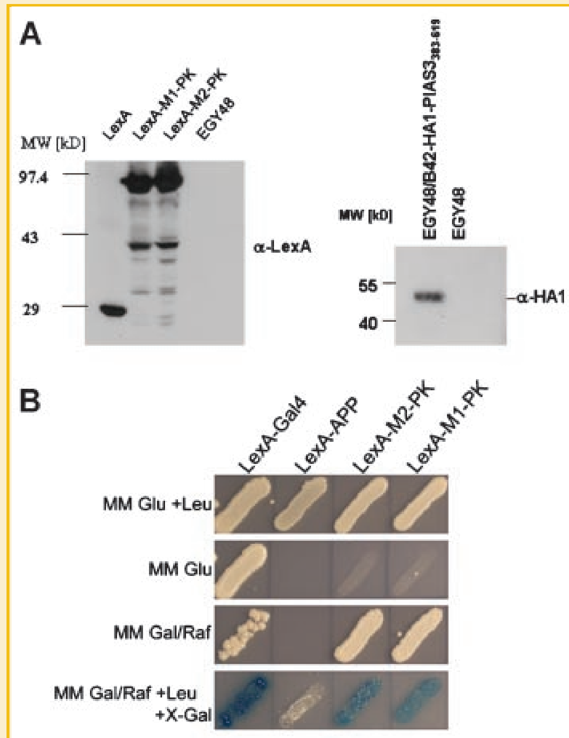

Fig. 1. Identification of PIAS3 as M2-PK-binding protein. A, Left panel: Expression of the baits LexA, LexA-M1-PK and LexA-M2-PK in the yeast strain EGY48 was detected by immunoblot analysis using a monoclonal anti-LexA antibody. A lysate of untransformed yeast strain EGY48 was loaded as control. Right panel: Expression of the B42-HA1-$PIAS3_{383-619}$ prey fusion protein in EGY48 as shown by immunoblot using anti-HA1 antibodies. B: Derivatives of yeast strain EGY48/pSH1834::URA3 (MATa, his3, ura3, trp1, leu2::lexAo6-pLEU2/LexAo8-Gal1-lacZ::URA3) expressing various LexA fusion proteins from pEG202::HIS3 vectors as indicated, were transformed with the plasmid pB42-$PIAS3_{383-619}$::TRP1. Transformants were selected for uracil, histidine, and tryptophane prototrophy and then streaked out onto each of four plates and incubated for 4 days at 30°C under the following nutrient conditions; MM Glu +Leu: Glucose minimal medium with leucine; all strains grow. MM Glu: Glucose minimal medium without leucine; selection for B42 fusion protein independent activation of the LexAo6-LEU2 reporter. MM Gal/Raf: Galactose minimal medium without leucine, selecting for B42 fusion protein dependent activation of the LexAo6-LEU2 gene. MM Gal/Raf +Leu +X-Gal: Galactose minimal medium with leucine and X-Gal, selecting for B42 fusion protein dependent activation of the LexAo8-Gal1-LacZ::URA3. Gal4 served as a positive control and LexA-APP as a negative control.

