The Interaction of Piasy with Trim32, an E3-Ubiquitin Ligase Mutated in Limb-girdle Muscular Dystrophy Type 2H, Promotes Piasy Degradation and Regulates UVB-induced Keratinocyte Apoptosis through NFκB

Received for publication, February 22, 2006, and in revised form, June 19, 2006 Published, JBC Papers in Press, June 30, 2006, DOI 10.1074/jbc.M601655200

Amador Albor†1, Sally El-Hizawi†, Elizabeth J. Horn1,2, Melanie Laederich3, Patrick Frosk3, Klaus Wrogemann3, and Molly Kulesz-Martin4,5

From the 4Department of Dermatology and 5Program in Cell and Molecular Biology, Oregon Health and Science University, Portland, Oregon 97239 and the 6Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, Manitoba R3E 0W3, Canada

Protein inhibitors of activated STATs (PIAS) family members are ubiquitin-protein isopeptide ligase-small ubiquitin-like modifier ligases for diverse transcription factors. However, the regulation of PIAS protein activity in cells is poorly understood. Previously, we reported that expression of Trim32, a RING domain ubiquitin-protein isopeptide ligase-ubiquitin ligase mutated in human limb-girdle muscular dystrophy type 2H (LGMD2H) and Bardet-Biedl syndrome, is elevated during the TRIM family may be located in the cytoplasm or nucleus and are thought to form large multimeric complexes, forming subcellular structures resembling spheres, speckles, or ribbons. Different TRIM family members are characterized by a specific carboxyl-terminal domain. In Trim32, the carboxyl terminus contains six repeats of the NHL (NCL-1, HT2A and LIN-41) motif, thought to mediate protein/protein interactions (3). TRIM family members have been implicated in oncogenic translocations, such as PML and RFP (4–6), carcinogenesis, such as estrogen-responsive finger protein and Trim32 (7, 8), and retroviral species-specific restriction, such as TRIM5α (9).}

Protein ubiquitination is a fundamental process in eukaryotic cells, controlling the degradation of proteins through the 26 S proteasome. E3 ubiquitin ligases catalyze the last step of the process and provide substrate specificity. The activity of many cellular factors is controlled by their abundance and stability in the cell, properties that depend on the rate of ubiquitination. By providing substrate specificity, E3 ligases become a point of control for ubiquitination and stability of many cellular factors. A variety of otherwise structurally unrelated E3 ligases contain a common RING domain that provides interaction with the E2 ubiquitin-conjugating enzyme (1). In Trim32, the RING domain is present in the amino terminus of the protein, as part of the RBCC (RING/B-Box/Coiled-coil) or tripartite motif that defines the TRIM family of proteins (2). Members of the TRIM family may be located in the cytoplasm or nucleus and are thought to form large multimeric complexes, forming subcellular structures resembling spheres, speckles, or ribbons. Different TRIM family members are characterized by a specific carboxyl-terminal domain. In Trim32, the carboxyl terminus contains six repeats of the NHL (NCL-1, HT2A and LIN-41) motif, thought to mediate protein/protein interactions (3). TRIM family members have been implicated in oncogenic translocations, such as PML and RFP (4–6), carcinogenesis, such as estrogen-responsive finger protein and Trim32 (7, 8), and retroviral species-specific restriction, such as TRIM5α (9).

Several TRIM family members, including Trim32, are also mutated in human hereditary conditions (10). Within the fourth NHL repeat of human Trim32, a mutation of an evolutionarily conserved aspartic acid to asparagine is linked to the development of limb-girdle muscular dystrophy type 2H (LGMD2H), an autosomal recessive myopathy also described as sarcotubular myopathy (11, 12). It has been reported that Trim32 has E3-ubiquitin ligase activity for actin in vitro (13).

*This work was supported by National Institutes of Health Grant CA098577 and Oregon Health and Science University Cancer Institute Core Grant CA69533. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.
1To whom correspondence may be addressed: Dept. of Dermatology, Mail Code OP06, Oregon Health and Science University, 3181 SW Sam Jackson Park Rd., Portland, OR 97239. Tel.: 503-418-4271; Fax: 503-418-4206; E-mail: albor@ohsu.edu.
2Present address: National Psoriasis Foundation, Portland, OR.
3To whom correspondence may be addressed. Tel.: 503-494-9933; Fax: 503-418-4266; E-mail: kuleszma@ohsu.edu.

4The abbreviations used are: E3, ubiquitin-protein isopeptide ligase; PIAS, protein inhibitors of activated STATs; SUMO, small ubiquitin-like modifier; LGMD2H, limb-girdle muscular dystrophy type 2H; BBS, Bardet-Biedl syndrome; TNFα, tumor-necrosis factor-α; GFP, green fluorescent protein; DAPI, 4,6-diamido-2-phenylindole; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; NLS, nuclear localization signal; NES, nuclear export signal; SAP, SAF-A/ASF-Acinus-PIAS; GST, glutathione S-transferase; CML, chronic myelogenous leukemia; PML, promyelocytic leukemia; RFP, RET finger protein; ATPγS, adenosine 5′-O-(thiotriphosphate).
However, no difference was found between wild type and LGMD2H-mutated Trim32 in terms of intrinsic E3-ubiquitin ligase activity or actin and myosin binding. Thus the molecular defect in LGMD2H mutated Trim32 remains unknown (13). Recently, a missense mutation in the coiled-coil domain of Trim32 has been linked to Bardet-Biedl syndrome (14), although it is not known how this mutation alters the function of Trim32. We have previously reported that mouse Trim32 expression is elevated during carcinogenic progression in a mouse skin carcinogenesis model, as well as in mouse skin tumors induced in vivo by UVB irradiation of SKH1 mice (8). We also demonstrated that Trim32 expression protects keratinocytes from apoptosis induced by combined UVB and TNFα treatment, both in vitro and in vivo, and that Trim32 has the hallmarks of an E3-ubiquitin ligase, including self-ubiquitination and interaction with ubiquitinated proteins in response to combined UVB/TNFα treatment (8).

The SUMOylation process is closely related to ubiquitination, resulting in covalent attachment of ubiquitin-like SUMO molecules to mostly nuclear proteins, such as transcription factors and other proteins involved in DNA repair and chromatin structure (15–17). Together with RanB2 and Pc2, the four members of the PIAS family are the only known mammalian E3-SUMO ligases. PIAS family members are characterized by the presence of an amino-terminal SAP domain, thought to target proteins to regions of actively transcribed chromatin (18, 19), and a SP-RING domain, a RING-like motif with a characteristic spacing of cysteine residues required for SUMO-E3 ligase activity that interacts directly with Ubc9 (20). Originally described as STAT-interacting proteins, PIAS family members are involved in the regulation of several transcription factors, including, in addition to STATs, NF-κB, SMADs, p53, p73, and the androgen receptor (21). In the developing mouse, Piasy (originally described as Piasy in mice (22)) is expressed in the central nervous system, epidermis (including whisker follicles and hair follicle placodes), muscle, and limb buds (22, 23). Human Piasy is down-regulated during the progression of human chronic myelogenous leukemia (CML), and reexpression of Piasy into CML cells induces apoptosis (24). Despite the large number of SUMOylation substrates, the role of SUMO modification in the control of transcription, and their involvement in tumorigenesis, little is known about the regulatory mechanisms of PIAS proteins and, in particular, Piasy.

The role of Trim32 in the progression of skin carcinogenesis, its anti-apoptotic activity, and its mutation in human disease (8, 11, 14) led us to investigate the biochemical function of Trim32 and to search for Trim32-binding proteins as potential substrates of Trim32 E3-ubiquitin ligase activity. We found that Trim32 binds to PIAS proteins and that the NHL mutation in Trim32 associated with LGMD2H abolished binding to PIAS proteins. Because of the expression of Piasy in the skin and the role of Piasy in tumorigenesis (24, 25), we further analyzed the binding of Trim32 to Piasy and the functional consequences of their interaction. We report that Trim32 interacts with and promotes the ubiquitination and degradation of Piasy. Treatment with the proteasomal inhibitor MG132 promoted the interaction and the subcellular colocalization of both proteins, in a process involving the redistribution of Piasy from the nucleus to the cytoplasm. Trim32 and Piasy have opposite effects on the transcriptional activity of NFκB, with Trim32 inducing and Piasy inhibiting NFκB. Furthermore, Piasy expression sensitized keratinocytes to apoptosis induced by TNFα and UVB treatment. Our results demonstrate a novel mechanism for regulation of PIAS proteins and implicate the interaction between Trim32 and Piasy in the modulation of keratinocyte apoptosis in response to cellular stress.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—Plasmid pGB-Trim32 was generated by cloning the mouse Trim32 cDNA into the EcoRI/BamHI sites of pGBKTK7 (BD Biosciences) for expression of Trim32 as a fusion protein to the GAL4 DNA binding domain. Plasmids pAC-Pi-asy, pAC-Piasxβ, and pAC-Pias1 express the cDNAs for the respective PIAS proteins as fusions to the GAL4 transactivation domain from vector pACT2 (BD Biosciences). A Myc-tagged ubiquitin protein was expressed from plasmid pEBB-myc-ubi. Plasmid pAC-Piasy was obtained from the yeast two-hybrid assay, whereas pAC-Piasxβ, pAC-Pias1, and pEBB-myc-ubi were provided by Dr. David Ransom (Oregon Health and Science University, Portland). GFP fusion proteins used in this study were expressed from plasmid pEGFP-C1 (BD Biosciences). FLAG fusion proteins were expressed from plasmid pCMVTag2 (Stratagene). Piasy protein with a His6 tag was expressed in mammalian cells from plasmid pCDNA6/His (Invitrogen) and in *Escherichia coli* (DH5α) from plasmid pROEX-HT (Invitrogen). A GST fusion protein to Trim32 was expressed in *E. coli* strain BL21(DE3)pLysS (Stratagene) from plasmid pGEX-2T (Amersham Biosciences). Plasmids pNF-κB-Luc and pRL-TK, used for the luciferase assays, were purchased from Stratagene and Promega, respectively.

