FAT10 is a proteasomal degradation signal that is itself regulated by ubiquitination

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ABSTRACT **FAT10 is a ubiquitin-like protein modifier that is induced in vertebrates following certain inflammatory stimuli. Its functions and the repertoire of its target substrates have remained elusive. In contrast to ubiquitin, its cellular abundance is tightly controlled by both transcriptional and posttranslational regulation, and it was reported to be rapidly degraded by the proteasome. Here we provide data to indicate that the degradation of FAT10 requires ubiquitination: degradation was inhibited in cells expressing a ubiquitin mutant that cannot be polymerized and in a mutant cell harboring a thermolabile ubiquitin-activating enzyme, E1. Of importance, FAT10 can serve as a degradation signal for otherwise stable proteins, and in this case, too, the targeting to the proteasome requires ubiquitination. Degradation of FAT10 is accelerated after induction of apoptosis, suggesting that it plays a role in prosurvival pathways.**

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INTRODUCTION

Protein degradation is essential for many cellular processes, including cell cycle, regulation of gene expression, and responses to stress. A major degradation pathway involves the modification of target proteins by ubiquitin followed by their proteasomal degradation (Glickman and Ciechanover, 2002). However, it is now known that posttranslational modification of proteins by ubiquitin also serves numerous nonproteolytic functions (Welchman *et al.*, 2005; Chen and Sun, 2009). Besides ubiquitin, several ubiquitin-like molecules have been discovered; they all have structural homology to ubiquitin, and many of them can also be conjugated to target proteins in order to modify their fate (Welchman *et al.*, 2005; Hochstrasser, 2009). However, little is known on the regulation of ubiquitin-like proteins, and in particular on the role of ubiquitination in controlling their level. Ubiquitin, for example, has a half-life of 9–10 h (Carlson and Rechsteiner, 1987; Hiroi and Rechsteiner, 1992) and can be degraded by the proteasome as a monomer, along with the substrate to which it is conjugated, but also when fused to a short peptide of at least 20 residues (Shabek *et al.*, 2009). Although ubiquitin was shown to be ubiquitinated (Bernal-Bayard and Ramos-Morales, 2009), it appears that its degradation does not require further ubiquitination (Shabek *et al.*, 2009). Two other ubiquitin-like proteins, NEDD8 and SUMO, are degraded faster than ubiquitin in a proteasome-dependent manner (Tanaka *et al.*, 2003; Hipp *et al.*, 2004; Tatham *et al.*, 2008), but a role for ubiquitination in this process has not been shown.

FAT10 appears to be unique among all other ubiquitin-like proteins. It is limited to vertebrates and is expressed following specific proinflammatory stimuli in particular tissues such as the thymus and the spleen (Fan *et al.*, 1996; Gruen *et al.*, 1996; Liu *et al.*, 1999). FAT10 is composed of two in-tandem ubiquitin-like domains that both have ~30% identity with ubiquitin (Liu *et al.*, 1999). Like ubiquitin, FAT10 harbors a C-terminal diglycine motif used for its activation by a specific E1 enzyme (E1-L2) and subsequent conjugation to its substrates, which have not been identified yet (Raasi *et al.*, 2001; Hipp *et al.*, 2005; Chiu *et al.*, 2007). FATylation of downstream proteins was hypothesized to target them to proteasomal degradation (Raasi *et al.*, 2001; Hipp *et al.*, 2005).

The importance of the regulation of FAT10 expression has been highlighted by different observations: 1) it is overexpressed in many types of cancer cells (Lee *et al.*, 2003; Lim *et al.*, 2006) and 2) its absence in transgenic mice leads to spontaneous apoptosis of lymphocytes (Canaan *et al.*, 2006). However, this prosurvival role has been contradicted by other studies showing that FAT10 overexpression leads to apoptosis of cultured cells (Raasi *et al.*, 2001; Ross *et al.*, 2006). Finally, upregulated FAT10 expression has also been

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Abbreviations used: CHX, cycloheximide; DHFR, dihydrofolate reductase; GFP, green fluorescent protein; IFNγ, interferon γ; STS, staurosporine; TNFα, tumor necrosis factor α; Ub, ubiquitin; WT, wild type.

associated with aberrant mitosis and aneuploidy in a colon carcinoma cell line (Ren *et al.*, 2006).

Although FAT10 expression is induced by inflammatory cytokines like interferon γ (IFNγ) and tissue necrosis factor α (TNFα; Liu *et al.*, 1999; Chiu *et al.*, 2007; Lukasiak *et al.*, 2008), retinoids (Dokmanovic *et al.*, 2002), or a viral infection (Fan *et al.*, 1996; Gruen *et al.*, 1996; Ross *et al.*, 2006), it is repressed by p53 (Zhang *et al.*, 2006). Moreover, FAT10 is also subjected to a cell cycle–dependent control (Lim *et al.*, 2006). The transcriptional regulation of FAT10 is accompanied by a posttranslational regulation involving fast proteasomal degradation (Raasi *et al.*, 2001; Hipp *et al.*, 2004, 2005). This rapid regulation appears to be unique to FAT10. Given the potential effects of FAT10 on cell growth and survival, it is particularly important to understand how cells remove this molecule when it is no longer required.

A few studies showed that FAT10 can serve as a degradation signal for itself and for downstream, otherwise stable substrates that are fused to it in a process that does not require further ubiquitination (Hipp *et al.*, 2004, 2005; Schmidtke *et al.*, 2006). In this study we show that the degradation of FAT10 by the proteasome requires its prior ubiquitination. Lysine-less FAT10 rapidly disappears, which may have led researchers to believe it is degraded without any further ubiquitination. Our study shows that the mutated protein is rapidly aggregated and precipitated in an insoluble fraction. In contrast, the wild-type (WT) protein appears to require polyubiquitination for its degradation. It is stabilized in cells expressing nonpolymerizable ubiquitin and in cells harboring a thermolabile mutation in the ubiquitin-activating enzyme, E1. FAT10 can serve as a degradation signal for otherwise stable proteins, but here, too, as for the degradation of FAT10 itself, further polyubiquitination is required. Finally, in response to induction of apoptosis, FAT10 is rapidly ubiquitinated and degraded, suggesting that it plays a role in prosurvival pathways.

