Human Kruppel-like Factor 5 Is a Target of the E3 Ubiquitin Ligase WWP1 for Proteolysis in Epithelial Cells

Received for publication, June 7, 2005, and in revised form, September 28, 2005. Published, JBC Papers in Press, October 13, 2005, DOI 10.1074/jbc.M506183200

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The transcription factor KLF5 plays an important role in human carcinogenesis. In epithelial cells, the KLF5 protein is tightly regulated by the ubiquitin-proteasome pathway. To better understand the mechanisms for the regulation of KLF5 protein, we identified and characterized an E3 ubiquitin ligase for KLF5, i.e. WWP1. We found that WWP1 formed a protein complex with KLF5 in vitro and in vivo. Furthermore, WWP1 mediated the ubiquitination and degradation of KLF5, and the catalytic cysteine residue of WWP1 is essential for its function. A PY motif in a transactivation domain of KLF5 is necessary for its interaction with WWP1. Finally, WWP1 was amplified and overexpressed in some cancer cell lines from the prostate and breast, which negatively regulated the function of KLF5 in gene regulation. These findings not only established WWP1 as an E3 ubiquitin ligase for KLF5, they also further implicated the KLF5 pathway in human carcinogenesis.

Human Kruppel-like factor 5 (KLF51–3/KLF/BTEB2) is an important transcription factor that plays a role in carcinogenesis in different human tissues (1–3). KLF5 has been demonstrated to regulate cell proliferation (4), differentiation (5), cell cycle regulation (6), and angiogenesis (7). Like other Kruppel-like factors, KLF5 regulates the expression of genes, and such regulation is often cell type-specific. For example, whereas some KLF5-regulated genes such as the platelet-derived growth factors (PDGFA, PDGFB) (8, 9), cyclin D1 (3), and PPARY (5) are relevant to epithelial cells, the induction of the smooth muscle myosin heavy chain B gene (10) and the SM22-α gene (11) is more specific to smooth muscle cells. The role of KLF5 in carcinogenesis also appears to be context-dependent. Although expression of KLF5 enhances cell proliferation in untransformed cells (3, 12) and even transforms normal fibroblasts (4), KLF5 appears to suppress cell growth in cancer cells (1–3). In addition, KLF5 is haplo-insufficient (7) and undergoes frequent genomic deletion in human cancer (1, 2), which indicates a loss of function for KLF5 during carcinogenesis. KLF5 apparently has an important role in cancer, though the molecular mechanisms of its function remain to be elucidated.

The activity of KLF5 is strictly regulated at both transcriptional and post-translational levels, and alterations in the regulation of KLF5 are associated with human cancer. KLF5 mRNA is highly expressed in normal epithelial cells such as those from the prostate, breast, and intestine, but is frequently down-regulated in cancer cells (1–3). KLF5 has also been demonstrated to be a responsive gene of H-Ras (6), Wnt-1 (13), and ERBB2 oncoproteins (14) in different cell models. Moreover, several carcinogens and growth factors including phorbol 12-myristate 13-acetate, sphingosine 1-phosphate, androgen, and fibroblast growth factor can induce KLF5 expression (9, 15, 16). On the post-translational level, phosphorylation of KLF5 at its CREB-binding protein (CBP) interaction region by protein kinase C increases its transactivation activity (17), and acetylation of KLF5 at its zinc finger domain is regulated positively by the acetylase p300 but negatively by the SET and the acetylase HDAC1 (18, 19). Recently, we demonstrated that the ubiquitin-proteasome pathway (UPP) is an important mechanism for the regulation of the KLF5 protein (20). We further found that the proteolysis of KLF5 appears to be more active in cancer cells than in untransformed epithelial cells (20).

