Constitutive and UV-induced Fibronectin Degradation Is a Ubiquitination-dependent Process Controlled by β-TrCP

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Fibronectin (FN), a matrix glycoprotein, plays important roles in cell adhesion, migration, growth, and differentiation (1). The functional FN consists of two 250-kDa subunits linked covalently near their C termini by a pair of disulfide bonds. There are two different forms of fibronectins: plasma “cold insoluble globulin” and tissue FNs. Both forms originate from the same gene by alternative splicing in three different regions (EIIIA, EIIIB, and IIICS), giving rise to ~20 isoforms (2). FN generally interacts with integrins, a major component of the extracellular matrix (ECM), and thereby facilitates bilateral exchange of information between cells and the matrix environment (3). It has been known for decades that malignant transformation modifies such cell-matrix interactions (4), and perturbed FN matrix assembly has been recognized as a hallmark of transformed cells (5, 6). During transformation cells lose their surface FN, which has so far been attributed either to transcriptional down-regulation or to proteolytic degradation (7, 8). However, it is obscure how post-translational modifications control the metabolism of FN. It is necessary to study FN protein stability because significant up-regulation of FN protein has been detected in metastatic breast cancer tissues, without significant increase in the transcript levels (9).

It has been well established that ubiquitination plays an important role in proteasomal degradation of proteins. Recent findings also emphasize the role of ubiquitination in endocytosis of plasma membrane-associated proteins followed by their sorting and lysosomal degradation (10). Several E3 ubiquitin ligases, such as c-Cbl/Hakai and Rsp5/NEDD4, have been implicated in such processes (11). More recently, another ubiquitin ligase, Homolog of Slimb (HOS), also known as β-TrCP2 or Fbw1b, was reported to mediate the ligand-induced ubiquitination of interferon-α receptor followed by its endocytosis and lysosomal degradation (12).

Although there is only limited information about ubiquitin-mediated control of FN, FN matrix assembly is regulated by physical interaction with tumor suppressor von Hippel-Lindau protein. At a molecular level von Hippel-Lindau protein forms complexes with cullin 2, elongin B, and elongin C, together known as the VEC complex (13). The cullin 2 and elongin C component of VEC complex is structurally similar to cullin 1/Cdc53 and Skp1, respectively, of the Skp1, Cullin, and F-box (SCF)-like ubiquitin ligases in yeast (14). It was thus speculated that ubiquitination might be playing an important role in FN matrix assembly. It was also shown recently that relaxin, a polypeptide hormone member of the insulin-like growth factor family, could increase the ubiquitin-dependent degradation of FN in vitro (15). Interestingly, FN has been recently reported to undergo continuous turnover through caveolin-1-dependent endocytosis followed by lysosomal degradation (16). According to these data, we speculate that the lysosomal degradation of FN may be regulated by ubiquitination. In this report, we have identified SCFβ-TrCP as a potent ubiquitin ligase that determines the rate of FN matrix turnover by promoting lysosome-mediated degradation.
EXPERIMENTAL PROCEDURES

Cell Culture—Human osteosarcoma U2OS cells, mammary epithelial MCF-10A cells, and human breast cancer cell lines MCF-7, T47D, MDA-MB-231, and MDA-MB-468 were obtained from the American Type Culture Collection. U2OS, MCF-7, MDA-MB-231, and MDA-MB-468 cells were cultured in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal bovine serum, and MCF-10A cells were cultured in DMEM/F12 mixed (1:1) medium supplemented with 5% horse serum. To inhibit proteasome-dependent degradation of proteins, cells were treated with 2 μM MG132 for 4 h. To inhibit lysosomal degradation, cells were treated with 20 mM methylamine hydrochloride for 4 h.

Small Interference RNA—Small interference RNAs (siRNAs) were custom synthesized by Dharmacon. The sequences of anti-β-TrCP1/β-TrCP2 siRNAs were described previously (17). Cells were transfected with 200 nM specific siRNAs or randomized cocktails of double-stranded RNA as a control, using the Oligofectamine reagent (Invitrogen). At 24 h post-transfection, cells were transfected again with the same RNA preparations to ensure efficient silencing effects.