RESULTS

Degradation of FAT10 is ubiquitination and proteasome dependent

To assess whether FAT10 is degraded by the proteasome, we induced its expression in HEK-293 with TNFα and IFNγ (Lukasiak *et al.*, 2008) and monitored its disappearance following the addition of the translation inhibitor cycloheximide (CHX; Figure 1A). The degradation of endogenous FAT10 was complete after <4 h ($t_{1/2}$ < 1 h) and could be inhibited by the proteasome inhibitor MG132. This inhibition by MG132 was maximal during the first 2 h of CHX treatment (Figure 1A, ii). Moreover, treating the cells with MG132 in the absence of CHX induced an accumulation of FAT10 (Figure 1A, i). These results show that FAT10 is rapidly degraded by the proteasome.

At that point it was interesting to demonstrate a physical association between FAT10 and the proteasome. Because FAT10 is a ubiquitin-like protein, it was logical to assume that it also associates with the proteasome. As can be seen in Figure 1B, when immunoprecipitated, hemagglutinin (HA)-FAT10 also coprecipitates the 20S proteasome subunit α6 and the 19S subunit RPT5. The association appears to be specific, as precipitation of an irrelevant HA-tagged protein, DHFR, did not pull down these proteasome subunits. To avoid FATylation, we also used a derivative of FAT10 in which the C-terminal Gly residue was substituted with Val (Raasi *et al.*, 2001; Hipp *et al.*, 2005). Furthermore, to avoid ubiquitination, we used a derivative of FAT10 in which all internal lysine residues were substituted with arginines (HA-FAT10-K0), thus making sure that we observe the association of a single molecule of FAT10 with the proteasome.

An unsolved problem is whether degradation of FAT10 requires polyubiquitination and, if so, whether the 17 lysines in FAT10 are important for its modification and subsequent degradation. To address this problem, we compared the degradation of HA-FAT10-GV and HA-FAT10-K0-GV. An initial examination of the CHX chase stability experiment revealed that the half-lives of HA-FAT10-GV and HA-FAT10-K0-GV were ~1 and 4 h, respectively (Figure 1C, i). It is important to emphasize that the inhibitory effect of the proteasome inhibitor MG132 was incomplete, although it was significantly more prominent with the WT protein compared with its K0 counterpart (fourfold-higher level of the WT protein compared with 20% higher level in that of the K0 species at time 4 h). In addition, unlike an increase in the level of HA-FAT10-GV in cells that were treated with MG132 but not with CHX, we could not observe a similar increase in cells expressing its K0 counterpart (Figure 1C, ii). These data suggested that the disappearance of the two species of FAT10 is partially attributed to its degradation but also partially to its accumulation in an insoluble form that is not sensitive to the proteasome. The WT species appears to be less susceptible to aggregation than K0 and therefore more sensitive to degradation by the proteasome. Indeed, as can be seen in Figure 1D, i, newly synthesized (as monitored following labeling with ^{35}S-methionine) HA-FAT10-K0-GV is accumulating faster in an insoluble form compared with the WT protein, and the process, as expected, is temperature sensitive, that is, it occurs faster at a higher temperature (37 vs. 30°C). Quantification of the data is depicted in Figure 1D, ii. This was also corroborated in a CHX chase experiment (Figure 1E). It should be noted that FAT10 was already reported to be partially localized to aggresomes (Kalveram *et al.*, 2008).

Thus, mutational substitution of lysines in FAT10 appears to render the protein more susceptible to aggregation, and its disappearance in different chase experiments (following addition of CHX of chasing radioactive labeling) does not reflect its degradation. Therefore it is difficult to conclude from such an experimental approach whether degradation of FAT10 requires further ubiquitination, and other approaches are necessary.

To demonstrate that the degradation of FAT10 is ubiquitin dependent, we used two experimental approaches. In the first, we overexpressed lysine-less ubiquitin that cannot be polymerized and demonstrated that it results in stabilization of FAT10, whereas the overexpression of WT ubiquitin (Ub) did not (Figure 2A). It should be noted that K0-ubiquitin modifies FAT10 (Supplemental Figure S1), competing with the modification by WT Ub, which probably explains its inhibitory effect on the degradation of the molecule. In this experiment, too, the degradation of FAT10 was sensitive to inhibition by MG132, demonstrating that a significant part of the WT protein is degraded rather than aggregated. In a second approach, we used the ts20 hamster mutant cell, which harbors a thermolabile, mutated, ubiquitin-activating enzyme E1 (Kulka *et al.*, 1988). As can be seen in Figure 2B, heat inactivation of E1 abrogates degradation of FAT10 in the mutant cell, but the high temperature has no effect on the degradation of the protein in the WT cell E36. Taken together, these results strongly suggest that degradation of FAT10 is ubiquitination dependent.

Thus, one should be careful in concluding that the degradation of any protein is ubiquitination-dependent or ubiquitination-independent by relying on mutational substitution of lysine residues. Indeed, based on a similar mutational analysis, it was reported that the degradation of FAT10 is ubiquitin independent, where we suspect that the protein tested was aggregated and therefore escaped solubilization and detection in the assay used (Hipp *et al.*, 2005). Our data using expression of lysine-less ubiquitin and the ts20 cells