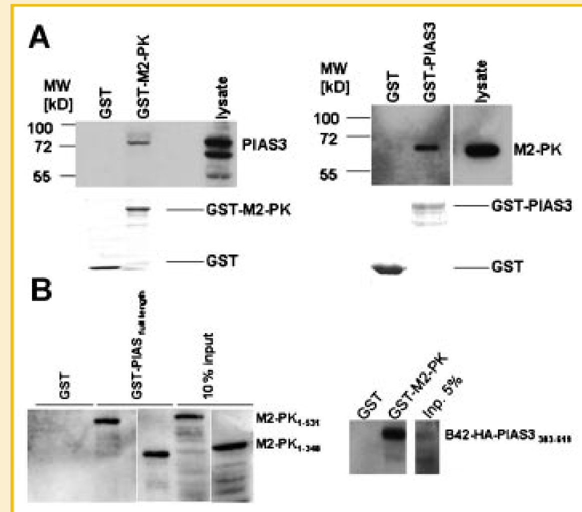

Fig. 2. M2-PK interacts with PIAS3 in vitro. A, Left panel: Purified GST or GST-M2-PK proteins immobilized on glutathione-Sepharose 4B beads were incubated with whole-cell extracts from human U-2 OS cells overexpressing the PIAS3 full-length protein. The amount of PIAS3 protein that was retained after washing was determined by immunoblotting using polyclonal anti-PIAS3 antibodies (top). Five percent of the lysate was loaded as input. Input of the GST proteins was controlled by Coomassie staining (bottom). Middle panel: Purified GST or GST-PIAS3 proteins immobilized on glutathione-Sepharose 4B beads were incubated with whole-cell extracts from U-2 OS cells. After washing, the amount of M2-PK protein that was retained was determined by immunoblotting using a monoclonal anti-M2-PK antibody (top). Five percent of the lysate was loaded as input. Input of the GST proteins was controlled by Coomassie staining (bottom). B: Mapping of the PIAS3/M2-PK binding domains. Left panel: Purified GST or GST-PIAS3 protein immobilized on glutathione-Sepharose 4B beads was incubated with extracts from yeast cells expressing B42-HA-M2-PK_{1-531} or B42-HA-M2-PK_{1-348} fusion proteins. After washing, the amount of M2-PK protein that was retained was determined by immunoblotting using a monoclonal anti-M2-PK antibody. M2-PK-input was loaded as indicated (for input of GST proteins see A). Right panel: Purified GST or GST-M2-PK protein immobilized on glutathione-Sepharose 4B beads was incubated with extracts from yeast cells expressing B42-HA-$PIAS3_{383-619}$. After washing, the amount of the bound B42-HA-$PIAS3_{383-619}$ protein was determined by immunoblotting using a monoclonal anti-HA1 antibody. Five percent of the input was loaded (for input of GST proteins see A).

a GST pull down experiment. GST-M2-PK bound specifically and with high affinity to B42-HA-$PIAS3_{383-619}$ (Fig. 2B, right), underlining that it is the C-terminal domain of PIAS3 that interacts with M2-PK. Collectively, these results map the interaction domains to the amino-terminal part of M2-PK (residues 1–348) and the carboxyl-terminal domain of PIAS3 (residues 383–619).

To show that the described interaction also occurs in cell extracts, co-immunoprecipitation experiments were conducted using human U-2 OS cells transiently transfected with a CMV promoter driven PIAS3 expression vector (Fig. 3). Endogenous M2-PK was specifically co-precipitated with PIAS3 using anti-PIAS3 antibodies, but not with an unrelated polyclonal control serum (Fig. 3, left). M2-PK antibodies precipitated M2-PK from U-2 OS extracts and co-immunoprecipitation of PIAS3 was observed, whereas an unrelated polyclonal control serum precipitated neither M2-PK nor PIAS3 (Fig. 3, right). These findings suggest that M2-PK can form a complex with the SUMO-E3 ligase PIAS3 in extracts from human cells.

A SUBFRACTION OF M2-PK IS SUMO-1-CONJUGATED

Computer-aided analysis of the human M2-PK sequence revealed eight putative SUMO-1 acceptor sequences in the M2-PK molecule (Supplementary Fig. 1), suggesting that it may be a sumoylation target. To examine whether endogenous M2-PK is covalently conjugated with SUMO-1, U-2 OS cell extracts were prepared in SDS-containing lysate buffer to avoid desumoylation, and M2-PK was immunoprecipitated from the extracts overnight. The precipitates were analyzed by Western blotting with antibodies against M2-PK and SUMO-1, respectively (Fig. 4A). The anti-M2-PK antibody recognized a specific band at 58 kDa, corresponding to

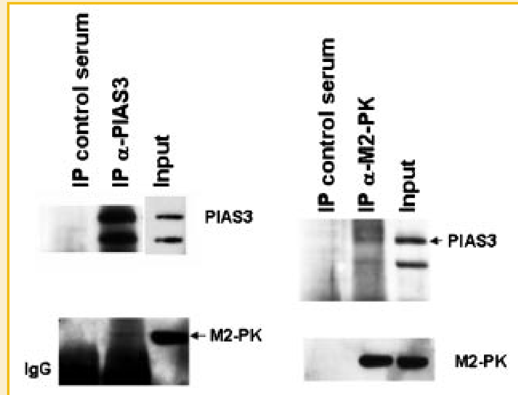

Fig. 3. M2-PK interacts with PIAS3 in U-2 OS cell extracts. Extracts were prepared from U-2 OS cells transiently overexpressing PIAS3 and subjected to immunoprecipitation with antibodies against M2-PK, PIAS3 antibodies and control antibodies, as indicated. Precipitated proteins were separated by SDS-gel electrophoresis and detected by Western blotting. Total cell lysate (100 μg) was loaded as input control. PIAS3 was immunoprecipitated by anti-PIAS3 antibodies (left, upper panel) and M2-PK was co-immunoprecipitated (left, lower panel). M2-PK was immunoprecipitated by M2-PK specific antibodies (right, lower panel) and PIAS3 was co-immunoprecipitated (right, upper panel). The species-specific preimmune sera (IP control serum) precipitated neither M2-PK nor PIAS3.