Reverse Transcription PCR—Total RNA was isolated from cells using the TRIzol reagent (Invitrogen). Total RNA (1 μg) was subjected to reverse transcriptase reaction using the SuperScript First-Strand synthesis system (Invitrogen). After the reverse transcriptase reaction, RNaseH was added to remove the RNA template from the reaction. Subsequently, PCR was performed in a total volume of 50 μl using 1 μl of the reverse transcriptase product. To confirm that the amounts of target mRNAs were within semiquantitative linear range of reaction, increasing amounts of RNA samples were examined in pilot experiments. The primers used for β-TrCP1 amplification were 5′-GGGGCCTATGATGGAAAAAT-3′ and 5′-TATGTTCGAGAAGGGGAACG-3′ and those for β-TrCP2 amplification were 5′-AAAACGGCTGGAATGTGGT-3′ and 5′-CAGTCATTGTGTAAGCGTATA-3′. The primers used for co-amplification (143 kb) of the control RPS14 ribosomal mRNA were 5′-GGGCAAGCCGAGATATCTCA-3′ and 5′-CAGTGTCAGGTCCTGAA-3′. The reaction was performed in the PTC-200 Peltier Thermal Cycler (MJ Research) at 94 °C for 2 min, followed by 23 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min. Amplified DNAs were analyzed by agarose gel electrophoresis, and signals in ethidium bromide-stained gels were quantified using the EDAS-290 Imaging System (Kodak).

Antibodies, Immunoblotting, Immunoprecipitation, and Immunostaining—Polyclonal anti-fibronectin (Ab-10) antibody was purchased from Neomarker, and mouse monoclonal anti-fibronectin antibody was purchased from Calbiochem. Anti-β-actin (AC-15) monoclonal antibody was purchased from Sigma. Horseradish peroxidase-conjugated anti-mouse and rabbit immunoglobulin secondary antibodies, fluorescein isothiocyanate-conjugated anti-mouse and Texas Red-conjugated anti-rabbit, as well as recombinant protein A-agarose beads and the Supersignal West Pico chemiluminescence reagent, were obtained from Pierce. To prepare cell lysates, cells were scraped off and lysed by sonication in a buffer including 50 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium orthovanadate, ×100 diluted protease inhibitor mixture (P8340; Sigma), 10% glycerol, and 0.1% Nonidet P-40. All the chemicals were purchased from Sigma. Immunoblotting and immunofluorescence staining were performed as described previously (18). Fluorescent-labeled cells were imaged on a Zeiss Axiosvert 200 inverted microscope followed by optical sectioning using Apotome slider module (Carl Zeiss Inc.). The signals on films were quantified by densitometric scanning using the GS-700 Imaging System and Molecular Analyst software (Bio-Rad).

Differential Separation of Matrix-associated FN—Matrix preparations were as described previously (19). In brief, MCF-10A cells were transfected with small interference RNA as described above. At 48 h post-transfection, cells were either left untreated or treated for 5 min with 20 mM ammonium hydroxide (NH₄OH). Released intracellular materials were washed completely with a flow of water followed by wash with phosphate-buffered saline. Matrix were collected by scraping using the
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A

siRNA:  

\[
\begin{array}{ccc}
\text{NH}_4\text{OH} & \text{NS} & \beta-\text{TrCP2} & \text{NS} & \beta-\text{TrCP2} \\
- & - & + & - & + \\
\text{WB: Fibronectin} & & & & \\
\end{array}
\]

B

\[
\begin{array}{cccc}
\text{NH}_4\text{OH} & \text{NS siRNA} & \beta-\text{TrCP2 siRNA} & \\
- & - & - & \\
\text{a} & \text{b} & \text{c} & \text{d} \\
\end{array}
\]