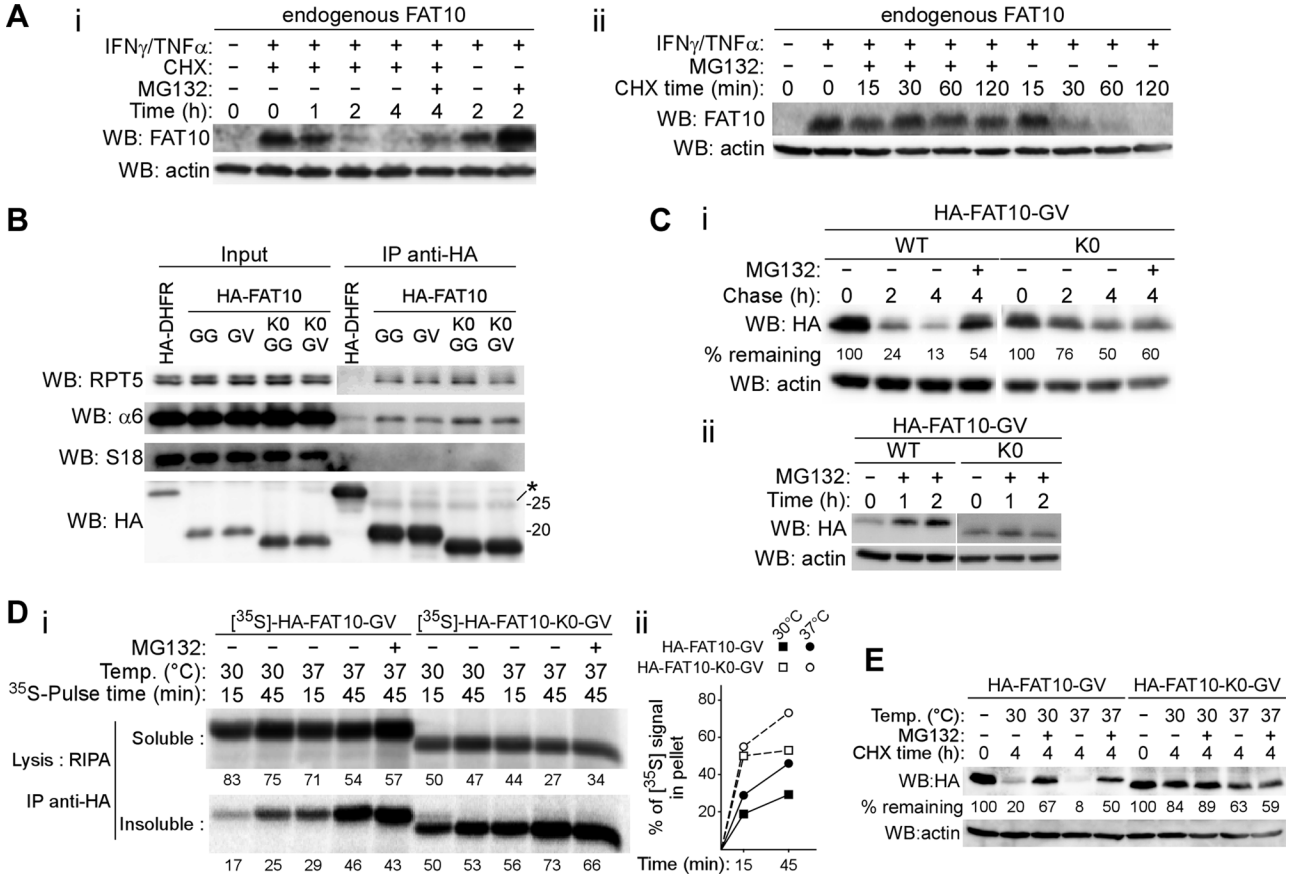

FIGURE 1: Proteasomal degradation of FAT10. (A) HEK-293 cells were treated for 24 h with TNFα and IFNγ to induce endogenous FAT10. (i) When indicated, cells were treated with cycloheximide (CHX) and MG132. (ii) After induction, cells were treated with CHX in the presence or absence of MG132 for the indicated times. Whole-cell lysates were resolved electrophoretically and subjected to WB with the indicated antibodies as described in *Materials and Methods*. (B) HEK-293 cells were transfected with pCS2-HA-DHFR or various pCS2-HA-FAT10 constructs as indicated. After 48 h, cells were lysed, and whole lysates were subjected to immunoprecipitation, using immobilized anti-HA. Total and immunoprecipitated proteins were analyzed by WB after SDS–PAGE using the indicated antibodies. Molecular weight markers and immunoglobulin light chains (*) are indicated. (C) HEK-293 cells were transfected with pCS2-HA-FAT10-GV or pCS2-HA-FAT10-K0-GV. (i) CHX was added to inhibit protein synthesis, and MG132 was added as indicated to inhibit the proteasome. (ii) A similar experiment aimed at monitoring the effect of proteasome inhibition without inhibiting protein synthesis. Proteins were detected as described for A. Numbers in (i) indicate the percentage of HA-FAT10 remaining at the different time points compared with time 0. (D) HEK-293 cells were transfected as in B, and newly synthesized proteins were radiolabeled as described in *Materials and Methods*. The labeling was carried out at 30 or 37°C for 15 or 45 min, and MG132 was added as indicated. Cells were lysed in RIPA buffer, and RIPA-insoluble material was dissolved by boiling in sample buffer. (i) HA-tagged FAT10 molecules were immunoprecipitated and analyzed by SDS–PAGE and autoradiography. The proportion of FAT10-associated radioactivity in the soluble and insoluble fractions is presented quantitatively in C, i. (ii) Representation of the time- and temperature-dependent accumulation of WT and lysine-less (K0) HA-FAT10-GV in the insoluble fraction. (E) HEK-293 cells were transfected as in B and treated at 30 or 37°C during 4 h with CHX. MG132 was added as indicated. Whole-cell lysates were resolved via SDS–PAGE and subjected to WB with the indicated antibodies as described in *Materials and Methods*. Numbers denote the percentage of remaining HA-FAT10 compared with time 0.

demonstrate that ubiquitination is required for the degradation of FAT10.

Following the demonstration that FAT10 degradation requires ubiquitination, it was important to isolate ubiquitin conjugates of FAT10. As can be seen in Figure 3A, using a cell-free ubiquitination system (Breitschopf *et al.*, 1998), we were able to show that FAT10 is indeed ubiquitinated in different cell extracts and in an ATP-dependent manner. Moreover, both ubiquitin-like domains of FAT10 underwent ubiquitination, suggesting that the full-length molecule probably contains several sites of ubiquitination. To corroborate this assumption, we conducted our in vitro assay in the presence of methylated or lysine-less ubiquitins, which are unable to polymerize. Both generated several discrete conjugates, demonstrating that ubiquitination targets indeed several lysine residues in FAT10 (Figure 3B).