In this study, we searched for the E3 ligase that targets KLF5 for UPP-mediated degradation and examined how degradation of KLF5 becomes hyperactive in cancer cells. We found that the E3 ubiquitin ligase WW1 targets KLF5 for proteolysis. Furthermore, overexpression of WWP1, primarily through the gain of gene copy number for WWP1 at 8q21, appears to be responsible for overdegradation of KLF5 in human cancer cells.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Expression plasmids for KLF5 mutants PY1, PY2, and PY1-2 were constructed from pcDNA3-KLF5–FLAG, in which a FLAG tag is attached to the C terminus of KLF5, using PCR-based approaches as described previously (20). Briefly, one PCR product was generated with the forward primer, F1 (5′-GAATCTAGATATGGC-CCAGTTCC-3′) (XbaI site is underlined) and the reverse primer, PY2R (5′-TGTAGACGGCTGGACATCCCTGCT-3′). Another PCR product was generated with the forward primer, PY2F (5′-GAATCTAGATATGGC-CCAGTTCC-3′) and the reverse primer, R1 (5′-TAGGGTCTGACAAATTTGCTC-3′) (the HpaI site is underlined.) These two PCR products, which overlap partially, were purified and used as templates to amplify a full-length PCR product using primers F1 and R1. The new PCR products were digested with XbaI and Hpal to prepare the insert, which was used to replace the counterpart in pcDNA3-KLF5–FLAG. Eight amino acids, i.e. NLTPPPSY from codon 321 to codon 328 (PY motif 2), were deleted in the resultant plasmid PY2. PY1 and PY1-2 were constructed using the same strategy based on pcDNA3-KLF5–FLAG and PY2. Five amino acid residues (PPTCY from
codon 282 to 286 or PY motif 1) were removed in the PY1 construct. In
the PY1-2 construct, both PY motifs were deleted. All clones were con-
firmed by DNA sequencing. Myc-tagged wild-type and mutant WWP1
constructs, i.e. Myc-WWP1 and Myc-WWP1C886S, have been reported
in a previous study (21). FLAG-WWP2 and FLAG-AIP4 con-
structs were kindly provided by Dr. Richard Longnecker from North-
western University (22). Nedd4-1 and Nedd4-2 constructs were gifts
from Dr. Olivier Staub at the University of Lausanne (23).

Cell Culture and Transfection—The 22Rv1 prostate cancer cell line
was maintained in RPMI 1640 medium supplemented with 5% fetal
bovine serum, HEPES (0.1 M), sodium pyruvate (1 mM), sodium bicar-
carbonate (0.15%), glucose (0.45%), and penicillin and streptomycin (1%).
The 293FT cell line was maintained in Dulbecco’s modified Eagle’s
medium supplemented with 5% fetal bovine serum. Plasmids were trans-
fected into the 22Rv1 cells using the Lipofectamine 2000 reagent
following the manufacturer’s manual (Invitrogen).

Western Blot and Immunoprecipitation—Western blot experiments
including those for ubiquitination analysis were performed as described
in our previous study (20). Co-immunoprecipitation using anti-Myc
antibody was conducted following the standard protocol (Cell Signal-
ing) with minor modifications. Briefly, to transfected 22Rv1 cells in each
antibody was conducted following the standard protocol (Cell Signal-
ing) and the manufacturer’s manual (Invitrogen).

Purified GST fusion protein was eluted into 10 mM reduced glutathione.

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The protein was analyzed by 10% SDS-PAGE and Coomassie Blue stain-
ing to determine the purity. The protein concentration was measured
by the Bradford method using bovine serum albumin as a standard
(Bio-Rad).

In Vitro Translation—DNA templates for wild-type KLF5 and its
mutants with one or both PY motifs were generated by PCR using the
forward primer: 5’-GGATCCTTAATACGACTCACTATAGGGAA-
GACCACATGGCTACAAGGTGCTGAG-3’ (the T7 promoter
sequence is underlined, the start codon ATG is in bold; this primer was
purified by SDS-PAGE after synthesis) and the reverse primer: 5’-TT-
TCTAGACTTTGTCATCGT CGTCTCTTGTAATCGTCGG-
TGCTCCTCATAT-3’ (The stop codon is in bold.) RNA transcrip-
tion and protein translation were performed in vitro in the presence
of [35S]methionine (Amersham Biosciences) using the TnT® Quick-cou-
plicated Transcription/Translation Systems (Promega) following the
accompanying protocol.

GST Pull-down Assay—Equal molar amounts of purified GST fusion
proteins (GST, GST-WWP1, or GST-WWP1C886S) were immobilized
to 50 μl of 50% glutathione-Sepharose 4B slurry beads (Amersham Bio-
sciences) in 0.5 ml of GST pull-down binding buffer (10 mM HEPES, pH
7.6, 3 mM MgCl2, 100 mM KCl, 5 mM EDTA, 5% glycerol, 0.5% C6360).
After incubation for 1 h at 4 °C with rotation, beads were washed three
times with GST pull-down binding buffer and resuspended in 0.5 ml of
GST pull-down binding buffer. 10 μl of 35S-labeled in vitro translated
protein (KLF5, PY1, PY2, or PY1-2) were added and mixed for 2 h at 4 °C
with rotation. The beads were then washed twice with 0.5 ml of ice-cold
radioimmune precipitation assay buffer and 1 ml of cold PBS buffer,
respectively. The bound proteins were eluted by boiling in 30 μl of
loading buffer. GST protein was visualized by Coomassie Blue stain,
and the 35S-labeled protein was detected by autoradiography.