FIGURE 2. β-TrCP2 siRNA-treated MCF-10A cells accumulate intracellular fibronectin without significant changes in extracellular FN matrix assembly. A, cells were either mock transfected or transfected with β-TrCP2 siRNA. At 48 h post-transfection, cells were either left untreated or treated with 20 mM ammonium hydroxide for 10 min. Total cell lysates or matrix-only-associated fractions were collected as described under “Experimental Procedures.” FN protein levels were determined by immunoblotting. B, immunostaining of FN in whole cell cultures (left panels) and extracellular matrix-only fractions (right panels). MCF-10A cells were transfected with indicated siRNAs, and samples were prepared.

RESULTS

Fibronectin Contains Putative β-TrCP Recognition Motifs—
FN was reported to undergo continuous endocytosis and degradation at the lysosome in a caveolin-1-dependent manner (16). In accordance with recent studies that have suggested the involvement of ubiquitination in endocytosis and lysosomal degradation of receptors (10), we first examined the involvement of the above ubiquitin ligases in our system. As β-TrCP was known to recognize its substrate through DpSG(X)2+nPS destruction motif, we analyzed human, mouse, and rat FN peptide sequences and identified two such destruction motifs (DSGVVYS and DSGSIVVS) that are conserved in all three species (Fig. 1A).

β-TrCP1/2 siRNA Increases Endogenous Fibronectin Levels—
To investigate whether FN is a substrate for SCFβ-TrCP, we transfected human mammary epithelial MCF-10A cells with β-TrCP1 and β-TrCP2 siRNA and analyzed total (cellular and matrix-associated) FN levels by Western blotting. As expected, a significant increase in the FN level was detected both in β-TrCP1- and β-TrCP2-siRNA treated cells (Fig. 1B). However, β-TrCP2 was more effective in stabilizing FN levels compared with β-TrCP1 (Fig. 1B). To evaluate the importance of β-TrCP in regulating FN levels in other cell line cultures, we tested the effect of β-TrCP2 knock down in a panel of human breast cancer cell lines (MDA-MB-468, MDA-MB-231, and T47D) and also in the human osteosarcoma U2OS cells. In all cases tested, β-TrCP2 knock down resulted in significant increases in endogenous FN levels within 48 h of siRNA transfection (Fig. 1C). Taken together, these data indicate that SCFβ-TrCP negatively regulates FN levels.

β-TrCP2 Suppression Results in Accumulation of Intracellular FN—
Because we consistently observed increased levels of total fibronectin in β-TrCP2 siRNA-treated cultures, we then examined the FN localization in control and β-TrCP2 siRNA-treated MCF-10A cells. To fractionate matrix-associated FN, we eliminated cellular proteins from culture preparations by osmotically lysing cells with NH4OH followed by repeated wash as described under “Experimental Procedures.” Immunoblotting demonstrated no major change in the amount of matrix-associated FN in β-TrCP2 siRNA-treated cultures compared with control cultures (Fig. 2A). Immunostaining of similarly treated cell cultures also showed comparable staining patterns, structure, and levels of matrix-associated FN (Fig. 2B, c and d). In sharp contrast, immunostaining of whole cell culture preparations showed that β-TrCP2 siRNA-treated cells had significantly increased levels of intracellular FN staining compared with control cultures (Fig. 2B, a and b). Therefore, β-TrCP appears to play an essential role in intracellular processing or disposal of FN and probably not in its matrix assembly; absence of such ubiquitin ligase activity could lead to intracellular accumulation of FN.

Fibronectin Undergoes Ubiquitination and Degradation upon UV Irradiation—
Treatment of cells with genotoxic agents such as UV irradiation, X-rays, and alkylating compounds is known to alter the synthesis as well as degradation of various extracellular matrix-associated proteins (20–23). Thus, we examined whether UV or γ-irradiation could alter FN protein levels. When U2OS cells were exposed to different doses (20–200 J/m2) of UV irradiation and further incubated for different time periods, progressive down-regulation of total FN levels was detected in a dose-dependent manner (Fig. 3B). Moreover, immunoprecipitation of FN followed by immunoblotting for ubiquitin showed a significant increase in FN ubiquitination upon UV treatment of U2OS cells, confirming the

lysis buffer described above, and for immunostaining, matrix were fixed using 10% buffered formalin.