The ΔRING and ΔNHL deletion mutants of Trim32 were obtained by PCR amplification, using primers to remove the first 102 or the last 210 amino acids of the mouse 655-amino acid-long Trim32 sequence, containing the RING and NHL domains, respectively. The ΔCC deletion mutant of Trim32 was generated by digestion of the mouse Trim32 cDNA with BbvCI, resulting in the removal of amino acids 143–317, containing the coiled-coil domain. The C21S and the LGMD2H mutants were obtained by site-directed mutagenesis of the mouse Trim32 cDNA, resulting in C21S and D489N missense mutations, respectively. These mutant Trim32 forms were expressed from the pEGFP-C1 and pACT2 plasmids.

Digestion of pAC-Piasy with EcoRI and BamHI yielded a fragment coding for amino acids 5–82 of the mouse Piasy sequence. This sequence was cloned into pGBKT7 and used to transfect AH109 cells carrying pGBKT7 and used to transfect AH109 cells carrying pGBKT7 and used to transfect AH109 cells carrying pGBKT7 (BD Biosciences) for expression of Trim32 as a fusion protein to the GAL4 DNA binding domain. Plasmids pAC-Pi-asy, pAC-Piasxβ, and pAC-Pias1 express the cDNAs for the respective PIAS proteins as fusions to the GAL4 transactivation domain from vector pACT2 (BD Biosciences). A Myc-tagged ubiquitin protein was expressed from plasmid pEBB-myc-ubi. Plasmid pAC-Piasy was obtained from the yeast two-hybrid assay, whereas pAC-Piasxβ, pAC-Pias1, and pEBB-myc-ubi were provided by Dr. David Ransom (Oregon Health and Science University, Portland). GFP fusion proteins used in this study were expressed from plasmid pEGFP-C1 (BD Biosciences). FLAG fusion proteins were expressed from plasmid pCMVTag2 (Stratagene). Piasy protein with a His6 tag was expressed in mammalian cells from plasmid pCDNA6/His (Invitrogen) and in *Escherichia coli* (DH5α) from plasmid pROEX-HT (Invitrogen). A GST fusion protein to Trim32 was expressed in *E. coli* strain BL21(DE3)pLysS (Stratagene) from plasmid pGEX-2T (Amersham Biosciences). Plasmids pNF-κB-Luc and pRL-TK, used for the luciferase assays, were purchased from Stratagene and Promega, respectively.

The ΔRING and ΔNHL deletion mutants of Trim32 were obtained by PCR amplification, using primers to remove the first 102 or the last 210 amino acids of the mouse 655-amino acid-long Trim32 sequence, containing the RING and NHL domains, respectively. The ΔCC deletion mutant of Trim32 was generated by digestion of the mouse Trim32 cDNA with BbvCI, resulting in the removal of amino acids 143–317, containing the coiled-coil domain. The C21S and the LGMD2H mutants were obtained by site-directed mutagenesis of the mouse Trim32 cDNA, resulting in C21S and D489N missense mutations, respectively. These mutant Trim32 forms were expressed from the pEGFP-C1 and pACT2 plasmids.

Yeast Two-hybrid Screening and Binding Assays—For yeast two-hybrid library screening, the full-length coding sequence of the Trim32 gene was cloned into pGBKTK7 and used to transform *MATa Saccharomyces cerevisiae* strain AH109. Cells of the *MATα S. cerevisiae* strain Y187 carrying inserts of a cDNA library from mouse testis (BD Biosciences) were mated to AH109 cells carrying pGBKTK7-Trim32. Two-hybrid colonies were screened under high stringency conditions, on quadruple
Trim32 Regulation of Piasy and Apoptosis Modulation

(--adenine/–histidine/–leucine/–tryptophan) synthetic SD minimal medium plates.

For analysis of protein interaction domains using the yeast two-hybrid assay, wild type and mutant Trim32 proteins were expressed from vector pGBKT7, and PIAS family members were expressed from vector pACT2. The human p53 protein, used as a positive control for PIAS proteins binding, was expressed from plasmid pGBK7. Plasmids were cotransformed into AH109 S. cerevisiae competent yeast cells (BD Biosciences), and grown on quadruple (--adenine/–histidine/–leucine/–tryptophan) synthetic SD minimal medium plates.

Cell Culture and Treatments—The mouse keratinocyte strain 291 was derived from neonatal mouse skin and displays normal regulation of proliferation and differentiation by extracellular Ca2+, characteristic of primary epidermal cultures (26). These cells were cultured on Eagle’s minimal essential medium without Ca2+ supplemented with 5% (v/v) Ca2+-chelaxed fetal calf serum, 10% (v/v) mouse dermal fibroblast conditioned media, 10 ng/ml epidermal growth factor (Upstate Biotechnology, Inc.), 1% (v/v) antibiotic/antimycotic, and 0.04 mM CaCl2 (8). 291-GFP and 291-Trim32 mouse keratinocyte cell strains were derived from 291 cells by stable retroviral transduction of 291 cells with vectors for expression of GFP and Trim32, respectively (8). 291-Trim32 cells stably express Trim32 at levels naturally expressed in initiated and transformed keratinocytes, i.e., ~3-fold the expression in 291 cells. Human dermal fibroblasts were cultured in α-minimum Eagle’s medium supplemented with ribonucleotides, ribonucleosides, and 20% (v/v) fetal calf serum. Cell transfections were performed overnight using the Lipofectamine (Invitrogen) reagent. UVB light irradiation (230 J/m2) was performed using two Westinghouse FS20T12 sun lamps (maximum emission at 310 nm). Reticulocyte mouse TNFα (Calbiochem) was dissolved in cell culture medium, and cycloheximide (Sigma) and MG132 (Peptides International) were dissolved in Me2SO.

Fluorescent Immunocytochemistry—Mouse keratinocytes (291) or human dermal fibroblasts were grown on microscopy cover glasses and transfected with plasmids for expression of GFP or FLAG fusions to Trim32 or Piasy. After treatment, cells were washed with phosphate-buffered saline and were fixed in 2% paraformaldehyde for 15 min at 4 °C. Cells were then permeabilized with PAB-T (phosphate-buffered saline, 0.5% bovine serum albumin, 0.1% sodium azide, and 0.05% Tween 20). Samples were incubated at 4 °C overnight, with a dilution of the primary antibodies in PAB-T. This was followed by incubation with a dilution of secondary antibodies (Alexa 588-conjugated anti-mouse or Texas Red-conjugated anti-rabbit antibody; Molecular Probes) in PAB-T. Samples were mounted on microscopy slides using FluorSave reagent (Calbiochem). Wide field microscopy images were obtained with a Leica DMI20 inverted fluorescence microscope. Confocal microscopy images were obtained with a Nikon TE200 inverted fluorescent microscope on an optical sectioning arrangement (DeltaVision). Confocal images were processed using DeltaVision deconvolution software.