Ubiquitin Conjugation Assay in Vitro—The Ubiquitin-Protein Conju-
agtion kit (BostonBiochem, Cambridge, MA) was used for the in vitro
ubiquitination assay. Briefly, 2 μl of rabbit reticulocyte lysate-translated
35S-labeled KLF5 was incubated in the absence or presence of bacterially
expressed GST–WWP1 (2.5 μg), 8 μg of fraction A, 8 μg of fraction B,
26 μg of ubiquitin, 4 μM ubiquitin aldehyde, and 25 μl of energy solution
(10X) in a 25 μl volume. After incubation at 37 °C for 30 min, the samples
were stopped with 25 μl of 3X sample loading buffer. Samples were elec-
trophoresed in 10% SDS-polyacrylamide gels for autoradiography.

RT-PCR and Real-time PCR—For gene amplification, primers were designed to flank exon/intron
boundaries. The primer sequences for the human WWP1 are 5’-
GATGATCGTAGAAG-3’ and 3’-GCTACCTGCTGGTCTGCT-
GTCGCGGGAGGAGAGGA-5’ (the start codon is in bold); these primer
was amplified by SYBR green real-time PCR in triplicate in an
ABI 7300 thermal cycler. The Ct values in log linear range representing the
detection threshold values were used for quantitating by the ΔΔCt
method.

siRNA Transfection—An siRNA with the sequence of 5’-GAGTT-
GATGATAGAGAGAGGA-3’ was designed to target WW1. The lucifer-
ase control siRNA was used as a negative control. PC-3 cells were trans-
fected with 200 nM chemically synthesized siRNA (Dharmacon,
Chicago, IL) using siPORT Amine (Ambion, Austin, TX) in 12-well plates. RNA and protein were collected 48 h after transfection.

Luciferase Assays—The PDGFA gene promoter was amplified from human genomic DNA by PCR. The primer sequences are: 5’-TTCTGGAGGGCGGGGGCG-3’ and 5’-TTAAGCTTGCAGGGCGCGTGTGGA-3’. Two restriction endonuclease recognition sites, XhoI and HindIII (underlined), were attached to the primers for subsequent cloning. The PCR product was purified from agarose gel, digested with XhoI and HindIII, and subcloned into the pGL3BASIC plasmid, which is a promoterless luciferase expression vector (Promega). The construct was verified by restriction digestion and DNA sequencing. The measurement of luciferase activity was described in our previous study (16).

RESULTS

WWP1 Targets KLF5 for Proteolysis through the Proteasome—We previously found that the KLF5 protein is significantly stabilized by the deletion of a 56-amino acid (codon 293–348) degradation signal adjacent to its transactivation domain (20). In the 56-amino acid sequence is a PY motif (PPPSY, codons 324–328), which could recruit WW domain-containing E3 ubiquitin ligases in the Nedd4 family, including Nedd4, WWP1 (AIP5), AIP4 (Itch), and WWP2 (AIP2) (24, 25). To search for KLF5-specific E3 ligases, we cotransfected KLF5 and each of the five E3 ubiquitin ligases from the Nedd4 family into 22Rv1 prostate cancer cells and examined the degradation of KLF5 protein by immunoblotting. We found that only WWP1 significantly degraded KLF5 protein (Fig. 1A), suggesting that WWP1 could be a specific E3 ligase for KLF5.

To further test whether WWP1 affects the degradation of KLF5 protein, the Myc-tagged mouse WWP1 and a FLAG-tagged human KLF5 were cotransfected into the 22Rv1 cells. As shown in Fig. 1B, expression of WWP1 led to remarkable KLF5 proteolysis in a dose-dependent manner, which was partly blocked by the presence of the proteasome inhibitor MG132. Furthermore, measurement of the KLF5 half-life indicated that expression of WWP1 dramatically decreased the half-life of KLF5 in 22Rv1 cells, as determined by the cycloheximide chase assay (Fig. 1, C and D).