unmodified M2-PK, and two slower migrating bands at approximately 80 and 110 kDa (Fig. 4A, left, lane 2). The anti-SUMO-1 antibody recognized the two slower migrating bands at 80 and 110 kDa (Fig. 4A, right, lane 2). The 110 kDa protein was also immunoprecipitated by isotypic control antibodies (Fig. 4A, right, lane 1), indicating that only the 58 and 85 kDa bands are specifically immunoprecipitated by the anti-M2-PK antibodies. These data suggest that the 80 kDa band is consistent with the conjugation of SUMO-1 to endogenous M2-PK. Most of the protein recognized by the anti-M2-PK antibodies runs at 58 kDa (Fig. 4A), suggesting that only a part of the endogenous M2-PK exists as SUMO-1 conjugate and/or that the M2-PK-SUMO-1 conjugates are relatively unstable. To address this question, the U-2 OS cells were harvested in a stabilizing SDS-containing lysis buffer and either immediately processed for Western blotting (Fig. 4B, lane 1) or incubated for 6 (Fig. 4B, lane 2) or 18 (Fig. 4B, lane 3) h at 4°C before processing. Over time the intensity of the high molecular weight bands recognized by the anti-M2-PK antibody was reduced (Fig. 4B, compare lanes 1–3)—especially after 18 h, the incubation time used for the immunoprecipitation experiment in Figure 4A. These data suggest that higher molecular weight M2-PK conjugates exist, but that these proteins are relatively unstable, explaining the weak signals obtained in the Co-IP (Fig. 4A).

To analyze whether PIAS3 specifically enhances M2-PK sumoylation, PIAS3 was transiently expressed in U-2 OS cells (Fig. 4C) and M2-PK was immunoprecipitated from PIAS3-overexpressing U-2 OS cells. When the immunoprecipitates were probed with anti-M2-PK (Fig. 4A, left, lane 3) and anti-SUMO-1 (Fig. 4A, right, lane 3) antibodies, it was found that the intensity of the 80 kDa M2-PK-SUMO-1 conjugate was slightly increased in the