Antibodies and Immunoprecipitation—Mouse monoclonal antibody B2 and rabbit polyclonal antibody (sc-8334), both specific for GFP, and anti-His6 mouse monoclonal antibody (H-3) covalently linked to horseradish peroxidase were purchased from Santa Cruz Biotechnology. Anti-FLAG epitope rabbit polyclonal antibody (E-7425), and anti-Myc epitope monoclonal antibody (9E10) were purchased from Sigma. Rabbit polyclonal antibody 608 is specific for Trim32 and has been described previously (8). Chicken anti-Trim32 antibodies were raised against a Trim32ΔNHL protein expressed in bacteria (Aves Laboratories). Both rabbit and chicken Trim32-specific antibodies were affinity-purified using immobilized recombinant Trim32ΔNHL. Anti-Hsc70 mouse monoclonal antibody (SPA-815) was purchased from StressGen. Rabbit polyclonal antibodies specific for human Piasy were purchased from Imgenex (IMG-290) and Abgent (Piasy1 N-term). A mouse polyclonal antibody specific for human Trim32 was raised against a human Trim32 polypeptide.5

For immunoprecipitation experiments, cells were lysed in buffer containing 50 mM Hepes, pH 7.8, 150 mM NaCl, 0.1% Triton X-100, and 20% glycerol and a mixture of protease inhibitors (phenylmethylsulfonyl fluoride, benzamidine, leupeptin, and pepstatin A). Protein concentration in the lysates was determined by the Bradford assay. For each experiment, equal amounts of total crude lysate protein were diluted to 1 μg of protein/μl in lysis buffer. The primary antibodies were then added to reach a final concentration of 1 ng/μl, and samples were incubated overnight at 4 °C. Antigen-antibody protein complexes were collected with protein A-Sepharose beads (Amersham Biosciences), recovered in denaturing protein electrophoresis sample buffer, and separated by SDS-PAGE. Proteins were transferred to nitrocellulose membranes and detected by immunoblotting with specific antibodies.

In Vitro Ubiquitination Assays—Purified recombinant Muc-ubiquitin, ubiquitin-activating enzyme (E1), and ubiquitin-conjugating enzymes (UbcH5a and UbcH6) were purchased from Boston Biochemical. GST-Trim32 was expressed in E. coli BL21(DE3)pLyS strain (Stratagene), and isolated from cells lysed in 10 mM Tris/HCl, pH 8.0, 10% glycerol, 1% Triton X-100, 100 mM NaCl, 10 mM MgCl2, 10 μM ZnCl2, and a mixture of protease inhibitors as above, using glutathione-Sepharose 4B columns (Amersham Biosciences) following the manufacturer’s recommendations. His6c-tagged Piasy was expressed in E. coli DH5α cells and isolated through nickel nitrilotriacetic acid-agarose columns (Invitrogen) following the manufacturer’s recommendations. In vitro ubiquitination reactions were carried out in a buffer containing 50 mM Tris/HCl, pH 8.0, 5 mM MgCl2, and 0.5 mM dithiothreitol. Where indicated, the reactions contained the following components: 3 mM Muc-ubiquitin, 160 nM E1, 300 nM UbcH5a or UbcH6, 50 nM GST-Trim32, and 200 nM His6c-Piasy. Reactions were started by addition of 5 mM ATP and 1.5 mM ATPγS and incubated at 37 °C for 120 min. Reactions were stopped by addition of Laemmli protein electrophoresis buffer, and products were separated by SDS-PAGE and detected by immunoblotting with 608 (for detection of Trim32), 9E10 (for detection of Myc-tagged ubiquitin), and Piasy1-N-Term (specific for human Piasy, cross-reacts with mouse Piasy when enough mouse protein is present).

5 K. Wrogemann, unpublished data.
Measurement of NFκB Transcriptional Activity by Luciferase/Renilla Assays—Cells were plated in 12-well dishes, grown to 40% confluence, and cotransfected with plasmids pNF-κB-Luc, pRL-TK, and plasmids for expression of Piasy proteins tagged to His₆, FLAG, or GFP sequences. Empty protein expression plasmids were included to control for total plasmid amount and transcription factor squelching. Following treatments, cell extraction and simultaneous determination of luciferase and Renilla activities were carried out using the dual luciferase reporter assay system (Promega), following the manufacturer’s recommendations.

Determination of Apoptosis—For determination of keratinocyte apoptosis, cells were transfected with GFP or GFP-Piasy expression plasmids, and 24 h later were treated with UVB, TNFα, or both. Mitotracker and Hoechst 33342 (both from Molecular Bioprobes) were added to the cultures 20 h after treatment, at a final concentration of 5 μg/ml and 10 nM, respectively. Cells were incubated for 1 h and then observed by fluorescence microscopy using a Leica DMIRE2 inverted fluorescence microscope. Cells were scored for GFP or GFP-Piasy expression and apoptotic status by combining the phase contrast, blue, red, and green fluorescence channels as described in the text.

RESULTS

Trim32 Interacts with Members of the PIAS Family—We performed a yeast two-hybrid assay using full-length Trim32 as bait to screen a mouse cDNA library from testis, where Trim32 is expressed at high levels (8). The screen isolated an almost complete cDNA sequence for the mouse Piasy gene (22), lacking only the first four amino acids on the amino terminus. Comparison of the mouse genomic region encompassing the Piasy gene with the human genome (27) revealed that Piasy is the murine ortholog of human Pias. Because of the high sequence similarity between PIAS family members, we tested binding of Trim32 to Pias1 and Piasxβ, which are also expressed in testis (28). Trim32 demonstrated binding to the three PIAS family members (Fig. 1A). The interaction between the two proteins in yeast was specific, and each protein in isolation could not support growth. Trim32 mutational analysis of binding revealed a complex interaction among the protein domains, as has been shown for other TRIM family proteins such as PML/Trim19 (29). Complete removal of the RING domain resulted in an enhancement of interaction (Fig. 1B); however, mutation of the conserved Cys²¹ residue in the RING motif to Ser, which would unfold its secondary structure (1), completely prevented binding. This suggests that removal of the RING domain stabilizes binding to Piasy, perhaps because the RING domain promotes ubiquitin-mediated degradation of Piasy in yeast, whereas the C21S mutant, which has an unfolded secondary structure, may prevent binding by steric interference. Removal of the coiled-coil domain also prevented binding. Because TRIM proteins (including Trim32) can oligomerize through their coiled-coil domains (2), this suggests that oligomerization of Trim32 is necessary for binding to Piasy. Interestingly, reproduction of the D487N mutation found in LGMD2H (D489N in mouse) also abolished binding to Piasy, as did complete deletion of the NHL domain. As a positive control, all PIAS proteins tested bound to p53, as expected because p53 activity is regulated by the three PIAS proteins (31–33).

We next proceeded to determine whether Trim32 and PIAS proteins could interact in mouse keratinocytes. Of the three PIAS proteins binding to Trim32 detected by yeast two-hybrid assays, only Piasy has been reported to be expressed in skin keratinocytes (22, 23). Furthermore, because elevated Trim32 activity can initiate skin carcinogenesis and protect keratinocytes from UVB/TNFα-induced apoptosis (8), we focused our analysis on Piasy because of its involvement in tumorigenesis and apoptosis induction (24, 25). Because we hypothesized that Piasy was a substrate for Trim32 E3-ubiquitin ligase activity involved in the control of UVB/TNFα-induced apoptosis, we observed the subcellular localization of both proteins in cells untreated or treated with the proteasome inhibitor MG132 and UVB/TNFα. To determine the interaction between the two proteins, we immunoprecipitated endogenous Trim32 protein from cell lysates of mouse keratinocytes growing under proliferative, nondifferentiating conditions and expressing either His₆-Piasy or GFP-Piasy (Fig. 1C). To control for nonspecific immunoprecipitation, we also determined the immunoprecipitation of the constitutively expressed chaperone protein Hsc70, which we have found to be abundantly expressed in mouse keratinocytes. For these experiments, Trim32 was immunoprecipitated using affinity-purified Trim32-specific chicken polyclonal antibody 74 (supplemental Fig. 1). As shown in Fig. 1C, Piasy, irrespective of the protein tag, specifically immunoprecipitated with endogenous Trim32, whereas Hsc70 was completely undetectable in the immunoprecipitates. Furthermore, their coimmunoprecipitation was strongly induced by treatment with MG132/UVB/TNFα, suggesting that Piasy is a substrate for Trim32 E3-ubiquitin ligase during the keratinocyte UVB/TNFα response. As expected, incubation of the MG132/UVB/TNFα-treated lysates with the corresponding chicken preimmune IgY (Fig. 1C, IP-Pre-I) failed to immunoprecipitate Piasy.