To test whether ubiquitination of FAT10 occurs also in cells, we transfected HEK-293 cells with a FLAG-ubiquitin (FLAG-Ub) construct along with HA-FAT10-GV or HA-FAT10-K0-GV (Figure 3C, i and ii). Conjugates were detected after immunoprecipitation, SDS–PAGE, and Western blotting (WB). The results clearly show that it is predominantly WT FAT10 that is ubiquitinated, suggesting again that ubiquitination precedes and signals degradation. It should be

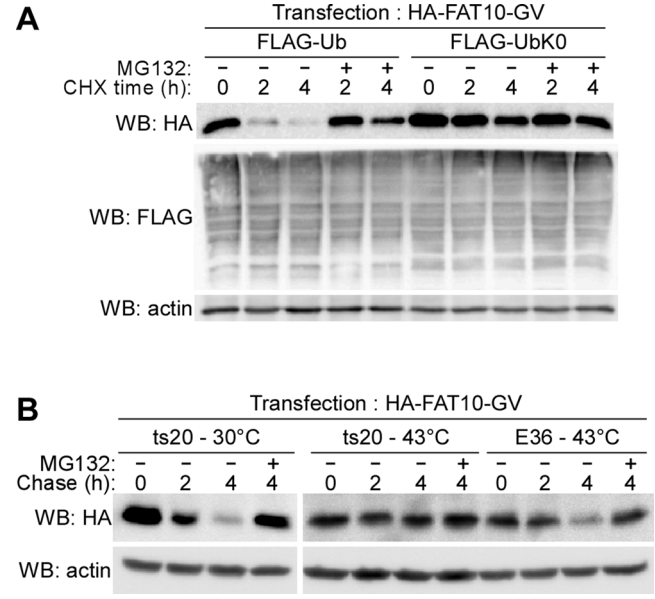

FIGURE 2: Degradation of FAT10 requires ubiquitination. (A) HEK-293 cells were transfected with pCS2-HA-FAT10-GV along with pCAGGS-FLAG-ubiquitin (FLAG-Ub) or pCAGGS-FLAG-lysine-less ubiquitin (FLAG-UbK0). After 48 h, cells were treated with CHX, and MG132 was added as indicated. Cells were harvested at the indicated times, and whole-cell lysates were subjected to SDS–PAGE and WB with the indicated antibodies as described in *Materials and Methods*. (B) CHO E36 and ts20 cells were transfected with pCS2-HA-FAT10-GV. After 42 h they were further incubated for 30 min at the permissive (30°C) or restrictive (43°C) temperature as described in *Materials in Methods*. CHX was added and the incubation continued at 30°C in the presence or absence of MG132. Cells were harvested at the indicated times, and whole-cell lysates were subjected to SDS–PAGE and WB with the indicated antibodies as described in *Materials and Methods*.

noted that the nonmodified WT-FAT10 and its lysine-less counterpart are also precipitated (Figure 3C, i). This can be due to the fact that FAT10 can interact with the proteasome or with ubiquitin-shuttling proteins to which ubiquitinated proteins are bound as well. It can also be nonspecific binding. Figure 3C, ii, describes a similar experiment in which FAT10 was immunoprecipitated and the resolved immunoprecipitated proteins were probed either with anti-HA, which detects FAT10, or anti-FLAG, which detects ubiquitin. In both cases we were able to detect specific ubiquitin conjugates of WT- but not K0-FAT10, similar to the experiment shown in Figure 3C, i, and further corroborating it.

FAT10 can serve as a proteasomal degradation signal

Because FAT10 binds to the proteasome, it was interesting to study whether it can serve as a degradation signal for otherwise stable proteins. Similar to the findings on FAT10, it was reported that FAT10 fused to GFP is degraded in a ubiquitination-independent manner, and it was suggested that FAT10 serves as the only degradation signal without requiring further ubiquitination (Hipp *et al.*, 2005). However, other studies on ubiquitin–green fluorescent protein (GFP) fusion proteins showed that, although ubiquitin can serve as a weak degradation signal for GFP, its further ubiquitination is required for efficient degradation (Qian *et al.*, 2006). Accordingly, we wondered whether FAT10 is similar to ubiquitin, that is, whether its ubiquitination is required to degrade FATylated substrates. We first demonstrated that FAT10 fused to the N-terminal residue of GFP, but not to its C-terminal residue, can target the protein for proteasomal degradation (Figure 4A, i and ii). Moreover, treatment of the cells with MG132 without inhibiting protein synthesis resulted in accumulation of FAT10-GV-GFP, suggesting that the protein is indeed short lived (Figure 4B). In agreement with these data, we demonstrated that FAT10-GV-GFP is also ubiquitinated compared with FAT10-K0-GV-GFP, the ubiquitination of which is significantly weaker (Figure 4C). Not unexpectedly, HA-FAT10-GV-GFP could also be found bound to the proteasome (Figure 4D). Examining the question of whether FAT10 needs to be further ubiquitinated in order to render the fused substrate susceptible for degradation, we demonstrated that expression of lysine-less (but not WT) ubiquitin significantly slows down the degradation of FAT10-GV-GFP (Figure 4E). It is still possible that FAT10 can serve as a partial degradation signal without requiring further ubiquitination, although it is clear that for efficient degradation, the generation of polyubiquitin chains is required.