WWP1 Interacts with KLF5 in Vivo and in Vitro—To test whether WWP1 directly interacts with KLF5 at the protein level, we cotransfected the KLF5-FLAG plasmid with the Myc-WWP1C886S plasmid into 22Rv1 cells. In the Myc-WWP1C886S expression construct, the catalytic cysteine at nucleotide 886 was replaced with a serine, which prevented the degradation of KLF5 mediated by WWP1 (see below for details) and made it possible to detect the interaction between KLF5 and WWP1. As shown in Fig. 2A, KLF5 was detected by immunoblotting with anti-FLAG antibody in the protein complexes immunoprecipitated from the vector cotransfected cells (lane 2). In the immunoprecipitates from the vector...
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KLF5 can be detected in the presence of MG132. As shown in Fig. 2A, the expression of WWP1 induced a smear of KLF5 proteins with higher molecular masses than wild-type KLF5 (57 kDa) with MG132 treatment, as determined by an anti-KLF5 Ab described previously (20). On the other hand, a FLAG-tagged control E3 ligase, FLAG-Parkin (26), or empty vector control did not induce KLF5 with higher molecular masses. We also co-transfected a HA-tagged ubiquitin expression construct with KLF5 and Myc-PP1 plasmids. Expression of WWP1 not only induced a smear of KLF5 with higher molecular masses (left panel in Fig. 3B), it also significantly induced the ubiquitination of KLF5, as detected by immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-HA antibody (Fig. 3B, right panel). These results indicate that WWP1 induces the ubiquitination of KLF5 in vivo.

WWP1 Promotes KLF5 Protein Ubiquitination in Vivo—WWP1 is an E3 ubiquitin ligase, which can ubiquitinate its substrate proteins. To test whether WWP1 ubiquitinitates KLF5 protein, we cotransfected the Myc-PP1 plasmid with the KLF5 expression construct into 22Rv1 cells. As shown in Fig. 3A, the expression of WWP1 induced a smear of KLF5 proteins with higher molecular masses than wild-type KLF5 (57 kDa) with MG132 treatment, as determined by an anti-KLF5 Ab described previously (20). On the other hand, a FLAG-tagged control E3 ligase, FLAG-Parkin (26), or empty vector control did not induce KLF5 with higher molecular masses. We also co-transfected a HA-tagged ubiquitin expression construct with KLF5 and Myc-PP1 plasmids. Expression of WWP1 not only induced a smear of KLF5 with higher molecular masses (left panel in Fig. 3B), it also significantly induced the ubiquitination of KLF5, as detected by immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-HA antibody (Fig. 3B, right panel). These results indicate that WWP1 induces the ubiquitination of KLF5 in vivo.

WWP1 Ubiquitinates KLF5 in Vitro—We further tested whether WWP1 can directly ubiquitinate KLF5 in vitro. In the presence of ubiquitination reagents from an in vitro ubiquitin protein conjugation kit, in vitro translated KLF5 was effectively ubiquitinated (lane 2 in Fig. 4). Incubation of in vitro translated KLF5 protein with purified GST-fused control (lane 1), however, no KLF5 was detectable (lane 1). In addition, a FLAG-tagged control protein, FLAG-Parkin, could not be co-immunoprecipitated with WWP1C886S, although the expression level of this protein was not lower than that of the KLF5-FLAG in cell lysates as determined by the anti-FLAG antibody (Fig. 2A). These results suggest that specific interaction between KLF5 and WWP1 proteins occurred in 22Rv1 prostate cancer cells when the genes for these proteins were co-transfected.

To examine whether WWP1 directly interacts with endogenous KLF5, we expressed the Myc-tagged WWP1C886S in 293FT cells in co-transfected 22Rv1 prostate cancer cells when the genes for these proteins were previously (20). On the other hand, a FLAG-tagged control protein, FLAG-Parkin (26), or empty vector control did not induce KLF5 with higher molecular masses. We also co-transfected a HA-tagged ubiquitin expression construct with KLF5 and Myc-PP1 plasmids.

acts with KLF5 at the protein level. To further test the interaction between WWP1 and KLF5, we transfected KLF5 and Myc-PP1C886S into 22Rv1 cells, and detected their proteins by immunofluorescence staining. Fig. 2D shows localization of KLF5 in the nucleus, which is consistent with our previous observation (20). The distribution of WWP1, however, is on the membrane as well as in the cytoplasm and nucleus. Co-localization of WWP1 and KLF5 in the nucleus was clearly detected. This finding further indicates that WWP1 interacts with KLF5 in vivo.