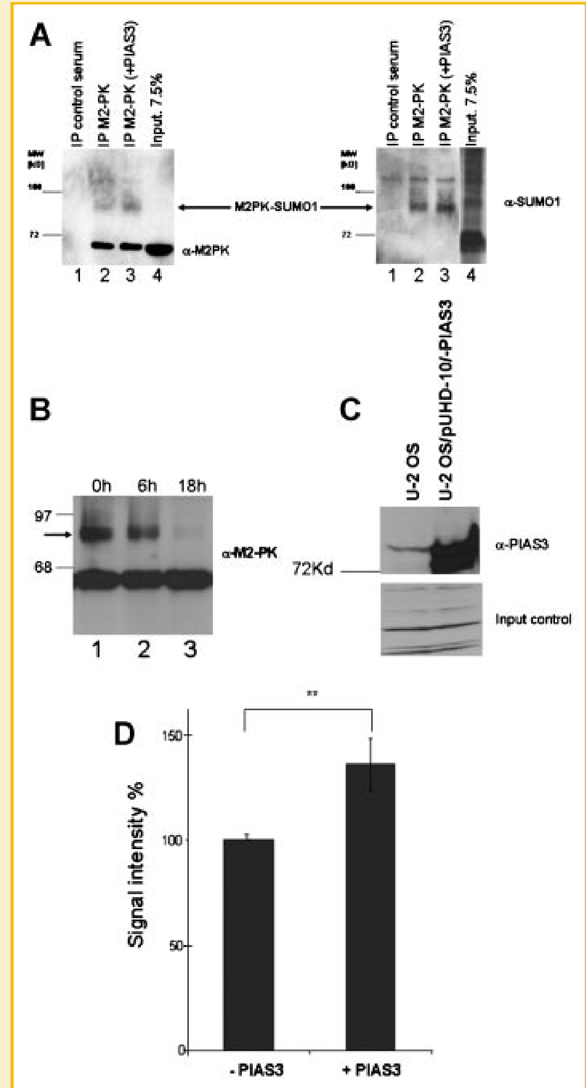

Fig. 4. Sumoylation of M2-PK. A: Detection of SUMO-1-M2-PK conjugates in U-2 OS cells. U-2 OS cells were transiently transfected either with empty vector or with a CMV-promoter driven expression vector for PIAS3. 30 h post-transfection, immunoprecipitations were performed as described in the sumoylation assay with M2-PK specific antibodies from PIAS3 non-overexpressing (lanes 2) or PIAS3 overexpressing U-2 OS cells (lanes 3). Immunoprecipitations were also performed with an unrelated control serum as negative control (lane 1). The precipitates were separated by SDS–PAGE and Western blotted using anti-M2-PK (left panel) and anti-SUMO-1 antibodies (right panel). Input controls are shown as indicated (lanes 4). B: Decrease of higher molecular weight M2-PK conjugates in U-2 OS cell lysates over time. Cells were lysed in an SDS containing buffer and the lysates placed for 6 and 18 h at 4°C or immediately (0 h) processed for Western blotting using anti-M2-PK antibodies. C: The levels of endogenous and ectopically overexpressed PIAS3 protein were analyzed in U-2 OS cells by Western blotting using anti-PIAS3 antibodies. D: M2-PK-SUMO-1 conjugates were co-immunoprecipitated as in (A), and the intensity of the immunoprecipitated 85 kDa band from PIAS3 non-overexpressing and PIAS3 overexpressing U-2 OS cells was quantified by densitometry (statistical significance was calculated using Student's *t*-test, $n = 4$, $^{**}P = 0.0056$).

PIAS3 co-expressing cells. The triple repetition of this immunoprecipitation experiment and quantification of the intensity of the 80 kDa M2-PK-SUMO-1 band from PIAS3 non-overexpressing and PIAS3 overexpressing U-2 OS cells by densitometry showed that PIAS3 produced a slight increase in M2-PK sumoylation (Fig. 4D).

EXPRESSION OF PIAS3 COINCIDES WITH NUCLEAR LOCALIZATION OF M2-PK

Since there is precedence that sumoylation can influence the subcellular localization of target proteins acting in carbohydrate metabolism, as has been shown for the glucose transporter GLUT4 and GLUT1 [Giorgino et al., 2000; Lalioti et al., 2002], we investigated the subcellular localization of M2-PK in PIAS3 overexpressing cells. To this end, we transiently transfected a PIAS3 expression vector into U-2 OS cells. Co-staining of PIAS3 with anti-PIAS3 antibodies and the DNA stain TO-PRO-3 revealed that PIAS3 (green) was predominantly localized in the nucleus (red), a nuclear speckled staining for PIAS3 was found, as monitored by indirect immunofluorescence experiments using a confocal laser-scanning microscope (Fig. 5A). Co-staining of M2-PK and TO-PRO-3 revealed that M2-PK (green) was also localized in the nucleus (red) in PIAS3 overexpressing cells (Fig. 5B); however, not in U-2 OS cells containing only empty vector (Fig. 5C). To analyze whether M2-PK and PIAS3 co-localize in the nucleus of PIAS3 overexpressing U-2 OS cells, we conducted a co-staining with anti-M2-PK and anti-PIAS3 antibodies and visualized the staining by either red (M2-PK) or green (PIAS3) fluorescence (Fig. 5D). U-2 OS cells transiently expressing PIAS3 (Fig. 5D, middle, green) showed a strong nuclear staining signal for M2-PK, although significant levels of M2-PK were still detectable in the cytoplasm (Fig. 5D, left). This is consistent with the finding that only a part of endogenous M2-PK is SUMO-1 conjugated (Fig. 4). In the PIAS3 overexpressing cells, M2-PK was distributed throughout the nucleus, whereas PIAS3 was predominantly detected in defined nuclear speckles, and co-localization between M2-PK and PIAS3 was observed predominantly at these nuclear structures (Fig. 5D, right, yellow). In mock-transfected U-2 OS cells, the anti-M2-PK antibodies stained only structures in the cytoplasm but not in the nucleus, and endogenous PIAS3 was not detectable (Fig. 5E). No staining of U-2 OS cells was observed when only the secondary anti-rabbit and anti-goat IgGs were added (Fig. 5F). To corroborate these findings, PIAS3 was transiently expressed in NIH3T3 mouse fibroblasts. Similar to our findings in U-2 OS cells, ectopic expression of PIAS3 resulted in co-localization of PIAS3 with M2-PK in the nucleus of NIH3T3 cells (Fig. 5G). Together these experiments demonstrate that PIAS3 and M2-PK specifically co-localize in the nucleus in PIAS3 over-expressing cells.