Nucleocytoplasmic Redistribution of Piasy and Interaction with Trim32 in the Cytoplasm—We and others have reported that Trim32 is localized in cytoplasmic speckles (2, 8), whereas Piasy and PIAS family members in general are considered nuclear proteins (34, 35). Thus, it was important to determine the subcellular localization of the interaction of Trim32 and Piasy. To this end, and to differentiate between the effects of MG132 and UVB/TNFα treatment on Trim32/Piasy interaction, cultured epidermal mouse keratinocytes expressing GFP-tagged Trim32 and FLAG-tagged Piasy were observed by wide field fluorescence microscopy after immunostaining (Fig. 2). In the absence of treatment, Trim32 localized in cytoplasmic speckles, usually concentrated around the nucleus (Fig. 2A), in agreement with previous reports (2). This was in contrast to Piasy, which, in the absence of treatment, presented predominantly nuclear staining, in agreement with previous observations of endogenous Piasy localization (34) and the general description of PIAS family members such as nuclear proteins (35). However, within 3 h of treatment with 20 μM MG132, many of the Piasy-positive cells showed a reduction in nuclear staining and an accumulation of Piasy in cytoplasmic granules. Overlapping the Piasy and Trim32 fluorescence signals...
revealed colocalization of these proteins, particularly around the nucleus as represented in cultured cells shown in Fig. 2A. Individual cells were observed under confocal fluorescence microscopy, with an optical section thickness of 600 nm. Representative examples are shown in Fig. 2B. The accumulation of Piasy in cytoplasmic granules, as well as in more diffuse cytoplasmic staining, was apparent with MG132 treatment. In some cases, as shown in the figure, Piasy was depleted from the nucleus after MG132 treatment. Cytoplasmic Piasy colocalized with Trim32, as shown in detail in Fig. 2B, inset a. Nonoverlapping areas of green or red fluorescence indicate that the colocalization signal is specific and not an artifact of red fluorescence signal leaking into the green channel after cytoplasmic accumulation of Piasy.

These colocalization experiments demonstrate that Piasy and Trim32 can interact in mammalian cells, and suggested
that Trim32 controls Piasy cellular levels by ubiquitination of Piasy in the cytoplasm. To determine the effect of combined TNFα and UVB treatment on Piasy cytoplasmic accumulation and colocalization with Trim32, the number of Trim32/Piasy double-positive cells displaying colocalization was counted under fluorescence microscopy and expressed as a percentage of total Trim32/Piasy double-positive cells. As shown in Table 1, combined treatment with UVB and TNFα increased the colocalization of Trim32 and Piasy. In the absence of any treatment, 2–5% of the double-positive cells showed evidence of colocalization of Trim32 and Piasy, increasing 5–10-fold after treatment with 20 μM MG132. TNFα/UVB treatment increased co-colocalization 2 to 6 relative to untreated control, and that percentage nearly doubled after combined treatment with MG132.

The cytoplasmic colocalization of Piasy and Trim32 induced by TNFα/UVB treatment and MG132 incubation suggests that redistribution of Piasy between the nucleus and cytoplasm was involved in the control of Piasy cellular levels both in steady state and in response to cellular stress induced by TNFα/UVB treatment. Analysis of the amino acid sequence of Piasy revealed that, in addition to a single putative carboxyl-terminal

![FIGURE 2. Piasy accumulates in the cytoplasm and colocalizes with Trim32 when proteasome-dependent degradation is inhibited by MG132 treatment. A, plasmids for expression of FLAG-Piasy and GFP-Trim32 protein were cotransfected into mouse keratinocyte strain 291. Three hours after treatment with UVB + TNFα (230 J/m² and 5 ng/ml, respectively), MG132 (20 μM), or both, cells were fixed and stained with DAPI and a FLAG epitope-specific antibody and observed by wide field fluorescence microscopy. Representative fields of untreated and UVB/TNFα + MG132-treated cells are shown. Panels labeled DAPI, Piasy, and Trim32 are the separated blue, red, and green fluorescent channels, respectively. Merge shows the three channels fused. B, to further analyze colocalization of Trim32 and Piasy fluorescence signals, individual cells were observed by confocal microscopy. Representative examples of treated and untreated cells are shown. An area within the treated cell is enlarged in inset a to show detail of signal colocalization.](image)

![TABLE 1. Increased colocalization of Trim32 and Piasy after combined UVB/TNFα treatment](table)
nuclear localization signal (NLS), which corresponds to a previously described NLS in Piasxβ (37), Piasy also possesses putative NLS and nuclear export signals (NES) in the amino terminus, near the SAF-A/B-Acinus-PIAS (SAP) element (18, 19) (Fig. 3A). The SAP element mediates attachment to nuclear DNA scaffold/matrix attachment regions and promotes the intranuclear localization to areas of active transcription (19). Together with the putative NES, composed of spaced hydrophobic residues (38, 39), and the putative NLS, composed of a cluster of basic residues (40), these elements are conserved among PIAS family members, including mammalian (mouse and human Piasy, Pias1, and Piasx), teleost (puffer fish), and yeast (Saccharomyces Siz1) PIAS proteins (Fig. 3B). To test if this domain was responsible for nucleocytoplasmic distribution of Piasy, the sequence from amino acids 5–82 of Piasy was fused to GFP, and its intracellular localization compared with a full-length Piasy fusion to GFP (Fig. 3C). Although the full-length Piasy protein fusion localized exclusively in the nucleus, the fusion of amino acids 5–82 to GFP conferred nucleocytoplasmic distribution to GFP, localizing both in the nucleus and in cytoplasmic speckles reminiscent of the Trim32 cytoplasmic localization (Fig. 3C). Virtually every cell transfected with GFP-Piasy-(5–82) displayed both nuclear and speckled cytoplasmic staining. Interestingly, the corresponding region in Saccharomyces Siz1, amino acids 1–103 (Fig. 3B), also confers nucleocytoplasmic distribution when fused to GFP (41).

We also determined that the interaction domain of Piasy was contained within the amino-terminal half of the protein, amino acids 5–289. This amino-terminal half contains the SAP box and putative NES and NLS elements but excludes the RING-like zinc finger, the putative carboxyl-terminal NLS, and the acidic tract. Ectopic FLAG-tagged Piasy-(5–289) and GFP-Trim32 were expressed in cultured mouse keratinocytes and observed by both wide field and confocal fluorescence microscopy (Fig. 4). In the absence of MG132, Piasy-(5–289) was very unstable and difficult to express at detectable levels in keratinocytes. After incubation with MG132, Piasy-(5–289) displayed intense accumulation, both nuclear and cytoplasmic. Under confocal microscopy, staining in the cytoplasm exhibited a mixed speckled and diffuse pattern, resembling the one observed with the full-length protein (Fig. 4B). Overlapping with the Trim32 signal under confocal fluorescence microscopy demonstrated colocalization of the proteins (Fig. 4B). As before, nonoverlapping areas of green or red fluorescence support the specificity of the respective protein signals and the colocalization of both proteins.

Homozygous Missense Mutation in the NHL Domain of Human Trim32 Found in LGMD2H Abolishes Binding to Human Piasy—We next sought to verify colocalization of endogenous Piasy and Trim32 proteins and their potential relevance to human disease. As mentioned above, missense point mutation within the NHL domain of human Trim32 occurs in familial LGMD2H (11). The results shown above indicate that the interaction domain in Piasy is present in the amino-terminal half of the protein, whereas in Trim32 the removal of the NHL domain or reproduction of the missense D487N mutation prevents binding to Piasy (Fig. 1B). To determine the effect of the LGMD2H missense mutation on the interaction between endogenous human Trim32 and Piasy proteins, we isolated human dermal fibroblasts from a healthy donor and from an LGMD2H patient, and we determined the interaction of Trim32 and Piasy by immunofluorescence microscopy. LGMD2H patients are homozygous for the autosomal recessive D487N mutation (corresponding to D489N in mouse). Cells were stained after control solvent or combined MG132/UVB/TNFα treatment (Fig. 5). In untreated fibroblasts from a healthy donor, Trim32 showed a cytoplasmic granular distribution, whereas most cells displayed nuclear Piasy staining. However, in treated cells Piasy could be detected both in the nucleus and cytoplasm, and colocalization of Trim32 and cytoplasmic Piasy was apparent in ~1 out of 4 Trim32-positive cells (Fig. 5A). In contrast, Piasy failed to accumulate in the cytoplasm of LGMD2H fibroblasts after treatment with UVB/TNFα plus MG132, exhibiting only nuclear localization under both control and treated conditions. Interestingly, Trim32 expression levels were very low in untreated LGMD2H fibroblasts and increased after treatment. The low basal steady-state levels in LGMD2H suggest that the D487N mutation may make Trim32 protein less stable, thus failing to accumulate in the cell. However, even after treatment, when mutant Trim32 is readily detectable in LGMD2H fibroblasts, no colocalization with Piasy could be detected, with Piasy detectable only in the nucleus. These results are quantified in Fig. 5B. Approximately 50% of fibroblasts from a healthy donor displayed Trim32 staining, and this percentage did not change significantly with treatment (Fig. 5B, healthy donor, solid bars). Nevertheless, treatment did affect the percentage of cells displaying Trim32 colocalization with Piasy, from about 4% in control to 24% under treatment conditions (Fig. 5B, healthy donor, solid bars). In contrast, in fibroblasts from LGMD2H patients only 4% of total cells showed Trim32 staining under control conditions, but treatment increased the proportion to 84% of cells (Fig. 5B, LGMD2H donor, solid bars). However, as stated above, no colocalization of Trim32 with Piasy was detected in these cells even under treatment conditions. As before, cytoplasmic colocalization of both proteins was confirmed by individual observation of healthy donor fibroblasts under confocal microscopy (Fig. 5C, and inset A), similar to the results obtained in mouse keratinocytes.