FIGURE 2. Protein association of WWP1 with KLF5 in vivo and in vitro. A, co-immunoprecipitation of KLF5 with WWP1. The 22Rv1 cells were co-transfected with FLAG-tagged KLF5 (KLF5-FLAG) and Myc-tagged mutant WWP1 (WWP1C886S) for immunoprecipitation (IP), which was followed by Western blotting. FLAG-tagged Parkin (FLAG-Parkin) was used as a negative control. Cells were treated with MG132 (20 μM) for 4 h before collection. The lower two panels were from cell lysates without IP. B, WWP1 interacts with endogenous KLF5 in 293FT cells. The 293FT cells were transfected with Myc-PP1C886S and treated with MG132 (20 μM) for 4 h before collection at 24 h. C, pull-down of in vitro translated KLF5 protein by both wild-type (GST-PP1) and mutant (GST-PP1C886S) WWP1 but not by GST alone. D, co-localization of KLF5 and WWP1 in the nucleus of 22Rv1 cells as determined by immunofluorescence staining and confocal microscopy.
wild-type WWP1 protein resulted in a significant increase in KLF5 ubiquitination (lane 4 in Fig. 4). In contrast, mutant GST-WWP1C886S suppressed KLF5 ubiquitination (lane 5 in Fig. 4). GST alone had no effect on KLF5 ubiquitination in vitro (lane 3 in Fig. 4). These results provide direct evidence that WWP1 is an E3 ubiquitin ligase for KLF5.

The Catalytic Cysteine Is Essential for WWP1 to Induce KLF5 Ubiquitination and Proteolysis—For WWP1, the HECT domain is known to be involved in the transfer of ubiquitin. Cys-890 (Cys-886 for mouse WWP1 used in this study) is the catalytic residue in the ubiquitin ligation reaction (27). To test whether the catalytic cysteine residue is essential for WWP1-mediated ubiquitination and degradation of KLF5, we co-transfected wild-type and mutant WWP1, i.e. Myc-WWP1 and Myc-WWP1C886S. In the latter the catalytic cysteine at 886 was changed into serine, with the KLF5 plasmid into 22Rv1 cells in the presence or absence of HA-ubiquitin. We then analyzed the ubiquitination and degradation of KLF5. The C886S mutation not only abolished the activity of WWP1 in the degradation of KLF5, it also caused more accumulation of KLF5 protein without MG132 treatment (Fig. 5A). Furthermore, we found that the C886S mutation also abolished the ability of WWP1 to ubiquitinate KLF5, as detected by immunoprecipitation with anti-FLAG antibody followed by immunoblotting with anti-KLF5 or anti-HA antibody (Fig. 5B). These findings indicate that the catalytic cysteine at 886 is essential for WWP1 to target KLF5 for ubiquitination and proteolysis.

A PY Motif in a Transactivation Domain of KLF5 Is Essential for WWP1 Binding in the Ubiquitination and Degradation of KLF5—It has been established that WWP1 binds to PY motifs of target proteins through its WW domains (25). One PY motif of KLF5, PY2 spanning codons 324–328, was detected in the 56-amino acid sequence that has been demonstrated to direct the degradation of KLF5 (20). Another PY motif in KLF5, PY1 (PPCTY), was detected at codons 282–286. To determine whether the two PY motifs mediate the interaction between KLF5 and WWP1, we constructed three KLF5 mutants in which either the essential promoter of PDGFA (8, 9). Knock-down of WWP1 in both PC-3 and MCF7 cells not only resulted in an increased level of KLF5 protein, it also caused an increase in the expression of PDGFA (Fig. 4). As expected, transfection of siRNA for WWP1 into PC-3 and MCF7 cells specifically reduced the RNA level for WWP1 but did not change the RNA for both KLF5 and WWP1 but a low level of protein for KLF5 (20). (siRNA) method in the PC-3 cell line, which expresses a high level of WWP1 naturally occurs in epithelial cells. To further test whether WWP1 regulates endogenous KLF5 protein, we knocked down the expression of WWP1 using the small interfering RNA (siRNA) method in the PC-3 cell line, which expresses a high level of WWP1 and Myc-WWP1C886S failed to efficiently ubiquitinate KLF5, as detected by immunoprecipitation (IP) with anti-FLAG antibody followed by immunoblotting (IB) with anti-KLF5 or anti-HA antibody (top two panels). The lower two panels represent Western blot analysis of cell lysates without immunoprecipitation, which indicates similar transfection efficiencies for WWP1 plasmids.