To test whether the increased nuclear localization of M2-PK depends on the catalytically active RING domain in PIAS3, we employed the catalytically inactive PIAS3 RING domain mutant Flag-mPIAS3 (C299S, H301A) described in Long et al. [2004], and investigated the impact of the Flag-mPIAS3 RING domain mutant and of Flag-mPIAS3 wild-type on nuclear localization of M2-PK in U-2 OS cells (Fig. 6). We transiently expressed both proteins in U-2 OS cells and detected nuclear M2-PK only in the Flag-mPIAS3

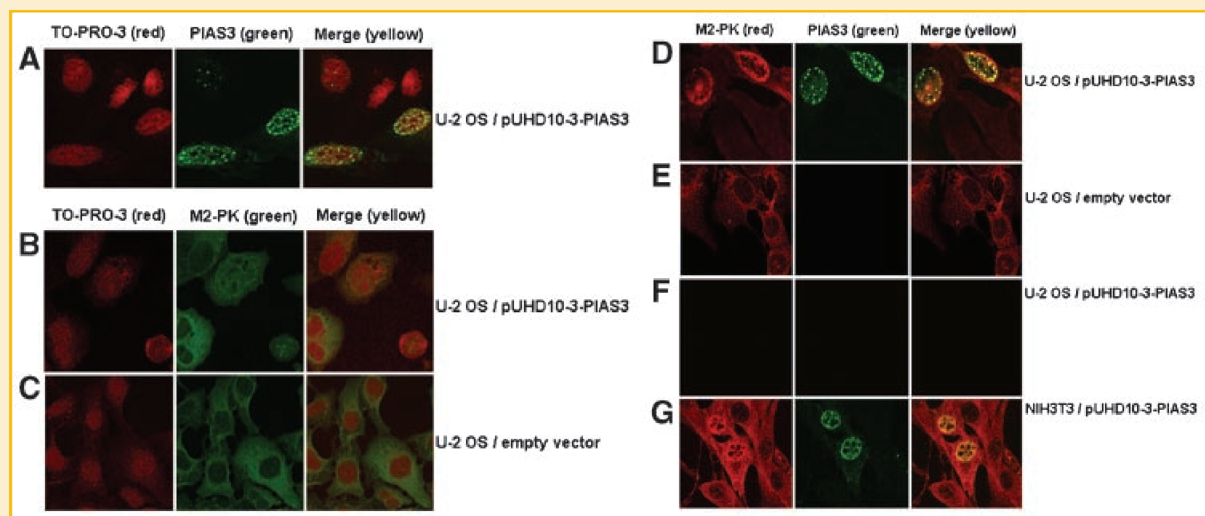

Fig. 5. Subcellular localization of PIAS3 an M2-PK. U-2 OS or NIH3T3 cells were transiently transfected with a CMV-promoter driven expression vector for PIAS3 or the empty vector as indicated. Thirty hours later, cells were fixed/permeabilized with 4% PFA/0.2% Triton X-100 and stained with anti-PIAS3 antibodies, the DNA-stain TO-PRO-3 or anti-M2-PK antibodies as indicated and processed for indirect immunofluorescence microscopy and viewed by using a confocal scanning system. A: Staining of PIAS3-overexpressing U-2 OS cells with TO-PRO-3 (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge (right panel, yellow). B: Staining of PIAS3-overexpressing U-2 OS cells with TO-PRO-3 (left panel, red) and anti-M2-PK antibodies (middle panel, green), Merge (right panel, yellow). C: Staining of U-2 OS/empty vector cells with TO-PRO-3 (left panel, red) and anti-M2-PK antibodies (middle panel, green), Merge (right panel, yellow). D: Staining of PIAS3-overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge (right panel, yellow). E: Staining of U-2 OS/empty vector cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge (right panel, yellow). F: Incubation of PIAS3-overexpressing U-2 OS cells only with the secondary anti-rabbit and anti-goat IgGs. G: Staining of PIAS3-overexpressing NIH3T3 cells with anti-M2-PK antibodies (left panel, red) and anti-PIAS3 antibodies (middle panel, green), Merge (right panel, yellow).