These results confirm the interaction of the Trim32 and Piasy in endogenous proteins. Combined with the lack of binding to Piasy in the yeast two-hybrid assay (Fig. 1B), these results suggest that the D487N mutation inactivates the Trim32 protein by reducing its overall cellular levels and preventing protein/protein interactions.

E3-Ubiquitin Ligase Activity of Trim32 and Destabilization of Piasy—The fact that the proteasomal inhibitor MG132 increased the detectable interaction between Trim32 and Piasy suggested that Piasy is a substrate for Trim32-mediated ubiquitination and degradation. Furthermore, Trim32 has been reported recently to have in vitro E3-ubiquitin ligase activity (13). To determine whether Trim32 could display enzymatic E3-ubiquitin ligase activity toward Piasy in vitro, we purified recombinant Trim32 and Piasy and tested the ability of Trim32 to catalyze the sequential addition of ubiquitin molecules to Piasy in a defined, reconstituted in vitro system with recombi-
nant ubiquitin, E1 and E2 enzymes. In agreement with the results of Kudryashova et al. (13), in the absence of substrate, a purified recombinant GST-Trim32 fusion protein displayed self-ubiquitination in the presence of recombinant UbcH5a or UbcH6 (Fig. 6A). Thus, when the reaction products were probed with an antibody specific for Trim32, at least mono- and

FIGURE 3. The amino terminus of Piasy promotes nucleocytoplasmic redistribution of Piasy. A, schematic representation of the Piasy protein, showing the location of the SAP domain, the zinc finger domain, the carboxyl-terminal NLS corresponding to the one described in Tussie-Luna et al. (37), and the acidic tract. The sequence from residues 14 to 73, containing the SAP domain and the putative NES and NLS, is expanded. B, the cellular localization motifs on the amino terminus of PIAS proteins are evolutionarily conserved among PIAS proteins. The amino acid sequences 12–79 of the indicated human (h), mouse (m), teleost fish (Fugu rubripes, Fr), and yeast (Saccharomyces cerevisiae, Sc) PIAS proteins are shown. Sequences were aligned using a sequence analysis application (Omiga, Oxford Molecular) and manual adjustment. The numbering above refers to the mouse and human Piasy protein. Numbering below corresponds to the yeast Siz1 protein. The critical residues for the SAP box, nuclear export, and localization signals (NES and NLS) are highlighted in green (SAP), red (NES), and blue (NLS), respectively. Lines above the sequence indicate the previously reported (30) NMR secondary structure between residues 1 and 65 in Pias1. C, full-length Piasy or amino acids 5–82 of Piasy were ectopically expressed as amino-terminal GFP fusion proteins in epidermal mouse keratinocyte strain 291. Cells were fixed, stained with DAPI, and observed by fluorescent confocal microscopy. Two representative fields are shown per protein. Untransfected cells can be recognized by the deep blue DAPI fluorescence of their nuclei alone. The nuclei of transfected cells show turquoise fluorescence, because of color fusion with the green GFP fluorescence in the merge image.
di-ubiquitinated Trim32 could be detected, in addition to a smearing of immunoreactive proteins in the higher molecular weight area (>120 kDa), which could not be individually resolved (Fig. 6A, lanes 2 and 3). This ubiquitination reaction required the presence of E1 (Fig. 6A, lane 1). When the reaction products were probed with an antibody specific for the Myc tag of ubiquitin, a more intense pattern of immunoreactivity could be detected in the higher molecular weight area, particularly with UbcH5a (Fig. 6A, lanes 6 and 7). These products may represent higher polyubiquitinated forms of Trim32 (which would be detected with much higher intensity with a ubiquitin antibody than with the Trim32-specific antibody), although we cannot rule out the possibility that other proteins present in the assay could be contributing to this pattern. In any case, this ubiquitination was strictly dependent on the presence of GST-Trim32 (Fig. 6A, lane 8) and E1 (lane 5), demonstrating the ubiquitin E3 ligase activity of Trim32.

Next, we introduced purified recombinant Piasy in the reaction and tested the capacity of Trim32 to catalyze the ubiquitination of Piasy. Fig. 6B shows the results obtained with UbcH6. Similar results were obtained with UbcH5a, although the total levels of activity were somewhat lower than with UbcH6. In the absence of Trim32, a small amount of monoubiquitinated Piasy could be detected, likely corresponding to random interactions between Piasy and UbcH6 in solution (Fig. 6B, lane 4). This was expected, because E2 enzymes do not possess substrate specificity. However, the presence of Trim32 in the reaction resulted in a sharp increase in the rate of ubiquitin transfer to Piasy, with a much stronger band of monoubiquitinated Piasy and fainter bands of higher order polyubiquitinated Piasy (Fig. 6B, lane 3). Furthermore, even after prolonged exposure of the films, no polyubiquitinated forms of Piasy are detectable in the absence of Trim32 (Fig. 6B, lane 4, overexposed). Whether in the presence or absence of Trim32, ubiquitination of Piasy was strictly
dependent on the presence of E1 enzyme in the reaction (Fig. 6B, lane 1).

The in vitro ubiquitination assays with purified components demonstrated that Trim32 is a bona fide E3-ubiquitin ligase for Piasy. This implies that in living cells Piasy should be less stable in the presence than in the absence of Trim32, and this effect should be dependent on the RING domain of Trim32, required for E3 ligase activity. To address this issue, mouse keratinocytes were cotransfected with His6-Piasy and GFP-tagged wild type Trim32 or a RING domain deletion mutant (GFP-T32/H9004 RING).

As shown in Fig. 7A, in the absence of MG132, His6-Piasy accumulated to much higher levels in the presence of GFP-T32/H9004 RING than in the presence of GFP-Trim32. Treatment with UVB/TNFα did not seem to have a discernable effect on steady-state accumulation of His6-Piasy in the presence of GFP-T32/H9004 RING. In the presence of GFP-Trim32, His6-Piasy protein was barely detectable even without UVB/TNFα treatment to activate Trim32.

In a second approach to determine the effect of Trim32 on Piasy stability, new protein synthesis was blocked by addition of cycloheximide, and Piasy levels were determined in crude lysates at increasing time points after cycloheximide block (Fig. 7B). We compared the effect of wild type Trim32 with the RING domain mutated on the critical residue C21S, required for RING domain secondary structure (GFP-T32C21S). For these experiments, we used a FLAG-tagged Piasy form, which was more stable than the His6-tagged form and could be readily detected in the absence of MG132. The results show that Piasy was far more unstable in cells cotransfected with wild type Trim32 than in cells cotransfected with the C21S RING mutant or with a plasmid control. Fig. 7C shows the results of densitometric quantification.
Trim32 Regulation of Piasy and Apoptosis Modulation

**A**

| myc-ubi | + | + | + | + | + | + | + | + |
|---------|---|---|---|---|---|---|---|---|
| E1      | + | + | + | + | + | + | + | + |
| UbcH5   | + | + | + | + | + | + | + | + |
| UbcH6   | + | + | + | + | + | + | + | + |
| GST-Trim32 | + | + | + | + | + | + | + | + |
| kDa     | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |

**B**

| myc-ubi | + | + | + | + | + | + | + | + |
|---------|---|---|---|---|---|---|---|---|
| E1      | + | + | + | + | + | + | + | + |
| UbcH6   | + | + | + | + | + | + | + | + |
| GST-Trim32 | + | + | + | + | + | + | + | + |
| 6xHis-Piasy | + | + | + | + | + | + | + | + |

**FIGURE 6.** *Trim32* catalyzes the ubiquitination of Piasy in vitro in a reconstituted assay with purified components. A, in vitro self-ubiquitination of recombinant Trim32. Purified GST-Trim32 was mixed with the indicated components, and reactions proceeded as described under “Experimental Procedures,” in a total volume of 30 μl. At the end of the incubation period, 28 μl were separated by SDS-PAGE and immunoblotted with a rabbit polyclonal antibody for Trim32, whereas 2 μl of the reaction were separated by SDS-PAGE and immunoblotted with a monoclonal antibody for the Myc epitope tag of ubiquitin. B, Trim32 catalyzes the in vitro ubiquitination of recombinant Piasy. Purified His6-Piasy was incubated with the indicated components, and reactions proceeded as described under “Experimental Procedures.” Reaction products were then separated by SDS-PAGE and detected by immunoblotting with an antibody specific for human Piasy (Abgent), which cross-reacts with mouse Piasy. A prolonged photographic exposure of the chemiluminescence reaction is shown to the right. Small tic marks indicate the location of mono- and polyubiquitinated forms of Piasy. Asterisks indicate the location of minute amounts of dimeric and trimeric Piasy produced during the expression and purification of the recombinant protein. WB: Western blot.