WWP1 Affects KLF5 Function in Gene Regulation—KLF5 is a transcription factor, and many of its target genes have been identified. To test whether WWP1 regulates endogenous KLF5 protein, we knocked down the expression of WWP1 using the small interfering RNA (siRNA) method in the PC-3 cell line, which expresses a high level of WWP1 (Fig. 7A). The protein level of KLF5, however, was significantly increased by WWP1 knock-down, as determined by Western blot analysis (Fig. 7B). These findings suggest that endogenous regulation of KLF5 protein by WWP1 naturally occurs in epithelial cells. To further test if altered regulation of KLF5 by WWP1 affects KLF5 function, we examined the expression of PDGFA, which is a well-characterized target gene for KLF5 (8, 9). Knock-down of WWP1 in both PC-3 and MCF7 cells not only resulted in an increased level of KLF5 protein, it also caused an increase in the expression of PDGFA (Fig. 7A). Furthermore, we cloned the essential promoter of PDGFA (~71 to +16) into a luciferase reporter plasmid, and examined the effect of KLF5 and WWP1 on the
promoter activities in the 22Rv1 cells. As shown in Fig. 7C, overexpression of KLF5 increased the promoter activity of PDGFA, and co-expression of wild-type but not mutant WWP1 abolished PDGFA promoter activity mediated by KLF5. These findings suggest that WWP1 directly affects KLF5 function in its transactivation in epithelial cells.

Overexpression of WWP1 Was Frequent in Prostate and Breast Cancer Cell Lines Hyperactive in KLF5 Degradation—In some cancer cell lines from the breast and prostate, the level of KLF5 protein is significantly lower than in normal cells even though they have a high level of RNA expression for KLF5 (20). This appears to be mediated by more severe degradation of KLF5 protein in these cancer cell lines (20), which could be mediated by a hyperactive E3 ligase for KLF5. To test this possibility, we first examined RNA expression of WWP1 by RT-PCR in prostate cancer cell lines PC-3 and MDAPCa2b, both of which are hyperactive for the degradation of KLF5 (20). We found that WWP1 was significantly up-regulated in the PC-3 but not in the MDAPCa2b cell line (data not shown). A real-time PCR assay further demonstrated that the level of WWP1 RNA increased four times compared with the average WWP1 expression level in three untransformed prostate cell lines (PZ-HPV-7, PWR-1E, and RWPE-1) (Fig. 8A). In breast cancer cell lines, overexpression of WWP1 was detected in four of nine cell lines that are hyperactive for the degradation of KLF5. Therefore, overexpression of WWP1 could be one of the major mechanisms for increased degradation of KLF5 protein in some cancer samples.

Gene Amplification May Mediate the Overexpression of WWP1 in Cancer Cells—The WWP1 gene is located at the q21 band of chromosome 8 (8q21). It is about 1-Mb apart from the PrLO/TDP52 locus, which is frequently amplified in human prostate cancer including the PC-3 prostate cancer cell line (28, 29). We examined the DNA copy number for WWP1 in the prostate and breast cancer cell lines that are hyperactive for KLF5 degradation, using the PC-3 cell line as a control. We found that WWP1 had an increased copy number (>2-fold) in 5 cancer samples (PC-3, BT549, MDA-MB361, HCC70, and MDA-MB134). None of the seven untransformed cell lines showed a copy number gain at WWP1. In agreement with the literature, PC-3 doubled the copy number for WWP1 when compared with normal samples. More importantly, each of the five cancer cell lines with WWP1 copy number gain also showed overexpression of its mRNA (Fig. 8).

DISCUSSION

Protein instability is a typical feature for many important proteins that regulate cell cycle, apoptosis, and transcription, including p53, p27, and p21. Hyperactive degradation of these proteins through overexpression of their ubiquitin ligases such as Mdm2 and Skp2 is common in human carcinogenesis (30–32). Many E3 ligases are either oncogenes or tumor suppressor genes. For example, the E3 ligase for transcription factor HIF1α, VHL, is a frequently mutated tumor suppressor gene in renal carcinoma (33), and cyclin E E3 ligase, Fbw7/Cdc4, is frequently mutated in pancreatic cancer (34). It was shown recently that targeting Mdm2 can restore the apoptotic response of prostate cancer cells to androgen deprivation therapy (35, 36). Therefore, E3 ubiquitin ligases are potential therapeutic targets, and it is worthwhile to identify cancer-related E3 ubiquitin ligases.