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role of ubiquitination in the process (Fig. 3C). In contrast, when cells were treated with γ-irradiation, we did not detect any significant change in the total FN level even at higher doses of γ-irradiation (10 grays) (Fig. 3B).

**UV-mediated Degradation of Fibronectin Is Mediated by Lysosomes**—As it was previously demonstrated that FN undergoes continuous turnover through a lysosome-dependent pathway, we examined whether UV-dependent FN down-regulation was mediated by either lysosomal or proteasomal degradation. U2OS cells were UV irradiated in the presence of the lysosomal inhibitor methylamine hydrochloride or the proteasomal inhibitor MG132. The UV-mediated FN degradation was almost completely protected by methylamine hydrochloride, whereas MG132 was ineffective (Fig. 4A). These results suggest that UV irradiation induces rapid ubiquitination and lysosomal degradation of FN.

**β-TrCP2 Is Involved in UV-mediated Degradation of FN**—Because it became clear that the UV-mediated FN degradation is a lysosomal event preceded by increased ubiquitination, we then assessed whether SCFβ-TrCP plays a key role in the process. Interestingly, previous studies suggested that SCFβ-TrCP can control not only proteasomal but also lysosomal degradation of the interferon-α receptor (12). Thus, we transfected U2OS cells with β-TrCP2 siRNA, and 48 h later cells were exposed to 20 J/m² UV irradiation. Total FN levels in cultures were measured by immunoblotting at 2 h post-irradiation. β-TrCP2 knockdown by siRNA significantly blocked the UV-mediated FN degradation, suggesting that β-TrCP2 is involved in the UV-mediated lysosomal degradation of FN (Fig. 4B).

**DISCUSSION**

ECM remodeling is known to play important roles in various biological events, including tissue homeostasis and malignant transformation of cells (24, 25). Among various regulators, FN matrix polymerization is a key regulator of ECM remodeling (26). Recent studies also indicated that FN matrix turnover is a critical determinant for ECM remodeling (16). Therefore, identification of factors regulating FN matrix turnover is essential for better understanding of the biology of ECM. Previous studies have shown that FN matrix turnover occurs through a caveolin-1-dependent endocytic process followed by lysosomal degradation (16). Although initially it was believed that the lysosomal degradation of proteins might not be precisely regulated, more recent studies showed that ubiquitination plays important roles not only in proteasomal degradation but also in lysosomal degradation (27). In this report, we attempted to identify the ubiquitin ligase for FN turnover. By locating the conserved amino acid motif DSG(X)₂₋riages and also by using siRNA technology, we could identify SCFβ-TrCP as a ubiquitin ligase promoting constitutive as well as UV-mediated FN degradation.

As ubiquitination has already been implicated for FN matrix assembly (13), we also investigated the effect of β-TrCP knockdown on FN matrix assembly. However, our data indicate that β-TrCP does not significantly affect FN matrix assembly. Thus, ubiquitination of FN by different E3 ligases may lead to distinct consequences, i.e., von Hippel-Lindau protein-mediated ubiqu-
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ubiquitination regulating matrix assembly (28) and β-TrCP-mediated ubiquitination promoting lysosomal degradation. A fine balance between the activities of the two ubiquitin ligases may determine the homeostasis of FN. At this point it is plausible to hypothesize that following endocytosis, FN has to be ubiquitinated by β-TrCP before being targeted to lysosome for degradation.