**C**

**FIGURE 7.** *Trim32* promotes the destabilization of Piasy, depending on the presence of a functional RING domain. A, *Trim32* destabilizes Piasy but not when the RING domain is deleted. Mouse epidermal keratinocyte strain 291 cells were transfected with plasmids for expression of the indicated GFP and His6 fusion proteins and treated with UVB + TNFα (230 J/m2 and 5 ng/ml, respectively). Whole cell lysates were prepared 4 h after treatment, and the levels of the His6-Piasy protein were determined by immunoblotting. The nitrocellulose membranes were then sequentially stripped and immunoblotted with antibodies for GFP and endogenous Hsc70 (for normalization of loading). B, expression of *Trim32* decreases the half-life of Piasy. Mouse keratinocytes (291 strain) were cotransfected with plasmids for expression of the indicated proteins and treated with cycloheximide (25 μM) to block new protein synthesis. At the indicated times after cycloheximide block, cells were lysed, and the levels of Piasy were determined by immunoblotting with anti-FLAG antibody. Hsc70 is shown as loading control. C, quantification of Piasy half-life when coexpressed with wild type or C21S RING domain Trim32 proteins. Mouse keratinocytes (291 strain) were cotransfected with plasmids for expression of FLAG-Piasy and GFP-Trim32 ( or ), GFP-T32C21S ( or ), or empty plasmid ( or ) and treated with combined UVB + TNFα (230 J/m2 and 5 ng/ml, respectively, closed symbols) or control (open symbols). Cycloheximide (25 μM) was then added to the cells. Whole cell lysates were prepared at 2 and 6 h after cycloheximide addition. The levels of Piasy protein were determined by immunoblotting as in B and quantified by densitometry. Data at each point post is expressed as percent of the amount of Piasy protein present before cycloheximide addition.
mutant, compared with the control with no exogenous Trim32 protein.

We next proceeded to determine the ubiquitination activity of the Trim32 protein, both against Piasy and itself. First, we analyzed the ubiquitination status of Trim32 in cells cotransfected with GFP fusion Trim32 protein and Myc-tagged ubiquitin expression plasmids (Fig. 8A). Ubiquitinated proteins were collected by immunoprecipitation, and the presence of ubiquitinated Trim32 or its RING domain deletion mutant (T32ΔRING) was determined by immunoblotting to the GFP tag. In the immunoprecipitates, bands of the same molecular weight as the native protein in crude lysates correspond to ubiquitinated Trim32 proteins. The signal from ubiquitinated proteins was notably higher molecular weight than the RING domain mutant (T32ΔRING). Bands of nonubiquitinated Trim32 proteins dimerizing with ubiquitinated Trim32 were not detected in lysates from cells transfected with plasmids (Fig. 8A, compare lanes 5 and 6 with lanes 7 and 8). In fact, ubiquitinated forms of Trim32, but not T32ΔRING, could be detected in the total lysates (Fig. 8A, black arrowhead). Furthermore, the signal from ubiquitinated proteins in the immunoprecipitates was stimulated after treatment with UVB/TNFα, but only in the case of full-length Trim32. The ubiquitinated forms detected with T32ΔRING are likely because of the endogenous Trim32 protein, expressed in keratinocytes (8), that can cross-ubiquitinate T32ΔRING.

Next, we determined whether the instability of Piasy induced by Trim32 was indeed because of ubiquitination (Fig. 8B). Wild type Trim32 was compared with the NHL domain mutant form found in LGMD2H (GFP-T32LGMD2H) and the RING deletion mutant (GFP-T32ΔRING). We determined the ubiquitination status of both the Trim32 proteins and Piasy, in order to compare Trim32 protein self-ubiquitination status with its ability to ubiquitinate Piasy. As before, ubiquitination of wild type Trim32, but not the RING deletion mutant (Fig. 8B, middle panel), was increased in response to UVB/TNFα treatment. Interestingly, the LGMD2H mutant displayed very little ubiquitination activity in these experiments.

Ubiquitination of Piasy could also be detected in lysates from these cells. In cells transfected with wild type Trim32, several bands corresponding to mono- and poly-ubiquitinated Piasy could be detected but only after UVB/TNFα treatment (Fig. 8B, lower panel). No ubiquitinated Piasy proteins could be detected in cells transfected with GFP-T32ΔRING, consistent with its lack of ubiquitination activity. The LGMD2H mutant was deficient in Piasy ubiquitination activity, compared with wild type. However, some Piasy ubiquitination could also be detected after UVB/TNFα treatment in the LGMD2H mutant. This is consistent with the fact that LGMD2H is a recessive, homozygous disease. It is likely that the activity seen with the T32LGMD2H mutant is attributable to the endogenous Trim32 protein in heterozygosity.

Trim32 Increases, although Piasy Decreases, the Transcriptional Activity of NFκB—The increase in Trim32 and Piasy interaction detected by immunofluorescence microscopy (Fig. 2) suggested that the process of destabilization of Piasy by Trim32 may be part of the UVB stress response of keratinocytes. In keratinocytes, NFκB is an anti-apoptotic, pro-survival factor in response to UVB light (42–44). Piasy has been reported to inhibit NFκB transcriptional activity in human embryonic kidney cells after stimulation of the Toll/interleukin1 receptor by cotransfection with the Toll/interleukin1 receptor adaptor protein TRIF (45). We hypothesized that the interaction of Trim32 and Piasy could be involved in the control of NFκB transcriptional activity in epidermal keratinocytes. If
such was the case, Trim32 and Piasy expression should have opposite effects on NFκB activity in epidermal keratinocytes. To test this hypothesis, the transcriptional activity of NFκB was determined in 291-GFP and 291-Trim32 mouse keratinocyte cell strains (8). We transfected these cells with Piasy protein expression plasmids (Fig. 9). Transcriptional activity was determined in 291-GFP and 291-Trim32 mouse keratinocyte cell strains. The indicated amounts of a Piasy-expressing plasmid were cotransfected with NFκB-dependent luciferase plasmid and constitutively expressed Renilla plasmid. Total plasmid amounts were equalized by cotransfection of the empty protein expression plasmid. Cells were lysed, and luciferase and Renilla activities in the extracts were determined with a luminometer as described under “Experimental Procedures.” Results are expressed as the ratio of luciferase to Renilla activity plotted against the amount of transfected Piasy plasmid in triplicate measurements. The results of two-tailed t test comparison are shown. n.s., not significant.

FIGURE 9. Trim32 and Piasy have opposite effects on NFκB transcriptional activity. A, dose-dependent effect of Piasy on NFκB transcriptional activity in 291D-GFP or 291D-Trim32 mouse epidermal keratinocyte cells strains. The indicated amounts of a Piasy-expressing plasmid were cotransfected with NFκB-dependent luciferase plasmid and constitutively expressed Renilla plasmid. Total plasmid amounts were equalized by cotransfection of the empty protein expression plasmid. Cells were lysed, and luciferase and Renilla activities in the extracts were determined with a luminometer as described under “Experimental Procedures.” Results are expressed as the ratio of luciferase to Renilla activity plotted against the amount of transfected Piasy plasmid in triplicate measurements. The results of two-tailed t test comparison are shown.

Trim32 Regulation of Piasy and Apoptosis Modulation

NFκB transcriptional activity was determined in 291-GFP and 291-Trim32 mouse keratinocyte cell strains. The indicated amounts of a Piasy-expressing plasmid were cotransfected with NFκB-dependent luciferase plasmid and constitutively expressed Renilla plasmid. Total plasmid amounts were equalized by cotransfection of the empty protein expression plasmid. Cells were lysed, and luciferase and Renilla activities in the extracts were determined with a luminometer as described under “Experimental Procedures.” Results are expressed as the ratio of luciferase to Renilla activity plotted against the amount of transfected Piasy plasmid in triplicate measurements. The results of two-tailed t test comparison are shown.

When cells were treated with TNFα and UVB, Trim32 expression maintained its stimulatory effect on NFκB transcriptional activity (Fig. 9B). Correspondingly, Piasy also inhibited NFκB activity under stimulated conditions. The ratio of NFκB activity between TNFα/UVB-treated and basal conditions was not significantly different for 291-GFP compared with 291-Trim32 (~3-fold). Together with the fact that NFκB is already elevated in 291-Trim32 under basal conditions, this suggests that the effect of Trim32 on NFκB activity is not at the level of the upstream cytoplasmic events leading to NFκB activation by TNFα and UVB but rather is downstream of these events. This would be consistent with a mechanism involving Trim32 destabilization of Piasy, because Piasy, an E3-SUMOylation enzyme for transcription factors, would be expected to interfere directly with NFκB transcriptional processes.