FAT10 is degraded following induction of apoptosis

Although FAT10 expression has been linked to regulation of cell death, its precise role has not been established. It has been reported that overexpression of FAT10 induces apoptosis in cells (Raasi *et al.*, 2001; Ross *et al.*, 2006). However, in lymphocytes, FAT10 appears to be antiapoptotic (Canaan *et al.*, 2006). Therefore, we decided to examine the effect of apoptosis on the stability of FAT10. Following induction of endogenous FAT10 in HEK-293 cells, we treated the cells with the apoptosis inducer staurosporine (STS). As shown in Figure 5A, the drug treatment resulted in apoptosis induction (visualized by the cleavage of the PARP-1 protein) accompanied by a complete disappearance of FAT10, which was proteasome dependent. Of interest, in this experiment we did not use CHX, which demonstrates that the degradation of FAT10 induced by STS is fast and efficient and exceeds significantly its synthesis. The same effect of STS could also be observed when we overexpressed HA-FAT10-GV (Figure 5B). Here, too, the STS effect was able to overcome the translation of HA-FAT10-GV. As expected, in the presence of CHX, STS induced a faster degradation of FAT10 compared with untreated cells (Figure 5C, i and ii). The induced degradation was sensitive to the two proteasome inhibitors MG132 and epoxomicin. Of importance, the increase in degradation of FAT10 was preceded by stimulation of its ubiquitination (Figure 5D). In line with the STS-induced ubiquitination and subsequent accelerated degradation of FAT10 is the finding that HA-FAT10-K0-GV is stable in the presence of STS (Figure 5E). The response of FAT10 to the apoptotic stimulus (stimuli) also can mediate the rapid degradation of a downstream protein fused to it, as shown in Figure 5F, where FAT10-GV-GFP is rapidly destroyed following addition of STS, even in the absence of CHX.

DISCUSSION

Degradation of proteins by the ubiquitin–proteasome system is a well-studied mechanism, but, paradoxically, little is known about the regulation of the components of the system. Among the many different modifying proteins, ubiquitin, SUMO, NEDD8, and FAT10 have been shown to be degraded by the proteasome, but the mechanisms that underlie these processes have remained elusive. We focused our work on FAT10, which appears to be the only short-lived ubiquitin-like protein. We confirmed previous data (Raasi *et al.*, 2001) showing that the half-life of FAT10 is ~1 h (Figure 1), compared with ~9 h for that of ubiquitin (Carlson and Rechsteiner, 1987; Hiroi and Rechsteiner, 1992). This tight regulation of FAT10 is probably linked to its expression profile: unlike ubiquitin, which is

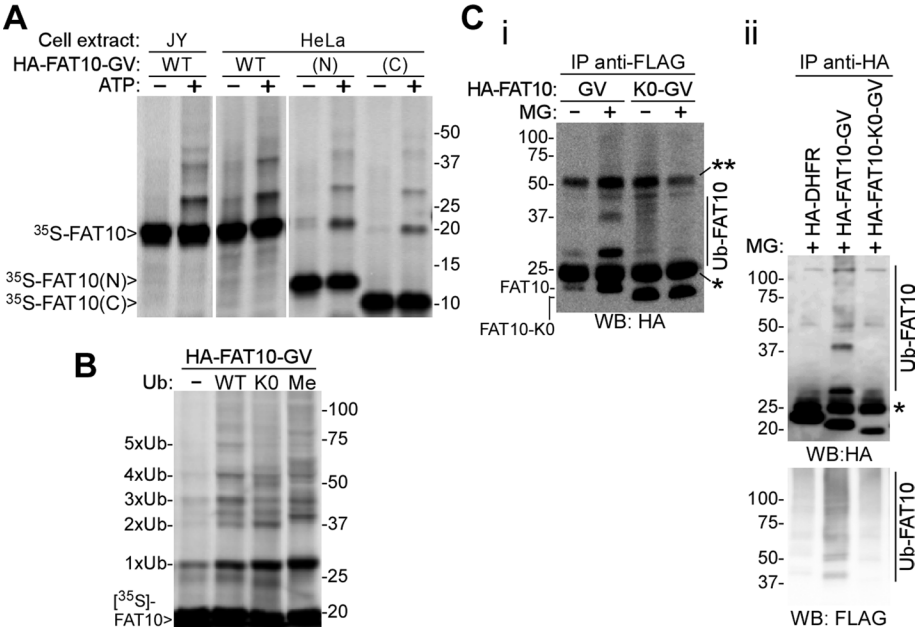

FIGURE 3: FAT10 is ubiquitinated both in a cell-free system and in cells. (A) In vitro translated HA-FAT10-GV, HA-FAT10(1-86) (N-terminal half of FAT10, referred to as N), and HA-FAT10(87-165)-GV (C-terminal half of FAT10, referred to as C) were subjected to a cell-free ubiquitination assay as described in *Materials and Methods*. Ubiquitination was carried out in JY or HeLa cell extracts as indicated. The ubiquitination reactions were analyzed by autoradiography following SDS–PAGE. Molecular weight markers are indicated. (B) Ubiquitination of in vitro translated, ^{35}S-methionine-labeled HA-FAT10-GV was carried out in HeLa cell extract in the absence (–) or presence of WT (+), methylated (Me), or lysine-less (K0) ubiquitin. Proteins were resolved and visualized as described for A. Bands corresponding to FAT10 conjugated with one to five ubiquitin molecules are indicated on the left, and molecular weight markers are indicated on the right. (C) (i) HEK-293 cells were cotransfected with pCAGGS-FLAG-Ub along with pCS2-HA-FAT10-GV or pCS2-HA-FAT10-K0-GV. After 45 h, cells were treated for 3 h with MG132 (MG) as indicated and lysed. Proteins were immunoprecipitated with an antibody directed against FLAG, resolved by SDS–PAGE, and detected following WB with an anti-HA antibody as described in *Materials and Methods*. (ii) Same as (i), except that cells were transfected also with pCS2-HA-DHFR as a control, and proteins were first immunoprecipitated with anti-HA antibody and then detected by anti-HA (top) or anti-FLAG (bottom) antibody. Ubiquitinated FAT10 molecules (Ub-FAT10), immunoglobulin light (*) and heavy (**) chains, and molecular weight markers are indicated.

expressed constitutively, FAT10 is induced only in specific tissues and under particular conditions, such as inflammation, and it has to be removed efficiently when its inducing signals are turned off.