Another possibility is that β-TrCP-mediated ubiquitination and degradation of FN is important for “ER quality control.” In eukaryotic cells the endoplasmic reticulum (ER) has the inherited ability to ensure that only correctly folded and assembled proteins are forwarded to their destination (29, 30). Most of the membrane-associated and secretory proteins are synthesized in the polypeptides bound to the rough ER. During and shortly after its synthesis, the ectodomain assumes proper conformation in the lumen of the ER. Proteins that fail to assume their correct final conformation in the lumen of the ER in most cases undergo ubiquitin-dependent degradation in the cytosol (31). For example, aggrecan, a major proteoglycan of the cartilage extracellular matrix, has already been reported to undergo such ubiquitination-dependent proteasomal degradation (32). It is possible that if FN protein is misfolded it may undergo ubiquitination-dependent lysosomal degradation and SCFβ-TrCP may play an essential role in the process. However, whether the intracellularly accumulated FN proteins in cells with β-TrCP knock down are misfolded, similarly to aggrecan, remains to be determined. Recent reports have shown that certain human cancers, e.g. metastatic thyroid carcinoma (33), display intracellular accumulation of FN, which may be associated with the more aggressive behavior of such tumors. Interestingly, in the peptide sequence of aggrecan we noticed the DSG(\(\chi\))2+nS motif, which can make this protein a probable substrate for β-TrCP. Thus, SCFβ-TrCP might play an essential role in ER quality control by degrading misfolded proteins.

It has been known for about a decade that cytoskeletal geometry plays a central role in activating intracellular signaling pathways that lead to cell cycle progression (34, 35). Proper FN matrix assembly in turn specifies the organization of the cytoskeleton and thereby can regulate cell proliferation. It is also well known that exposure of cells to UV or γ-irradiation can cause arrest of cell proliferation. A major emphasis has been placed on DNA damage-activated G1, S, or G2 checkpoints as mechanisms for such arrest (36). Although only a few studies have addressed the effect of radiation on ECM rearrangements, such studies were able to demonstrate significant alterations on ECM gene expression and protein degradation upon irradiation (20–22, 37–39). However, whether there is any cross-talk between matrix rearrangement and intracellular signaling events leading to activation of cell cycle checkpoints has, to our knowledge, not been explored. Recent studies by Cordes et al. (37) implied that the optimum cellular response to irradiation-induced growth arrest requires a coordinated interaction between cell matrix and cell growth factors. However, such issues have so far been poorly addressed.

Previous studies have demonstrated FN expression was significantly altered at both the transcript and protein levels in response to irradiation (20, 38). In this study we have shown a significant time- and dose-dependent decrease in FN protein levels when cells were treated with UV irradiation. Such degradation was significantly protected by lysosomal inhibitor, but not by proteasomal inhibitor. Such lysosomal degradation was preceded by increased ubiquitination, regulated by β-TrCP. In contrast, γ-irradiation did not show any significant alteration in FN levels, which is consistent with the previous report (20). According to previous studies, Cdc25A, a phosphatase critical for cell cycle progression, undergoes ubiquitination-dependent proteasomal degradation in a SCFβ-TrCP-dependent manner upon DNA damage by UV or γ-irradiation (40). Here we have shown that FN also undergoes SCFβ-TrCP-dependent ubiquitination followed by lysosomal degradation in response to UV irradiation. Therefore, it would be interesting to check whether UV irradiation triggers any significant alteration in FN-integrin signaling, which is a previously unexplored field of research with respect to damage-induced cell cycle arrest. As β-TrCP recognizes its substrate in a phosphorylation-dependent manner, efforts are ongoing in our laboratory to identify the kinase(s) responsible for FN phosphorylation. Although FN is known to undergo phosphorylation (41), the responsible kinase remains to be determined.

To conclude, our study has identified β-TrCP as a ubiquitin ligase regulating the lysosomal degradation of FN. β-TrCP-dependent FN degradation could regulate turnover of matrix FN and/or disposal of misfolded FN. Further investigation is necessary to fully understand the intracellular regulation of FN metabolism that could be perturbed in various human malignancies.

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