Piasy Sensitizes Keratinocytes to Apoptosis Induced by TNFα and UVB—Because of the involvement of NFκB in keratinocyte apoptotic survival (42–44), we predicted that Piasy expression, through inhibition of the NFκB activity, would make keratinocytes more sensitive to apoptosis induced by TNFα and UVB treatment. This would be opposite to the effect of Trim32, which we have shown previously to protect mouse epidermal keratinocytes from apoptosis induced by TNFα/UVB treatment (8). To address this issue, we transfected mouse keratinocytes with GFP or GFP-Piasy expression plasmids, and we determined the percentage of apoptotic cells after treatment with TNFα and UVB. Preliminary experiments confirmed that the addition of a GFP tag does not alter the ability of Piasy to inhibit NFκB transcriptional activity, as determined in luciferase assays (not shown). Because inhibition of NFκB is known to sensitize keratinocytes to apoptosis induced by TNFα treatment alone, which otherwise does not induce keratinocyte apoptosis (42), we determined the effect of TNFα and UVB both separately and in combination. Apoptosis of GFP-positive cells was determined using phase contrast microscopy and by mitochondrial and DNA staining with Mitotracker and Hoechst 33352, respectively. Mitotracker is a nonfluorescent hydrophobic compound that diffuses freely across cell membranes and, upon oxidation in the mitochondria, yields a positively charged red fluorescence compound that accumulates in intact mitochondria. A hallmark of apoptosis is mitochondrial membrane permeabilization, which releases Mitotracker and eliminates the bright red fluorescence.

Fig. 10A illustrates the detection of apoptotic GFP- and GFP-Piasy-expressing cells. GFP-expressing cells displayed diffused nuclear and cytoplasmic green fluorescence, whereas GFP-Piasy expression was restricted to the nucleus in the majority of cells. Nonapoptotic green fluorescence cells, either GFP- or GFP-Piasy-expressing, spread on the plate, exhibited smooth borders, and displayed intense red Mitotracker fluorescence concentrated in a punctated halo around the nucleus. In contrast, green fluorescence apoptotic cells (marked by a white arrow in Fig. 10A) had ruffled and irregular surface and a dim, diffuse red fluorescence that contrasts with the bright punctated halo fluorescence of nonapoptotic cells. Using these criteria, GFP and GFP-Piasy cells were scored for the percentage of green apoptotic rela-
Piasy clearly induced apoptosis in response to TNFα/H9251, UVB, and combined TNFα/H9251/UVB treatments, as expected from its effect on NFκB activity. This was particularly evident in the case of cells treated with TNFα alone, which did not induce measurable apoptosis levels compared with the control in GFP-transfected cells but induced apoptosis in almost 70% of cells expressing GFP-Piasy.

The opposing effects of Trim32 and Piasy on NFκB transcriptional activity and TNFα/UVB-induced apoptosis suggest that the interaction between Trim32 and Piasy is involved in the control of keratinocyte apoptosis in response to stress, which may be relevant both for the normal homeostatic control of the skin as well as for skin carcinogenesis initiation and progression.

**DISCUSSION**

Our initial characterization of Trim32 as a carcinogenesis-associated gene and the presence of a RING domain in Trim32, characteristic of E3-ubiquitin ligases, led us to search for interacting partners as putative ubiquitination substrates of Trim32. Of the members of the PIAS family detected in our screen (Piasy, Piasx, and Pias1), we centered our studies on Piasy for the following reasons. First, during mouse embryonic development and in adult human skin, Piasy is expressed preferentially in epidermal keratinocytes and in hair follicles, and its mRNA expression increases during keratinocyte differentiation suggesting a role in skin development and maintenance (22, 23). In addition, Piasy mRNA is also highly expressed in brain and in testis, matching the expression pattern of Trim32 mRNA (8, 11, 22, 28). Second, we found that expression of Trim32 increased...
Trim32 Regulation of Piasy and Apoptosis Modulation

epithelial cell transformation and resulted in hyperplasia in vivo. Furthermore, elevated expression of Trim32 was present in mouse tumors induced by UVB irradiation, and loss of human Piasy expression has been associated with late stages of chronic myelogenous leukemia (8, 24), suggesting that Trim32 and Piasy have opposing roles in tumorigenesis. Third, increased Trim32 expression protects keratinocytes from apoptosis induced by combined UVB/TNFα treatment (8), whereas increased expression of Piasy promotes apoptosis (24, 47), suggesting opposing effects in apoptosis regulation. Our results demonstrate that interaction between the two proteins in the cytoplasm, detectable mainly after incubation with the proteasome inhibitor MG132, is induced by treatment of keratinocytes with TNFα and UVB and that Trim32 controls the stability of Piasy. Furthermore, we show that Piasy and Trim32 have opposing effects in NFκB activity and TNFα/UVB-induced keratinocyte apoptosis. Piasy stimulated keratinocyte TNFα- and UVB-induced apoptosis as expected, given its inhibitory effect in keratinocyte NFκB activity. Based on these results, we propose a model for the regulation of Piasy (and possibly other PIAS proteins) through the control of its stability by cytoplasmic Trim32-mediated ubiquitination. According to this model, cytoplasmic redistribution of Piasy is enhanced by cellular stress signals (such as UVB/TNFα), depleting Piasy and promoting survival through stimulation of NFκB transcriptional activity.

Piasy redistribution from the nucleus to the cytoplasm is mediated by sequences present in the amino terminus of Piasy, based on the sufficiency of the Piasy fragment containing amino acids 5–82 to confer nucleocytoplasmic localization to a GFP molecule. Putative NES and NLS are present in this sequence, which are conserved among vertebrate PIAS proteins and in the S. cerevisiae PIAS homolog Siz1. Furthermore, the corresponding region in Siz1 also confers nucleocytoplasmic distribution to fused GFP molecule (41). Future work will be required to characterize the putative NES and NLS located within this sequence. Additional sequences relevant for protein localization may be present in Piasy, such as the PINIT motif (amino acids 235–239), which has been proposed as a PIAS family member involved in nuclear localization of Pias3 (48). However, the PINIT contribution to nuclear localization may be specific for individual PIAS family members. Thus, the PINIT motif has little contribution to nuclear localization of Siz1, which instead required the amino-terminal region (41), and Wong et al. (49) found that a splice variant of Piasy, lacking the PINIT motif, showed nuclear localization identical to full-length Piasy.

Our current findings with Piasy protein may also apply to Pias1 and Piasx proteins, due to the sequence similarities among members of the PIAS family and the results of our yeast two-hybrid assays. Another TRIM family member, RFP (or Trim27), has also been reported to bind to PIAS family members (50). However, interaction with RFP is through the carboxy-terminal end of the PIAS proteins, in contrast with Piasy/Trim32 interaction, which localizes to the amino-terminal half of Piasy. RFP is SUMOylated and colocalizes with PML in nuclear bodies (50, 51). Thus, it is likely that RFP interacts with PIAS proteins as a SUMOylation substrate, and it is not involved in PIAS protein ubiquitination. In contrast, our analysis of Trim32 protein ubiquitination revealed an absence of canonical SUMOylation signals, which together with Trim32 cytoplasmic localization argue against Trim32 being a SUMOylation substrate for Piasy.

Destabilization of Piasy offers a plausible mechanism for the observed anti-apoptotic effect of Trim32 expression on UVB/TNFα-treated keratinocytes and its role in carcinogenesis, and our results provide a possible downstream effector, NFκB. The question remains as to how Piasy may inhibit NFκB activity. SUMO-1 modification of components of the NFκB activation cascade NEMO (IKKγ) and IκBε has been implicated both in activation and inhibition of the NFκB pathway, respectively (52, 53). It remains to be determined whether Piasy can function as an E3-SUMO ligase specific for IκB, NEMO, or yet another, so far unidentified, member of the NFκB activation cascade.

The D487N mutation (D489N in mice) is associated with LGMD2H (11), a mild form of muscular dystrophy, with a variable presentation in homozygous carriers, from absence of symptoms to decreased stamina (54). The lack of other obvious organ alterations in LGMD2H patients, despite the more general tissue expression of Trim32, and the slow and mild progression of the disease suggest that other E3 ligases may be at least partially redundant for Trim32 function in tissues other than muscle. Our results show that Trim32 carrying the LGMD2H-associated mutation is defective in interaction with Piasy. In this respect, the involvement of NFκB inhibition in the etiology of LGMD2A (55, 56) is intriguing. However, additional experiments are needed to determine whether Piasy is the critical substrate for Trim32 activity in the muscle. For instance, although Piasy is reported to be expressed in the trapezius muscle of developing mice, Piasy knock-out mice did not display any obvious organ abnormality (23, 49). In any case, our conclusion that the LGMD2H mutation in the NHL domain inactivates Trim32 is consistent with published reports of other NHL-containing TRIM family proteins. Thus, Drosophila mei-P26, a putative Trim32 homolog, is inactivated by a missense mutation at a conserved residue in its NHL domain, leading to a neurological seizure phenotype (46). Similarly, missense point mutations in the NHL domain of Caenorhabditis LIN-41 also inactivate the protein, leading to an altered developmental phenotype (36).