In our previous study, we showed that the KLF5 transcription factor is degraded through the UPP in prostate and breast epithelial cells (20). A 56-amino acid degradation signal was identified in a transactivation

FIGURE 6. The PY2 motif in a transactivation domain of KLF5 is essential for efficient association of KLF5 with WWP1 as well as ubiquitination and degradation of KLF5 mediated by WWP1 in 22Rv1 cells. A and B, deletion of the PY2 motif in KLF5 protein significantly reduced its physical association with WWP1, as determined by co-immunoprecipitation followed by immunoblotting experiments (A) and by GST pull-down assay (B). C, deletion of the PY2 motif in KLF5 significantly impaired the ubiquitination of KLF5 by WWP1, as detected by co-immunoprecipitation (IP) with anti-FLAG antibody followed by immunoblotting (IB) with anti-HA (top panel) or anti-KLF5 (middle panel) antibody. The lower three panels represent direct Western blot analysis of cell lysates and serve as experimental controls. D, deletion of the PY2 motif in KLF5 made KLF5 resistant to WWP1-mediated degradation, as detected by Western blot analysis with different antibodies (indicated at the left).

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VOLUME 280 • NUMBER 50 • DECEMBER 16, 2005
domain of KLF5 for its role in directing the ubiquitination and degradation of KLF5, and the degradation of KLF5 was more active in a subset of human cancer cell lines. In this study, we tested different members of the Nedd4 E3 ligases that WW domains found in KLF5 for their role in the degradation of KLF5. We found that WWP1, but not other members of the family, mediated the degradation of KLF5 and shortened the half-life of KLF5 protein (Fig. 1). By using co-immunoprecipitation and GST pull-down assays, we found that the WWP1 protein physically formed a complex with the KLF5 protein in vivo and in vitro (Fig. 2, A–C). Association of KLF5 with WWP1 was further supported by their co-localization in the cell nucleus (Fig. 2D). More direct evidence for WWP1 as an E3 ligase for KLF5 came from finding that expression of WWP1 induced a significant increase in the ubiquitination of KLF5 in vivo (Fig. 3) and in vitro (Fig. 4) and that endogenous KLF5 also forms a complex with WWP1 (Fig. 2B).

WWP1 belongs to the HECT domain family of E3 ligases, which share a region of homology at the C terminus containing an E2 binding site and a cysteine that catalytically transfers ubiquitin to its substrate.

FIGURE 7. WWP1 affects the function of KLF5 in gene regulation in epithelial cells. A and B, knock-down of WWP1 by siRNA transfection in PC-3 and MCF7 cells did not affect the expression of KLF5 RNA but increased the expression of KLF5 protein and PDGFA RNA, as determined by RT-PCR (A) and Western blot analysis with anti-KLF5 antibody (B). Each group was in duplicate. β-Actin is used as a loading control, and the knock-down of WWP1 is visible (A). The WWP1 protein was not detected in B because of the lack of good anti-WWP1 antibody. C, wild type but not mutant WWP1 diminished KLF5 transactivation activity, as detected by a promoter-luciferase reporter assay for the PDGFA gene in 22Rv1 cells.

FIGURE 8. Increased RNA expression and DNA copy number for WWP1 in some prostate and breast cancer cell lines that are hyperactive for the degradation of KLF5, as determined by real-time PCR assay. Glyceraldehyde-3-phosphate dehydrogenase was used as a control to normalize the reading for WWP1 in each sample. The left panel is for prostate cancer cell lines and the right panel is for breast cancer lines. The immortalized epithelial cell lines labeled with asterisks are controls.
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(37). When the catalytic cysteine 886 in WWP1 was mutated, WWP1 lost its ability to ubiquitinate and degrade KLF5 (Fig. 5). On the other hand, the mutant WWP1 still binds to KLF5 efficiently (Fig. 2). Therefore, WWP1 directly binds to and ubiquitinates KLF5, and the domains for binding and ubiquitinating in WWP1 are likely distinct from each other. These findings indicate that the WWP1P886S mutant could play a dominant negative role in the degradation of KLF5.

It has been established that the WW domains in WWP1 and other members of Nedd4 E3 ligase family are responsible for protein binding between an E3 ligase and its substrate (38, 39). Although it remains to be determined how the WW domains in WWP1 are involved in the interaction and if any other domains are also involved, we found that the PY2 motif in a transactivation domain of KLF5 plays a significant role. Deletion of the PY2 motif in KLF5 not only impaired its physical binding with WWP1 (Fig. 6, A and B), but also diminished the ubiquitination and degradation of KLF5 mediated by WWP1 (Fig. 6, C and D). Deletion of another PY motif in KLF5, PY1, also affected the association between KLF5 and WWP1 and reduced WWP1-mediated ubiquitination and degradation of KLF5. However, the effect of PY1 deletion was much weaker than that of PY2 deletion (Fig. 6). Weak interaction between WWP1 and KLF5 was also detectable with both PY1 and PY2 deleted (Fig. 6, A and B), suggesting that other domains in KLF5 may also have a role. Therefore, the interaction of KLF5 with WWP1 in the ubiquitination and degradation of KLF5 mainly occurs at the PY2 motif of KLF5, although other domains may also be involved.