Previous data suggested that FAT10 degradation is proteasome dependent, however ubiquitin independent (Hipp *et al.*, 2005), and the process probably requires NUB1L as a cofactor (Hipp *et al.*, 2004; Schmidtke *et al.*, 2006). Because FAT10 is made of two in-tandem ubiquitin domains, one may find similarity between the ubiquitin-independent degradation of FAT10 and the efficient ubiquitin-independent degradation of C-terminally extended ubiquitin (Shabek *et al.*, 2009; Verhoef *et al.*, 2009). In this context, one may compare the N-terminal ubiquitin-like domain of FAT10 to ubiquitin that serves as the binding component of the molecule to the proteasome, and the C-terminal domain to the tail that allows penetration into the 20S catalytic chamber of the 26S proteasome. We show here that a nonubiquitinable, lysine-less form of FAT10 is rapidly aggregated and cannot serve therefore as a tool to study the requirement for ubiquitin in the proteolytic process (Figure 1). Thus, it is possible that the lysine-less FAT10 disappearance observed by Hipp *et al.* (2005) was due to its aggregation. Even WT FAT10 was shown to be partially localized in insoluble structures (Kalveram *et al.*, 2008; Figure 1D). Therefore we used two alternative and independent experimental approaches to examine whether the degradation of FAT10 requires polyubiquitination: 1) we overexpressed in cells nonpolymerizable ubiquitin and 2) we tested the degradation of FAT10 in cells harboring a thermolabile mutation in the ubiquitin-activating enzyme, E1. In both approaches we found that FAT10 degradation is dependent on further ubiquitination (Figure 2). It appears that our results are not in line with those of Hipp *et al.* (2005), which suggested, using also the E1 temperature-sensitive mutant, that the degradation of FAT10 is ubiquitin independent. We believe that the discrepancy is due to the different experimental conditions used by the two groups. We incubate the cells for a short time (30 min) at 43°C (which we showed is sufficient to inactivate E1 significantly), and then monitor the degradation of FAT10 at 37°C. Hipp *et al.* (2005) inactivated E1 by incubating the cells for 2 h at 42°C, followed by a long incubation of 5 h at 39.5°C. Our study shows that the higher the temperature, the faster is FAT10 aggregation (Figure 1D). Thus, we suspect that the FAT10 disappearance they observed was indeed ubiquitin independent (as they claimed) but probably was not due to proteasomal degradation; more likely it was due to aggregation of the protein. It would have been helpful if they had shown in their article an MG132 inhibition point in the ts20 cell line experiment. In our study, we used MG132 and showed that it inhibits FAT10 disappearance in the ts20 cell line at the low permissive temperature (Figure 2B, last lane), suggesting the disappearance is due to proteasomal degradation.

Our attempts to identify the FAT10 lysines modified by ubiquitin failed, as the lysine-less molecule is rapidly aggregated. Single substitutions of all FAT10 lysine residues did not affect its degradation, suggesting that there is not a single specific residue that is modified and also that ubiquitination can occur simultaneously on several lysines (data not shown). This notion was further corroborated in the experiments shown in Figure 3, where ubiquitination could be observed on each ubiquitin-like domain of FAT10 expressed independently, and also with the multiple adducts observed when nonpolymerizable ubiquitin was used to conjugate FAT10 in a cell-free conjugation assay. It was tempting to think that FAT10 ubiquitination mediates its interaction with the proteasome, but we observed that lysine-less FAT10 also interacts with the protease (Figure 1B), suggesting that its ubiquitin-like domain mediates the association, but the association is futile, and the substrate can be degraded only if it is ubiquitinated. In this case, ubiquitination would constitute the selective marker of the FAT10 molecules that have to be degraded.

Of interest, the fusion protein model FAT10-GFP displayed a behavior similar to that of FAT10 alone. Like ubiquitin, FAT10 was able to induce the proteasomal degradation of GFP but only when it was fused to its N-terminus, and the degradation was mostly