While this manuscript was in preparation, a missense point mutation of the coiled-coil domain of Trim32 was reported in association with Bardet-Biedl syndrome (BBS) (14), a genetically heterogeneous disease characterized by combination of symptoms, including renal failure, retinopathy, polydactyly, hypogonadism, and obesity. LGMD2H and BBS have no obvious symptoms in common, and it is evident that the missense point mutations found in LGMD2H and BBS must have different effects on the function or tissue specificity of Trim32. Further work will be required to determine the effect of the BBS-associated missense mutation in the function of Trim32 and its mechanistic involvement in BBS.

In summary, our data support a role for Trim32 in the regulation of Piasy, and possibly other PIAS proteins, by promoting Piasy ubiquitination and degradation and place this interaction in the physiological context of the ultraviolet light/stress.
responses of keratinocytes through modulation of NFκB and the apoptotic outcome. Our results also suggest that the LGMD2H-associated mutation inactivates Trim32. These findings suggest a model whereby activation of Trim32 plays a role in carcinogenesis by increasing initiated cell survival, thus increasing the target population of cells for subsequent events and tumorigenicity, whereas inactivation of Trim32 plays a role in LGMD2H. The current data raise intriguing questions about whether inactivating a survival mechanism of cells in response to stress or increasing apoptosis mediated by Piasy mediate the LGMD2H defect. However the involvement of Piasy in the etiology of LGMD2H, or the involvement of alternative substrates, requires further analysis. Furthermore, our previous data showing that Trim32 promotes epidermal transformation (8) and the characterization of Piasy down-regulation as a common event in the progression of CML (24) suggest that preventing the interaction of Trim32 with Piasy, and thus increasing Piasy levels, may have therapeutic potential by promoting apoptosis of cancer cells.

Acknowledgments—We thank Drs. Rosalie Sears and David Ransom for helpful discussions, James Lagowski for technical assistance, Aurelie Snyder for help with confocal microscopy, and Lao Novina-Sapinski and James Lagowski for assistance in the preparation of this manuscript. We also thank Yetao Jin and Jayme Gallegos of the Lu laboratory for technical advice with the in vitro ubiquitination assays.

REFERENCES

1. Jackson, P. K., Eldridge, A. G., Freed, E., Furstenthal, L., Hsu, J. Y., Kaiser, B. K., and Reimann, J. D. (2000) Trends Cell Biol. 10, 429–439
2. Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., Riganelli, D., Zanaria, E., Messali, S., Cainerca, S., Guiffanti, A., Minucci, S., Pelacci, P. G., and Ballabio, A. (2001) EMBO J. 20, 2140–2151
3. Slack, F. J., and Ruvkun, G. (1998) Trends Biochem. Sci. 23, 474–475
4. Goddard, A. D., Borrow, J., Freemont, P. S., and Solomon, E. (1991) Science 254, 1371–1374
5. Saenko, V., Rogounovitch, T., Shimizu-Yoshida, Y., Abrosimov, A., Lushchak, V., Zelent, A., Shintani, K., and Minna, J. D. (1992) Mutat. Res. 257, 81–90
6. Takahashi, M., Ritz, J., and Cooper, G. M. (1985) Cell 42, 581–588
7. Fujiwara, T. M., and Wrogemann, K. (2002) J. Biol. Chem. 277, 663–672
8. Frosk, P., Weiler, T., Nylén, E., Sudha, T., Greenberg, C. R., Morgan, K., Fujivara, T. M., and Wrogemann, K. (2002) Ann. J. Hum. Genet. 70, 663–672
9. Frosk, P., Greenberg, C. R., Tension, A. A., Lamont, R., Nylén, E., Hirst, C., Frappier, D., Roslin, N. M., Zaki, M., Bushby, K., Straub, V., Zate, M., de Paula, F., Morgan, K., Fujivara, T. M., and Wrogemann, K. (2005) Hum. Mutat. 25, 38–44
10. Kudryashova, E., Kudryashov, D., Kramerova, I., and Spencer, M. J. (2005) J. Mol. Biol. 354, 413–424
11. Chen, A. P., Beck, J. S., Yen, H. J., Tayeh, M. K., Schweitz, T. E., Swiderski, R. E., Nishimura, D. Y., Braun, T. A., Kim, K. Y., Huang, J., Elbedour, K., Carmi, R., Slusarski, D. C., Casavant, T. L., Stone, E. M., and Shepherd, V. C. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6287–6292
12. Gill, G. (2004) Genes Dev. 18, 2046–2059
13. Muller, S., Ledl, A., and Schmidt, D. (2004) Oncogene 23, 1998–2008
14. Seeler, J. S., and Dejean, A. (2003) Nat. Rev. Mol. Cell Biol. 4, 690–699
15. Aravind, L., and Koonin, E. V. (2000) Trends Biochem. Sci. 25, 112–114
16. Tan, J. A., Hall, S. H., Hamil, K. G., Grossman, G., Petrusz, P., and French, F. S. (2002) J. Biol. Chem. 277, 16993–17001
17. Takahashi, Y., Kahyo, T., Toh, E., Yasuda, H., and Kikuchi, Y. (2001) J. Biol. Chem. 276, 48973–48977
18. Shuai, K., and Liu, B. (2005) Nat. Rev. Immunol. 5, 593–605
19. Takahashi, M., Ritz, J., and Cooper, G. M. (1985) Cell 42, 581–588
20. Shuai, K., and Liu, B. (2005) J. Biol. Chem. 280, 48973–48977
21. Takahashi, Y., Kahyo, T., Toh, E., Yasuda, H., and Kikuchi, Y. (2001) Oncogene 20, 8249–8257
22. Gross, M., Liu, B., Tan, J., French, F. S., Carey, M., and Shuai, K. (2001) Oncogene 20, 3880–3887
23. Jensen, K., Shieks, C., and Freemont, P. S. (2001) Oncogene 20, 7223–7233
24. Okubo, S., Hara, F., Tsuchida, Y., Shimotakahara, S., Suzuki, S., Hatanaka, H., Yokoyama, S., Tanaka, H., Yasuda, H., and Shindo, H. (2004) J. Biol. Chem. 279, 31455–31466
25. Urano, T., Saito, T., Tsukui, T., Fujita, M., Hosoi, T., Muramatsu, M., Ouchi, Y., and Inoue, S. (2002) Nature 417, 871–875
26. Horn, E. J., Albor, A., Liu, Y., El Hizawi, S., Vanderbeek, G. E., Babcock, M., Bowden, G. T., Hennings, H., Lozano, G., Weinberg, W. C., and Kulesz-Martin, M. (2004) Carcinogenesis 25, 157–167
27. Stremlau, M., Owens, C. M., Perron, M. J., Kiesling, M., Autissier, P., and Sodroski, J. (2004) Nature 427, 848–853
28. Meroni, G., and Drez-Roux, G. (2005) BioEssays 27, 1147–1157
29. Chin, A. P., Beck, J. S., Yen, H. J., Tai, C. M., Schaefer, T. E., Swiderski, R. E., Nishimura, D. Y., Braun, T. A., Kim, K. Y., Huang, J., Elbedour, K., Carmi, R., Slusarski, D. C., Casavant, T. L., Stone, E. M., and Shepherd, V. C. (2006) Proc. Natl. Acad. Sci. U. S. A. 103, 6287–6292
30. Gill, G. (2004) Genes Dev. 18, 2046–2059
52. Desterro, J. M., Rodriguez, M. S., and Hay, R. T. (1998) Mol. Cell 2, 233–239
53. Huang, T. T., Wuerzberger-Davis, S. M., Wu, Z. H., and Miyamoto, S. (2003) Cell 115, 565–576
54. Schoser, B. G., Frosk, P., Engel, A. G., Klutzny, U., Lochmuller, H., and Wrogemann, K. (2005) Ann. Neurol. 57, 591–595
55. Baghdiguian, S., Martin, M., Richard, I., Pons, F., Astier, C., Bourg, N., Hay, R. T., Chemaly, R., Halaby, G., Loiselet, J., Anderson, L. V., Lopez, D. M., Fardeau, M., Mangeat, P., Beckmann, J. S., and Lefranc, G. (1999) Nat. Med. 5, 503–511
56. Baghdiguian, S., Richard, I., Martin, M., Coopman, P., Beckmann, J. S., Mangeat, P., and Lefranc, G. (2001) J. Mol. Med. 79, 254–261