(wild-type) (Fig. 6B) but not in the Flag-mPIAS3 (C299S, H301A) overexpressing cells (Fig. 6C). For this reason, we conclude that the intact RING domain in PIAS3 is important for nuclear localization of M2-PK.

DISCUSSION

In this study we have identified the SUMO-E3 ligase PIAS3 [Chung et al., 1997] as interaction partner of the glycolytic isoenzymes M2-PK and M1-PK in a two-hybrid screen. The interaction between M2-PK and PIAS3 was further corroborated in GST-pull down assays and by co-immunoprecipitation of PIAS3 and M2-PK from extracts of human U-2 OS cells. Moreover, we found that a substantial fraction of endogenous M2-PK is covalently conjugated to SUMO-1 and we present data that M2-PK co-localizes with PIAS3 in nuclear structures in cells transiently overexpressing PIAS3. These data suggest a functional link between PIAS3 and M2-PK.

M2-PK AS SUMOYLATION SUBSTRATE

Since SUMO-1 has an apparent molecular mass of approximately 20 kDa [Matunis et al., 1996] and the monosumoylation of RanGAP1 increases its apparent MW by roughly 25 kDa [Matunis et al., 1996], our identification of an additional M2-PK band with an apparent MW of 80 kDa in Figure 4, which is also recognized by SUMO-1 antibodies, is consistent with the addition of one SUMO-1 per M2-PK. Using the SUMO plot program (www.abgent.com/doc/sumo-plot), we identified eight potential sumoylation motifs (YK*X*D/E; Y bulky amino acid, K attachment site lysine, X any amino acid) in M2-PK (Supplementary Fig. 1). Moreover, analysis of the X-ray structure of human M2-PK [Dombrauckas et al., 2005] showed that all eight lysine residues are exposed on the surface of the enzyme. This underlines our finding that M2-PK is sumoylated. The identification of the sumoylated lysine residue(s) in M2-PK requires further study.

The identification of human pyruvate kinase as target for SUMO-1 conjugation in this study is reminiscent of similar findings in yeast. Thus, the *S. cerevisiae* homolog of pyruvate kinase Pyk1/Cdc19 has been identified as a SUMO substrate in mass spectrometry-based proteome-wide approaches [Panse et al., 2004; Wohlschlegel et al., 2004; Zhou et al., 2004; Denison et al., 2005; Hannich et al., 2005], suggesting that sumoylation of pyruvate kinase, as described here, represents an evolutionarily conserved process.

The two human M-type pyruvate kinase isoenzymes consist of 531 amino acid residues and differ only in 22 amino acid residues in a 45 residues-long domain encoded by the respective exons [Dombrauckas et al., 2005]. The type-specific domains are important for the interaction of the M1 and M2 pyruvate kinase subunits, respectively, and although structurally very similar [Wooll et al., 2001; Dombrauckas et al., 2005], the non-allosteric M1 isoenzyme exists only as tetramer, while M2-PK is an allosteric enzyme that can exist in different conformations. All hypothetical sumoylation motifs are localized in domains common to M1- and M2-PK (Supplementary Fig. 1), suggesting that M1-PK may also be subject