In the PC-3 prostate cancer cell line, which overexpresses WWP1 (Fig. 8), RNAi-mediated knock-down of endogenous WWP1 expression resulted in increased accumulation of endogenous KLF5 protein. An increased KLF5 protein level resulted in an increase in the expression of PDGFA transcripts through an induced promoter activity for PDGFA (Fig. 7), a gene known to be regulated by KLF5 (8, 9). Similar results were observed in the MCF7 breast cancer cell line. These functional results suggest that alterations in the expression of WWP1 can modulate the function of KLF5. Based on our expression analysis, the WWP1 gene is overexpressed in 8 of 12 prostate and breast cancer cell lines that are hyperactive for KLF5 degradation (Fig. 8), suggesting that overexpression of WWP1 is common but not the only reason for more severe degradation of KLF5 in cancer cells. The WWP1 gene is located at the q21 band of chromosome 8 (8q21), a region with frequent copy number gain in prostate and breast cancers (40, 41). It is only 1-Mb apart from the recently characterized PrlZ/TPD52 gene, which is amplified in prostate cancer (28, 29). Our fluorescence in situ hybridization analysis of prostate cancer cell lines LNCaP, DU 145, and PC-3 using a DNA fragment spanning the PrlZ gene indicated that PC-3 has duplicated the copy number for this gene at 8q21 (data not shown). In eight cancer cell lines with WWP1 gene overexpression, five showed copy number gain as determined by real-time PCR (Fig. 8). Overexpression of WWP1 has also been detected in cancer cell lines from different tissues (27). Therefore, WWP1 could be a target gene of amplification at 8q21 that functions as an oncogene in human cancer. We are currently testing this hypothesis. WWP1 is structurally related to E3 ubiquitin ligases for Smads and TGF-β superfamilies receptors, and has been demonstrated to negatively regulate TGF-β tumor suppressor signaling in cooperation with Smad7 (27, 42). These findings support the concept of WWP1 as an oncogene in human cancer.

Post-translational modification of KLF5 may be necessary for its ubiquitination and degradation mediated by WWP1. In the detection of WWP1-mediated KLF5 ubiquitination in an in vitro assay (Fig. 4), the ubiquitin conjugation system contained not only the full complements of conjugation enzymes (E1, E2s, and E3s) but also modification enzymes such as kinases and phosphatases. When only purified components including E1, GST-UbcH5b (E2), GST-WWP1 (E3), ubiquitin, ATP, and KLF5 were used, no ubiquitination of KLF5 was detected (data not shown). Therefore, other post-translational modifications of KLF5 may be required for its ubiquitination. Phosphorylation and acetylation of KLF5 have been reported in previous studies (17–19), and phosphorylation of serine and threonine residues adjacent to a PY motif enhances protein ubiquitination by the Nedd4 E3 ligases (43). It remains to be determined if and how post-translational modification of KLF5 affects its ubiquitination and degradation mediated by WWP1.

Although E3 ligases are more specific than E1-activating enzyme and E2-conjugating enzyme in the ubiquitination-proteasome pathway (44), KLF5 is not the only substrate for WWP1, as other proteins including KLF2, Tjp1, Smad2, and Smad4 have been shown to be degraded by WWP1 (21, 42, 45). On the other hand, WWP1 appears to be the only Nedd4 family E3 ligase for KLF5 because WWP2, AIP4, Nedd4-1, and Nedd4-2 do not promote KLF5 protein degradation, although all of them bear WW domains (Fig. 1A).

In summary, we established WWP1 as an E3 ligase for KLF5 by demonstrating a specific physical interaction between WWP1 and KLF5 and by detecting the ubiquitination and degradation of KLF5 mediated by WWP1. We also validated a PY motif critical for the interaction between WPP1 and KLF5. Furthermore, WWP1 was overexpressed in some prostate and breast cancer cell lines, likely through genomic amplification of 8q21 in these cancers, which affected the function of KLF5 in gene regulation. These findings are useful for understanding the role of KLF5 and WWP1 in the development and progression of human cancer.

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