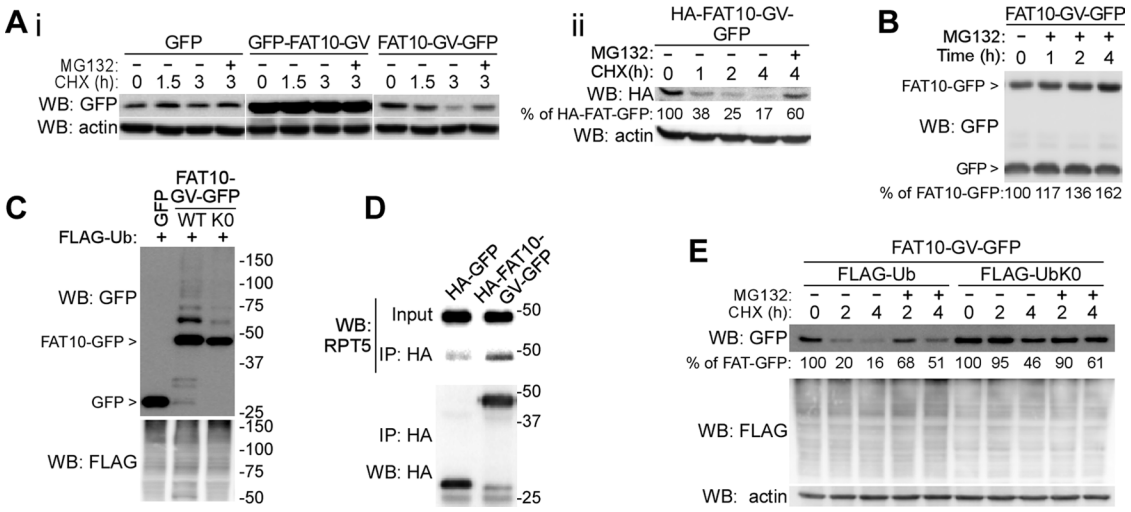

FIGURE 4: FAT10 can serve as a degradation signal. (A) HEK-293 cells were transfected with the indicated constructs and treated for the indicated times with CHX. MG132 was added as indicated along with CHX. Whole-cell lysates were resolved via SDS–PAGE and proteins visualized after WB with anti-actin and anti-GFP (i) or anti-HA (ii) antibodies. Numbers in (ii) denote the percentage of HA-FAT10-GV-GFP remaining compared with time 0. (B) HEK-293 cells were cotransfected with pCS2-GFP and pCS2-FAT10-GV-GFP. After 44 h MG132 was added for the indicated times, following which cells were lysed. Cell lysates were resolved via SDS–PAGE and proteins visualized after WB with an anti-GFP antibody. Numbers denote the level of FAT10-GV-GFP in the lysates compared with time 0. (C) HEK-293 cells were cotransfected with pCAGGS-FLAG-Ub along with pCS2-FAT10-GV-GFP or pCS2-FAT10-K0-GV-GFP. After 44 h, MG132 was added for 4 h, and the cells were lysed in RIPA buffer. Cell lysates were resolved via SDS–PAGE and proteins visualized after WB with the indicated antibodies. Molecular weight markers are indicated. (D) HEK-293 cells were transfected with pCS2-HA-GFP or pCS2-HA-FAT10-GV-GFP. After 48 h, the cells were lysed and the lysates subjected to the immunoprecipitation as described in the legend to Figure 1B. Total and immunoprecipitated proteins were resolved by SDS–PAGE and visualized after WB using the indicated antibodies. Molecular weight markers are indicated. (E) HEK-293 cells were cotransfected with pCS2-GFP and pCS2-FAT10-GV-GFP along with pCAGGS-FLAG-Ub or pCAGGS-FLAG-UbK0. After 44 h, CHX was added for the indicated times, after which cells were lysed. MG132 was added along with CHX when indicated. Cell lysates were resolved via SDS–PAGE and proteins visualized after WB with the indicated antibodies. Numbers indicate the percentage of FAT10-GV-GFP remaining compared with time 0.

dependent on ubiquitination (Figure 4). The residual degradation of FAT10-GFP observed in the presence of a nonpolymerizable mutant of ubiquitin can be explained by the fact that the interaction of FAT10 with the proteasome is sufficient to promote, at least partially, the degradation of a downstream fused protein. This is in line with previous findings showing that FAT10 can constitute a degradation signal without further ubiquitination (Hipp *et al.*, 2005). However, we think that ubiquitination of FATylated proteins is probably required to achieve their complete degradation. The question of why FATylation of proteins is needed in order to bring them to the proteasome requires further investigation, but it is tempting to speculate that FAT10 promotes ubiquitination of proteins that are normally not targeted by this modification. Given that FAT10 is a degradation signal for downstream proteins, its efficient ubiquitination and subsequent degradation could serve to control the degradation of a subset of proteins involved in specific processes, such as the inflammatory response.

The effect of FAT10 on cell growth has not been dissected mechanistically, but it was shown that deregulation of its expression is associated with chromosomal instability and cell transformation (Lee *et al.*, 2003; Lim *et al.*, 2006; Ren *et al.*, 2006). These observations seem to underline a prosurvival role for FAT10, so we decided to investigate the behavior of the molecule in the context of cell death. Induction of apoptosis resulted in fast degradation of FAT10 that was preceded by its increased ubiquitination (Figure 5), further highlighting the requirement for ubiquitination for FAT10 degradation and the role of FAT10 in prosurvival pathways.

MATERIALS AND METHODS

Cell culture and transfection

HEK-293 and CHO cells were grown at 37 and 30°C, respectively, in DMEM supplemented with 10% (HEK-293) or 20% (CHO) fetal calf serum, 0.2 mM L-glutamine, 10 U/ml penicillin, and 10 µg/ml streptomycin. Transfections were carried out in 100-mm dishes using the standard calcium phosphate method (HEK-293 cells) or the jetPEI (Polyplus-transfection, Illkirch, France) transfection reagent (CHO cells). Endogenous FAT10 expression was induced in HEK-293 cells by a 24-h treatment with TNFα and IFNγ at 100 ng/ml each.

Plasmids

All FAT10 and GFP cDNAS were subcloned into pCS2 (Invitrogen, Carlsbad, CA), which allows both cellular expression and in vitro translation. FLAG-ubiquitin and MDM2 cDNAs were subcloned into pCAGGS (Niwa *et al.*, 1991).

Chemicals

Tissue culture sera and media, TNFα, and IFNγ were from Biological Industries (Beit HaEmek, Israel). CHX, STS, and the proteasome inhibitors MG132 and epoxomicin were from Calbiochem (La Jolla, CA). The L-[^{35}S]methionine for in vitro translation was from PerkinElmer (Waltham, MA). Materials for SDS–PAGE, including prestained molecular weight markers. were from Bio-Rad (Hercules, CA). All other reagents and chemicals were from Sigma-Aldrich (St. Louis, MO).