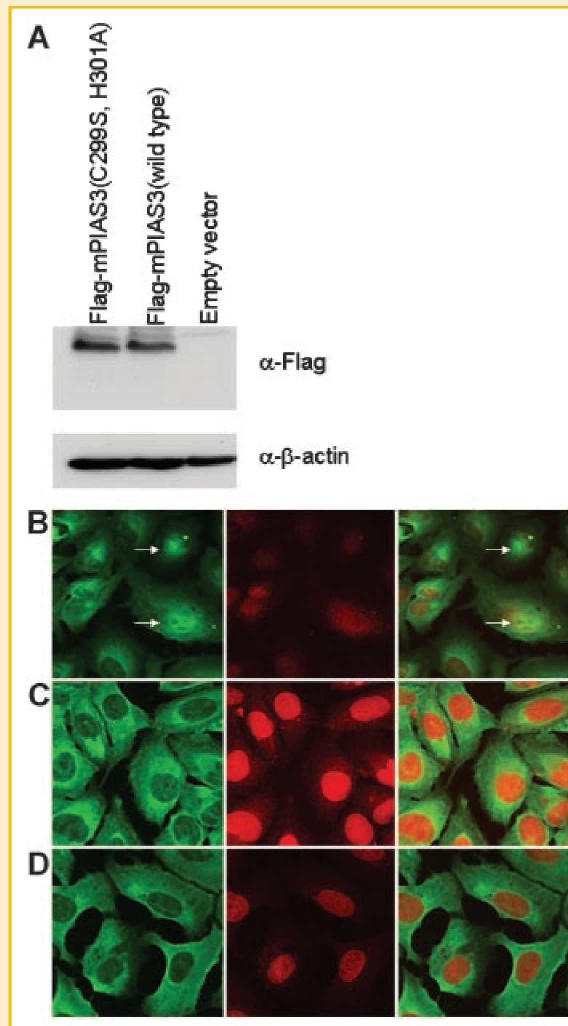

Fig. 6. Subcellular localization of endogenous M2-PK in cells overexpressing PIAS3 wild-type or the catalytically inactive RING domain mutant PIAS3 (C299S, H301A). U-2 OS cells were transiently transfected with CMV-promoter driven expression vectors for Flag-mPIAS3 wild-type, Flag-mPIAS3 (C299S, H301A) or the empty vector as indicated. Twenty-four hours later, cells were fixed/permeabilized with 4% PFA/0.2% Triton X-100 and stained with anti-M2-PK antibodies and the DNA-stain TO-PRO-3 as indicated and processed for indirect immunofluorescence microscopy and viewed by using a confocal scanning system. A: Expression of the Flag-mPIAS3 wild-type, Flag-mPIAS3 (C299S, H301A) proteins in U-2 OS cells as shown by immunoblot using anti-Flag antibodies, β-actin served as input control. B: Staining of Flag-mPIAS3-overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red), Merge (right panel, yellow). Cells with nuclear M2-PK are marked by arrows. C: Staining of mFlag-PIAS3 (C299S, H301A) mutant-overexpressing U-2 OS cells with anti-M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red), Merge (right panel, yellow). D: Staining of empty vector containing U-2 OS cells with anti-M2-PK antibodies (left panel, green), TO-PRO-3 (middle panel, red), Merge (right panel, yellow).

to sumoylation. This is in keeping with our finding that PIAS3 specifically binds to both isoenzymes, M2-PK and M1-PK in a yeast two-hybrid assay (Fig. 1), and further underlined by our finding that the amino-terminal domain, which is common to both M-type isoenzymes, is sufficient for the PIAS3/pyruvate kinase interaction

(Fig. 2). While these findings suggest that PIAS3 interacts with both M1-PK and M2-PK subunits, one cannot rule out that the quaternary structure of pyruvate kinase influences the strength of the M2-PK/PIAS3 interaction. It is conceivable that also the other two pyruvate kinase isoenzymes, L-PK and R-PK, can exist as sumoylated proteins in cells expressing these PK isoforms. This question needs, however, further studies.

M2-PK AS PIAS3 TARGET PROTEIN

We found that overexpression of PIAS3 induces a moderate increase in the sumoylation of M2-PK. This is in line with the finding that only a fraction of the endogenous M2-PK was detected in a complex with PIAS3 in the co-immunoprecipitation experiments and modified by SUMO. These results suggest that PIAS3 enhances sumoylation of M2-PK, but that the M2-PK/PIAS3 interaction occurs between a rather small fraction of the two proteins. However, the SUMO-E2 enzyme Ubc9 can directly bind the consensus sequence YK*X*D/E in a given substrate and Ubc9 together with the SUMO-E1 enzyme are sufficient to efficiently sumoylate substrates in vitro in the absence of an E3 [Johnson, 2004; Reverter and Lima, 2005], indicating that E3 enzymes can enhance sumoylation without being indispensable. Moreover, there are examples in which the effect of PIAS proteins on substrate function is independent of their SUMO ligase activity (their RING finger), suggesting that the role of PIAS proteins extends beyond modifying the outputs of the SUMO pathway [Johnson, 2004; Palvimo, 2007]. Thus, the interaction between PIAS3 and M2-PK, as shown here, could reflect either an enzyme–substrate complex with a short half-life or another type of regulatory interaction. In fact, only a few proteins are quantitatively sumoylated. Instead, most targets appear to be modified to a small percentage at steady state (usually less than 5%) [Geiss-Friedlander and Melchior, 2007]. It is important to consider that targets can undergo rapid cycles of modification and demodification. Although the equilibrium might lie on the side of the unmodified form, the whole pool of a given protein might be affected by sumoylation in a short window of time and there is precedence that low-level sumoylation can cause large effects [Geiss-Friedlander and Melchior, 2007]. When endogenous PIAS3 was partially depleted by siRNA-mediated gene knockdown, no discernable differences in the degree of M2-PK were observed (data not shown). When the catalytically inactive mouse PIAS3 RING domain mutant PIAS3 (C299S, H301A) [Long et al., 2004] was expressed in U-2 OS cells instead of wild-type PIAS3, no nuclear localization of M2-PK was observed (Fig. 6). Thus, while the role of PIAS3 for sumoylation of M2-PK remains to be defined, our data suggest that PIAS3 specifically induces nuclear localization of M2-PK and this effect depends on the RING domain of PIAS3.