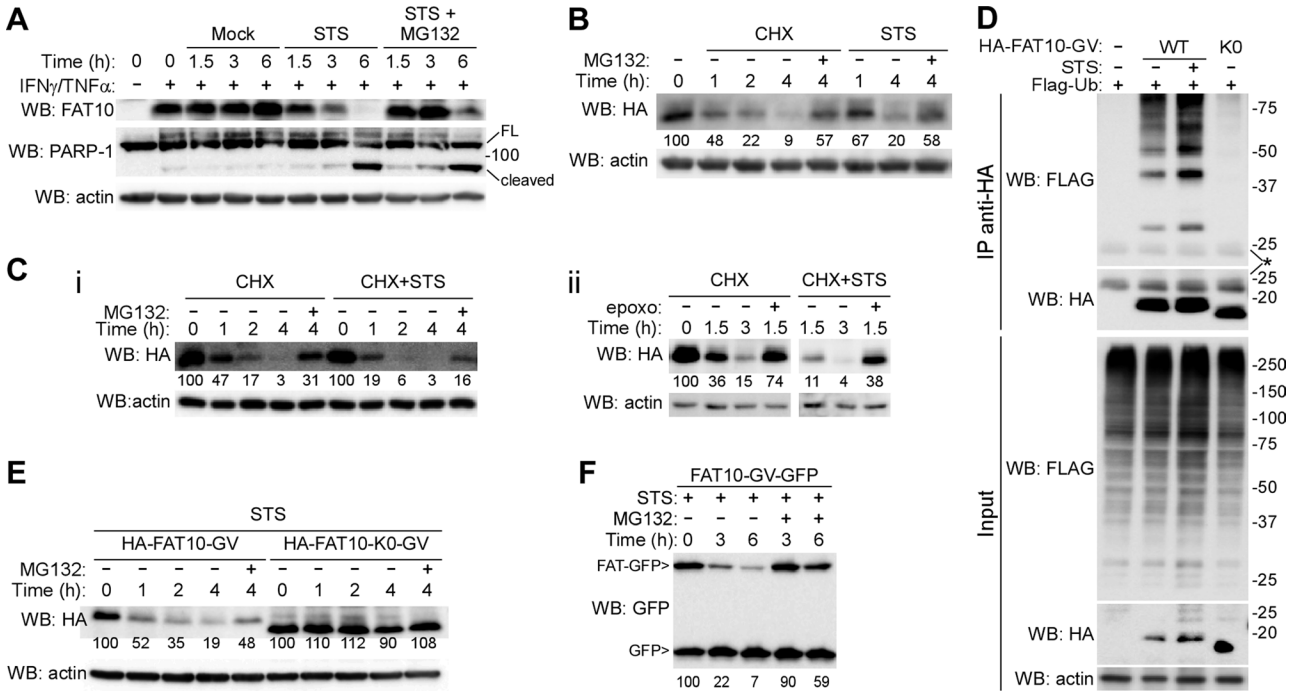

FIGURE 5: Apoptosis induces ubiquitination and degradation of FAT10. (A) Endogenous FAT10 expression was induced in HEK-293 cells by TNFα and IFNγ as described in *Materials and Methods*. STS was added along with MG132 (when indicated), and the cells were further incubated for the indicated times. Cells extracts were resolved via SDS–PAGE and proteins visualized following WB using the indicated antibodies. Positions of full-length (FL) and cleaved PARP-1 and the 100-kDa molecular weight marker are indicated. (B, C) HEK-293 cells were transfected with pCS2-HA-FAT10-GV and were treated for the indicated times with CHX or STS (B) or CHX with and without STS (C). The proteasome inhibitors MG132 (B and C, i) and epoxomicin (C, ii) were added as indicated. Cell lysates were resolved via SDS–PAGE, and proteins visualized after WB using the indicated antibodies. (D) HEK-293 cells were cotransfected with pCAGGS-FLAG-Ub and pCS2-HA-FAT10-GV or pCS2-HA-FAT10-K0-GV and were treated for 3 h with MG132 in the presence or absence of STS. RIPA lysates of the cells were then subjected to immunoprecipitation using immobilized anti-HA. Total and immunoprecipitated proteins were resolved via SDS–PAGE and proteins visualized after WB using the indicated antibodies. Molecular weight markers and immunoglobulin light chains (*) are indicated. (E) HEK-293 cells were transfected with pCS2-HA-FAT10-GV or pCS2-HA-FAT10-K0-GV and were treated for the indicated times with STS in the presence or absence of MG132. Cell lysates were resolved via SDS–PAGE and proteins visualized after WB using the indicated antibodies. (F) HEK-293 cells transfected with pCS2-FAT10-GV-GFP were treated and analyzed as in E. Numbers in B, C, E, and F indicate the percentage of remaining FAT10 (B, C, E) or FAT10-GFP (F) compared with time 0.

Monitoring protein stability in HEK-293 cells

At 42 h after transfection, cells were treated with 100 μg/ml CHX and harvested at different times after addition of the inhibitor. When indicated, a proteasome inhibitor was added (MG132 at 20 μM or epoxomicin at 5 μM), and STS was added at 1 μM.

Monitoring protein stability in CHO cells

At 42 h after transfection, cells were further incubated at 30°C or shifted to 43°C for 30 min in the presence of 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, pH 7.2. CHX (150 μg/ml) was added, and incubation continued at 30°C for the indicated times. MG132 (20 μM) was added when indicated.

Cell lysates and immunoprecipitations

All cell lysates were prepared in RIPA buffer (50 mM Tris HCl, pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with protease inhibitors (Complete; Roche, Indianapolis, IN). For ubiquitination experiments, 5 mM *N*-ethylmaleimide and 5 mM iodoacetamide were also added. Cell lysates from 100-mm dishes were incubated for 20 h at 4°C with 5 μl of immobilized anti-HA (clone 3F10; Roche) or 10 μl of immobilized anti-FLAG (clone M2; Sigma-Aldrich). The beads were then washed twice for 10 min in 1 ml of RIPA buffer, and proteins were eluted by boiling in sample buffer for 10 min.

Immunoblotting and antibodies

Cell lysates and immunoprecipitations were resolved via 11.5% SDS–PAGE, followed by transfer onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). Proteins were visualized after Western blotting, using appropriate antibodies, which were directed against HA epitope (clone 16B12; Covance, Berkeley, CA), FLAG epitope (clone M2; Sigma-Aldrich), GFP (sc-8334; Santa Cruz Biotechnology, Santa Cruz, CA), actin (clone C4; Millipore), FAT10 (PW9680; Enzo Life Sciences, Plymouth, PA), PARP-1 (MCA1522G; AbD Serotec, Raleigh, NC), the ribosomal protein S18 (clone E13; Santa Cruz Biotechnology). and human proteasome subunits α6 (PW8100; Enzo Life Sciences) and Rpt5 (clone 1B9; Sigma-Aldrich). Protein bands were quantified using ImageJ software (National Institutes of Health, Bethesda, MD), and measurements were normalized according to the corresponding actin band signal.

In vitro translation and ubiquitination of proteins

Proteins were translated in vitro in the presence of ^{35}S-methionine using the TNT wheat germ–based kit for coupled

transcription-translation (Promega, Madison, WI). In vitro conjugation assays were carried out essentially as described previously (Breitschopf *et al.*, 1998). Briefly, in vitro translated proteins (~30,000 cpm) were incubated at 37°C for 1 h in the presence of HeLa or JY cell extract (4 μg/μl), ubiquitin (0.4 μg/μl), ubiquitin-activating enzyme E1 (20 ng/μl), ATPγS (0.5 mM), and the isopeptidase inhibitor Ub-aldehyde (8 ng/μl; Hershko and Rose, 1987). Reactions were terminated by adding a fourfold sample buffer. Following boiling, samples were resolved by SDS–PAGE, and proteins were visualized using a PhosphorImager (GE Healthcare, Piscataway, NJ).

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