The interaction of M2-PK with PIAS3 involves the intact carboxyl-terminal region of PIAS3 from amino acid residues 383–619. Although the approximately 400 residues-long conserved amino-terminal region of PIAS3 is well characterized—it contains the RING finger-like zinc-binding domain mediating the SUMO-E3 ligase activity of PIAS proteins [Kotaja et al., 2002] and binds directly to Ubc9 [Kahyo et al., 2001]—the exact functions of the less conserved carboxyl-terminal region remain to be defined. This region contains an acidic motif, a serine/threonine rich region, and is involved in the interaction with specific substrate proteins [Shuai and Liu, 2005], as has been shown for some transcriptional regulators [Jimenez-Lara et al., 2001; Nakagawa and Yokosawa, 2002; Tirard et al., 2004]. Although PIAS proteins are localized predominantly in the nucleus and transcription is a major process regulated by these SUMO-E3 ligases, additional processes are regulated by sumoylation and PIAS proteins have been shown to interact with various cellular proteins, for instance enzymes involved in DNA-replication or proteins of the cytoplasmic septin family of cytoskeletal proteins [Johnson, 2004]. Whereas SUMO conjugation is important for the targeting of glucose transporters (GLUT4 and GLUT1) from intracellular sites to the cytoplasmic membrane [Giorgino et al., 2000; Lalioti et al., 2002], the direct interaction of a SUMO-E3 ligase with a glycolytic enzyme, along with its subcellular relocalization, has not previously been shown.

In this study it is shown, that overexpression of PIAS3 leads to nuclear localization of M2-PK and the co-localization of this enzyme with PIAS3 in the nucleus of different cell types.

Little is known about M2-PK beside its important metabolic function as glycolytic enzyme in the cytosol [reviewed in Mazurek et al., 2005]. A monomeric form of M2-PK with low enzymatic activity was previously described as a cytosolic thyroid hormone-binding protein [Kato et al., 1989], and a subsequent study suggested that monomeric M2-PK has a role in the regulation of thyroid hormone receptor-dependent transcription [Ashizawa and Cheng, 1992]. Previous work also provided evidence for DNA-binding and histone H1 kinase activity of M2-PK [Guminska et al., 1988], and recent work demonstrated that M2-PK can exist as a nuclear protein. Thus, interleukin-3 induces nuclear translocation of M2-PK and this enhances cell proliferation [Hoshino et al., 2007]. Another study showed that M2-PK translocates into the nucleus after apoptotic stimuli and contributes to the induction of programmed cell death [Steták et al., 2007]. Furthermore, a role in transcriptional regulation was described for nuclear M2-PK [Lee et al., 2008]. The present study underlines that M2-PK exists as nuclear protein and suggests a link between the SUMO system and M2-PK.

Nuclear localization and/or functions have been previously shown for other glycolytic enzymes (hexokinase, aldolase, glyceraldehyde 3-P dehydrogenase (GAPDH), phoshoglycerate kinase and enolase) [reviewed in Sirover, 2005]. In yeast, hexokinase 2 acts as a nuclear factor in glucose induced transcriptional repression [Rodriguez et al., 2001]. A nuclear alternative splicing form of enolase 1 has been demonstrated to be involved in transcriptional repression of the c-Myc gene in HeLa cells [Ghosh et al., 1999]. It was shown that GAPDH can modulate the activity of DNA–polymerase-α-primase complex [Grosse et al., 1986], and nuclear GAPDH plays a role in control of apoptotic cell death in several human cell types [Sawa et al., 1997].

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